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PhD Thesis

STUDIES ON LINEAGE SHIFT RESPONSES OF HUMAN PERIPHERAL BLOOD MULTIPOTENT CELLS

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ABSTRACT

Stem cell therapy is gaining momentum as an effective treatment strategy for degenerative diseases. As embryonic stem cells pose a lot of ethical issues, adult stem cells, isolated from various sources like cord blood, bone marrow or adipose tissue, are being considered as a realistic option due to their well documented therapeutic potentials. In our lab, we have standardised a method to isolate human fibroblastic multipotent stem cells (hPBMCs) from human peripheral blood, that are able to sustain long term in vitro culture and differentiate towards adipogenic, chondrogenic and osteogenic lineage.

In this work, hPBMCs were stimulated to obtain in vitro neuronal and myogenic-like cells. Moreover, their restorative potential in degenerative diseases of skeletal muscle and nervous tissue was evaluated using in vivo models. In order to test the neuronal differentiation potential, the cells were seeded ($1x10^4$) on gelatin coated dishes and cultured for 7 days in neurobasal medium with EGF and FGF followed by Retinoic acid and NGF for next 7 days. Myogenic induction was carried out using IGF and ascorbic acid for 14 days. At different time points, morphological studies were performed by SEM and specific neuronal and myogenic marker expression were evaluated using RT-PCR, flow cytometry and western blot. hPBMCs showed characteristic dendrite like morphology and expressed specific neuronal markers both at mRNA and protein level. The calcium flux activity of hPBMCs under stimulation with KCl 56 mM and the secretion of the neurotransmitter, noradrenalin, a precursor in the dopamine synthesis confirmed their ability to acquire a functional phenotype. When premarked by a cell tracker Qdot 800 and injected stearotactically into a rat brain, hPBMCs showed to be migratory and proliferative as detected after 10 and 20 days of injection. No tumor mass was identified. The myogenic potential of hPBMCs were confirmed by their ability to form syncitium like structures in in vitro culture and to express typical myogenic markers both at early and late phases of differentiation. hPBMCs were showed to integrate within the host tissue and to take part in tissue repair as demonstrated in a bupivacaine induced muscle damage model.

RIASSUNTO

Il trapianto di cellule staminali è una strategia terapeutica che sta conoscendo uno sviluppo sempre maggiore come possibile approccio clinico per il trattamento delle malattie degenerative. Considerando i problemi di carattere etico sollevati dall'impiego delle cellule staminali embrionali, le cellule staminali adulte isolate da varie fonti (sangue cordonale, midollo osseo, tessuto adiposo) rappresentano una realistica alternativa, in virtù della loro potenzialità rigenerativa ben documentata. Nel nostro laboratorio è stato standardizzato un metodo per isolare cellule staminali fibroblastoidi multipotenti (*Peripheral Blood Multipotent Cells*, hPBMC) da sangue periferico umano, che possono essere espanse *in vitro* durante la coltura a lungo termine e sono in grado di differenziare in senso adipogenico, condrogenico e osteogenico. Nel lavoro di tesi del Dott. Senthilkumar Rajendran, le cellule hPBMC sono state stimolate per l'ottenimento *in vitro* di cellule simil-neuronali e -muscolari. Inoltre è stato valutato il loro potenziale rigenerativo nel trattamento di malattie degenerative del muscolo scheletrico e del tessuto nervoso attraverso la sperimentazione *in vivo* su modelli animali. Al fine di testare il potenziale di differenziazione neuronale, le cellule sono state seminate ($1x10^4$) su *coating* di gelatina e coltivate per i primi 7 giorni in *Neurobasal medium* addizionato con EGF e FGF, e per i 7 giorni successivi in terreno basale contenente acido retinoico e NGF. L'induzione miogenica è stata effettuata utilizzando IGF e acido ascorbico per 14 giorni. Ad ogni *time point*, sono stati realizzati studi morfologici mediante SEM e analisi di espressione di specifici marcatori neuronali e miogenici mediante RT-PCR, citofluorimetria e western blot. Le cellule hPBMC hanno mostrato una caratteristica morfologia simil-dendritica e l'espressione di specifici marcatori neuronali a livello sia di mRNA che di proteine. Lo studio del flusso del calcio dopo stimolazione con KCl 56 mM e l'attività di secrezione del neurotrasmettitore noradrenalina, precursore nella sintesi della dopamina, hanno confermato la capacità delle cellule hPBMC di acquisire un fenotipo funzionale. Dopo marcatura con il *tracker* cellulare Qdot 800 e iniezione per stereotassi in un cervello di ratto, le hPBMC hanno dimostrato un elevato potenziale migratorio e proliferativo dopo 10 e 20 giorni dall'impianto. Non è stata identificata alcuna massa tumorale. Il potenziale miogenico delle popolazioni isolate è stato confermato dalla loro capacità di formare strutture simil-sinciziali durante la coltura *in vitro* e di esprimere marcatori tipici della linea miogenica, sia a tempi precoci che nelle

fasi tardive del differenziamento. Infine, testate in un modello animale di danno muscolare indotto con bupivacaina, le cellule hPBMC sono state in grado di integrarsi all'interno del tessuto ospite e di prendere parte nella riparazione dei tessuti.

INTRODUCTION

1. Degenerative disorders: a clinical challenge

Degenerative diseases are a poorly understood and largely untreatable set of pathologies that take a heavy toll in disability and death and have far-reaching socioeconomic impacts. They can be considered a biological phenomenon, which can be defined and understood in molecular and physiological terms. Much of biomedical research focuses on discovering the molecular basis of degenerative diseases to define and understand their natural history, etiology and pathogenesis. Thanks to that, modern medicine is accumulating an ever-increasing arsenal of molecular-based diagnostics and therapeutics for a large number of these disorders (Kolodny and Fattal-Valevski, 2005). Despite increasing knowledge on this field, degenerative disorder treatment still have to face some specific clinical challenges, which include: a) improving diagnostic capability, particularly early diagnosis; b) developing tools to measure changes accurately over time; c) understanding more about mechanisms of disease; d) developing new therapies aimed at slowing progression or preventing these disorders.

As a starting point, degenerative disorder classification can be useful to understand their molecular origin and mechanism. To simplify the topic, these conditions can be classified according to three principal mechanisms responsible for their pathology and resulting clinical symptomatology (Kolodny and Fattal-Valevski, 2005):

- *Storage disorders*. These are gradually progressive disorders in which large molecules are stored (i.e., glycolipid and glycoprotein), leading to multiorgan involvement according to the site of the storage material (i.e., bone, liver, spleen, bone marrow, central nervous system [CNS]).
- *Disorders with cellular intoxication*. These tend to involve small molecule diseases (amino and organic aciduria, urea cycle diseases) and have an acute presentation, usually at a young age or even in the neonatal period. Clinical symptoms are nonspecific and are typical for toxic encephalopathy (vomiting, lethargy, hypotonia, and respiratory abnormalities).
- *Disorders with energy deficiency*. These disorders mainly affect organs with high-energy requirement such as the CNS, skeletal muscle, cardiac muscle, and liver. They present with intermittent metabolic crises precipitated by stress.

Among degenerative disorders, some of the most common ones are pathologies which affect CNS (Alzheimer's and Parkinson's disease, multiple sclerosis), skeletal muscle (muscular dystrophies), heart muscle (myocarditis) bone (osteoporosis) and joints (osteoarthritis, rheumatoid arthritis).

1.1 Degenerative neural disorders

Neurodegenerative diseases result from the gradual and progressive loss of neural cells, leading to nervous system dysfunction (Brown et al., 2005). The hallmark of several degenerative disorders in the central nervous system (CNS), such as amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis, and Alzheimer's disease, is the massive loss of one or several types of neurons. Although neurological disorders manifest with different clinical features, the disease processes at the cellular level appear to be similar. For example, Parkinson's disease affects the basal ganglia of the brain, depleting it of dopamine. This leads to stiffness, rigidity and tremors in the major muscles of the body, typical features of the disease. In Alzheimer's disease, there are deposits of tiny protein plaques that damage different parts of the brain and lead to progressive loss of memory. Research focuses on the similarities in neurodegeneration that occur in all of these diseases. This can provide clues in the development of new therapies and therapeutic strategies that may benefit patients in any of the three conditions. Cell death and deposition of abnormal proteins and plaques, for example, is a feature common to most neurodegenerative disorders (Chesselet, 2001).

The development of new therapeutic strategies was once complicated by the fact that the nerve path was first thought to be static, immobile, and incapable of regeneration. In the last decade, much evidence demonstrates that generation of new neurons, namely neurogenesis, is not entirely restricted to prenatal development, but continues throughout adult life in certain regions of the mammalian brain (Gage, 2002). This may open new perspectives in clinical research and therapy of degenerative neural disorders.

1.2 Degenerative skeletal muscle disorders

A degenerative muscle disease is a condition marked by the progressive deterioration of muscle tissue that causes weakness and impairs normal function. There are various types of degenerative muscle diseases, and each one may affect different muscle groups. Usually, degenerative muscle diseases are marked by problems with walking, balance, and coordination, and many affect speech, swallowing, and even breathing. Some

examples of diseases that cause muscle deterioration include muscular dystrophy, which is inherited, and amyotrophic lateral sclerosis, which eventually causes the death of the patient (Tabebordbar et al., 2013).

Current treatment options for degenerative muscular disorders are disappointingly limited and focus mainly on managing symptoms and suppressing the immune and inflammatory response. Therapeutic approaches that aim instead to cure these diseases have been a subject of research for many decades and can be grouped broadly into two categories on the basis of their strategic approach. The first category seeks to repair or replace the mutated gene, whereas the second aims to reduce the impact of the mutation by activating alternative pathways or intervening downstream to correct the pathological consequences. Each of these strategies presents unique advantages and challenges, and past experiences have helped inform and focus the direction of future research and the design of future clinical trials (Partridge, 2011).

2. Degeneration and Regeneration

Advances in basic knowledge and clinical therapy of degenerative diseases have led to new and compelling ideas about treating these disorders with novel tissue regeneration strategies.

2.1 Mechanisms of tissue regeneration

Effective functioning of the body's tissues and organs depends upon innate regenerative processes that maintain proper cell numbers and replace damaged cells after injury. In many tissues, regenerative potential is determined by the presence and functionality of dedicated populations of stem and progenitor cells, which respond to exogenous cues to initiate tissue repair when needed (Figure 1). However, in many instances, resident precursor cells suffer declining activity in response to advancing age, leading to reduced repair potential and chronic degenerative disease. If a tissue is chronically or widely injured, normal and efficient repair can no longer occur and exogenous regenerative strategies have to be developed (Yamskova et al., 2010). For this purpose, regenerative medicine therapies, fueled by advances in stem cell biology and technologies, seek to direct inherent non-healing injuries towards full restoration of tissue structure and subsequent function. Numerous studies have demonstrated that - when recruitment of endogenous stem cells is not enough - exogenous administration of a number of stem

cell populations to injured and pathological tissues has resulted in structural regeneration as well as functional improvement (Baraniak and McDevitt, 2010).

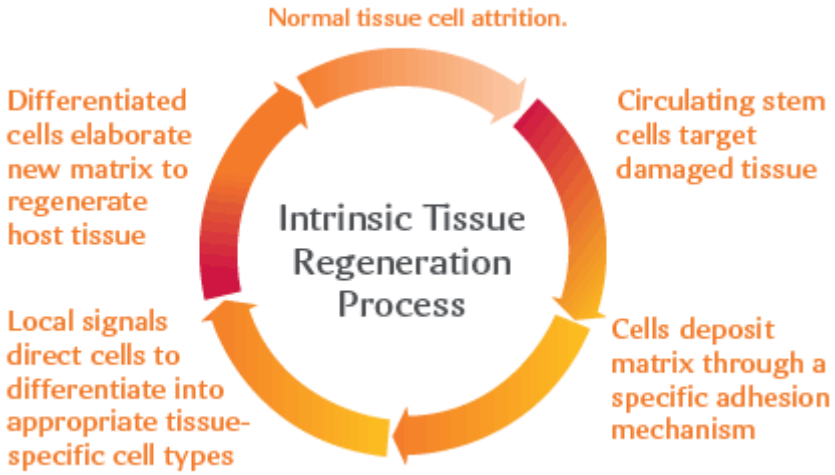


Figure 1. Mechanism of tissue regeneration

2.2 Transdifferentiation and lineage switching

Transdifferentiation has attracted great controversy in recent years, mostly in regards to whether stem cells from the bone marrow can colonize other tissues after transplantation. However, the general subject area of tissue-type switching is much wider than this specific controversy. It embraces some fascinating biological and pathological phenomena that deserve more attention than they have received. There has been a recent tendency to use the word transdifferentiation to mean 'conversion of anything into anything else'. But it is preferable to reserve the term for its original meaning — transformation of one differentiated cell type into another — and to use the term 'metaplasia' for the more general transformation of one tissue type into another. This is because tissues generally consist of several differentiated cell types and metaplasia often involves the transformation of undifferentiated stem or progenitor cells such that they produce a repertoire of cell types that are characteristic of a different tissue (Alison et al., 2004). Recently, there has been interest in the possibility of reprogramming cells from a differentiated state back to a pluripotent state that resembles the embryonic stem (ES) cell, shown here as ES transformation. In this article, the term 'metaplasia' will be used for any tissue-typeswitching (Figure 2) event and 'transdifferentiation' will be used only when both the precursor and the product are differentiated cell types. The phenomena discussed here are mostly metaplasias in which the starting cell type is an undifferentiated cell of some sort, which indicates that

it might be easier to convert a committed but undifferentiated cell to another lineage than to convert one differentiated cell type into another differentiated cell type. But are stem or progenitor cells really more labile than terminally differentiated cells? The numerous experiments involving grafts of haematopoietic stem cells into irradiated hosts did not seem, in the end, to have involved the large-scale reprogramming of cells. But there is probably a small residue of genuine metaplasia after the various other processes, such as marker gene transfer or cell fusion (Slack et al., 2007).

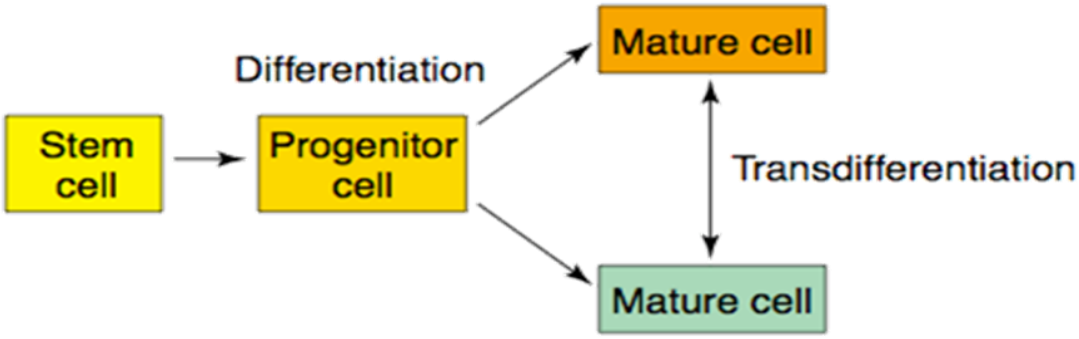


Figure 2. Transdifferentiation process

2.3 Role of Adult Stem Cells in Tissue Repair

While some tissues, such as the blood, skin, gut, respiratory tract and testis, must perpetually renew, the majority of cells and tissues in adult mammals exhibit very low turnover under normal circumstances; some of these respond poorly to regenerative pressure (e.g., heart), while other respond quite well (e.g., liver). These observations have been interpreted as indicating the existence and function of stem cells within some highly regenerative tissues and a lack of stem cell function in other tissues. Nonetheless, multiple organs, including the brain and heart, once thought of as non renewing, postmitotic tissues, actually have been shown to exhibit previously unappreciated cell turnover (Altman and Das, 1965; Kajstura et al., 1998; Kuhn et al., 1996). These observations have opened the door for studies aimed at identifying additional tissue-resident adult stem cell populations and evaluating their regenerative potential; however, the degree to which particular tissues depend upon replenishment of mature cells from relatively undifferentiated stem and progenitor cells is in many instances uncertain. Injury/repair studies provide strong support for essential stem cell function in the hematopoietic system, intestinal epithelium, dermal epithelium, and skeletal muscle, but whether endogenous stem cells play a significant role in tissue homeostasis

or responses to damage in other systems is unclear. In addition, in many cases the mechanisms by which such cells contribute to tissue regeneration are poorly defined, and in particular, whether regeneration of adult tissues from endogenous stem cells exploits the same molecular pathways used to establish that tissue during development has yet to be elucidated.

2.4 Crossing the Germ Layers

Hematopoietic stem cells (HSC), which reside predominantly in the bone marrow of adult mice and humans, normally function to generate all of the lineages of mature blood cell types necessary for maintaining proper hematopoietic function (Kondo et al., 2003). The concept that adult HSC function solely to maintain hematopoietic cell lineages was challenged by a series of papers suggesting that unfractionated bone marrow cells, or bone marrow cells enriched by various methods for hematopoietic stem cell activity, could be seen to contribute at low levels to multiple nonhematopoietic tissues following transfer into lethally irradiated, and often injured, recipient mice or humans (Herzog et al., 2003). Such studies have reported the expression of donor-derived genetic markers in nonhematopoietic cells within the skin (Krause et al., 2001), lung epithelium (Theise et al., 2002), intestinal epithelium (Krause et al., 2001), kidney epithelium (Kale et al., 2003), liver parenchyma (Krause et al., 2001, Lagasse et al., 2000), pancreas (Ianus et al., 2003), skeletal muscle (Brazelton et al., 2003), endothelium (Grant et al. 2002), myocardium (Jackson et al., 2001), and CNS neurons in the cortex and cerebellum (Priller et al., 2001). Such findings were extended by some to a general hypothesis of adult stem cell plasticity (Figure 3), wherein adult stem cells from one tissue were considered to be roughly equivalent in developmental potential to adult stem cells in another tissue, with the outcome of stem cell differentiation largely determined by different microenvironments encountered following differential trafficking from the bloodstream (Blau et al., 2001). BM cell contributions to nonhematopoietic tissues, including myocardium (Orlic et al., 2001) and skeletal muscle (Ferrari et al., 1998), also have been reported following direct delivery of cells to injured tissues in unirradiated recipients. The frequency with which such unexpected events have been detected has varied widely, from less than 0.1% to almost 20% of differentiated cells (reviewed in Herzog et al., 2003). In most cases where BM contributions to nonhematopoietic tissues have been detected, significant tissue injury has been necessary, but some have reported incorporation of cells into tissues without substantial additional injury aside from that

induced by the irradiation required for hematopoietic cell transplantation (Krause et al., 2001). With a few notable exceptions, in which contribution of transplanted cells to recovery of liver (Lagasse et al., 2000) or kidney (Kale et al., 2003) function has been documented, most reports of BM or HSC plasticity have not evaluated the tissue-specific function of putatively transdifferentiated cell types. Such determinations clearly will be important in assessing the biological relevance and clinical utility of such events. Disconcertingly, a significant number of studies also report a failure to detect BM or HSC contributions to nonhematopoietic tissues in similar experimental systems (Castro et al., 2002); the reasons for this apparent irreproducibility of results in different laboratories are not entirely clear, but may relate in part to differences in injury models, detection strategies and identification of donor markers, and/or cell purification techniques (Goodell, 2003). Given that in most cases the mechanism(s) and cell types involved in reported instances of BM or HSC plasticity have not been clearly defined, multiple alternative explanations for such observations remain, and must now be evaluated.

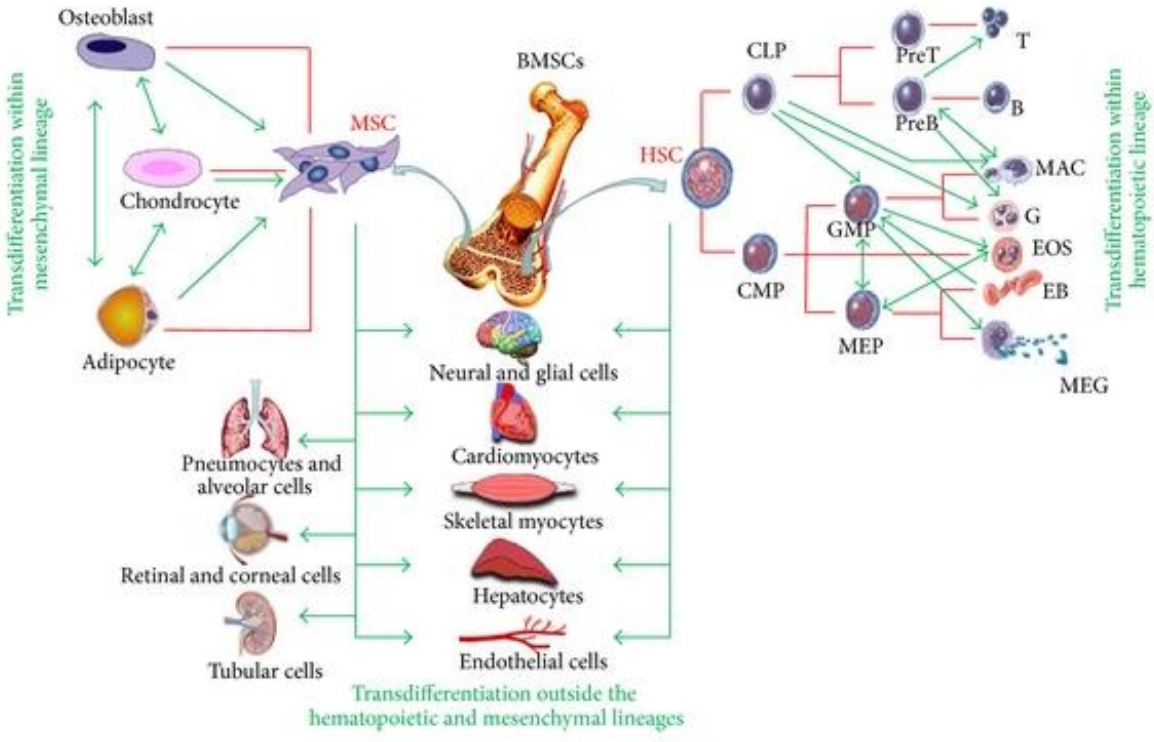


Figure 3. Crossing the lineage barrier

2.5 Non hematopoietic to Hematopoietic switch

Some studies have suggested that brain or muscle-derived stem cells may harbor hematopoietic potential. These include the observation that cultured neurosphere cells,

presumed to derive from central nervous system stem cells (CNS-SC), when injected into lethally irradiated recipient mice were seen to contribute to mature cells of various blood lineages (Bjornson et al., 1999), although these findings have proved difficult to reproduce in other laboratories (Morshead et al., 2002). In addition, muscle mononuclear cells (Jackson et al., 1999) or muscle SP cells (Gussoni et al., 1999) exhibited hematopoietic activity when injected intravenously into lethally irradiated recipient mice. Although such experiments have been interpreted to indicate the transdifferentiation of neural or muscle stem cells, it is important to note that HSC constitutively circulate in the blood of normal animals, potentially at very high rates of flux (Wright et al., 2001). Such blood-borne HSC are fully functional and can stably re engraft BM at distinct sites, contribute to ongoing hematopoiesis, and maintain the ability to competitively reconstitute lethally irradiated recipients. Thus, circulating or itinerant HSC likely contaminate many nonhematopoietic tissues, and may confound interpretation of experiments designed to test the hematopoietic potential of these tissues. In fact, subsequent experiments have demonstrated that the muscle SP population is actually a heterogeneous mixture of stem and progenitor cells and that all muscle SP cells with hematopoietic reconstituting activity are in fact committed hematopoietic precursors, expressing the panhematopoietic marker CD45 (McKinney-Freeman et al., 2002). Hematopoietic stem and progenitor cells appear to seed the muscle from BM cells following BM transplant (Issarachai et al., 2002), indicating that the hematopoietic activity of muscle cells likely derives from itinerant HSC, and does not in fact constitute an example of stem cell transdifferentiation.

2.6 Peripheral blood-lineage shift source

Bone marrow has been considered for ages the main hematopoietic and multipotent stem cell source, but its clinical use faces some practical difficulties, such as extraction procedures, which are highly invasive for the donor and cell yield, which is highly variable and dependent on the donors age (Stenderup et al., 2003). That is the reason why in the last decade alternative sources have been studied, such as umbilical cord (Rubinsteinet al., 1993), adipose tissue (Zuk et al., 2002) and peripheral blood (Seta and Kuwana, 2007). In particular, peripheral blood stem cell research is knowing great development because of many advantages offered by this novel ASC source: large availability, non invasive extraction practice and autologous cell origin.

Cells of fibroblastic morphology with clonogenic and proliferative potential have been identified in human peripheral blood (He et al., 2007) and adult animals such as guinea pig (Kuznetsov et al., 2001) , rabbit (Wan et al., 2006) , dog (Huss et al., 2000) , mice (Kuznetsov et al., 2001) and rat (Wu et al., 2003) . Cultured ex vivo, these populations tend to adhere to plastic and form typical colonies with fibroblastic morphology (CFU-F). PB CFU-F (Peripheral Blood Colony Forming Unit - Fibroblast) (Maximow, 1982) or PBMSC (Peripheral Blood-Derived Multipotent Mesenchymal Stromal Cells) (He et al., 2007), express the typical hematopoietic lineage markers (CD14, CD45), endothelial cell line (CD117/c-Kit) and histocompatibility complex (HLA-DR). Within the population of PB CFU-F were also identified CD133 positive cells (Tondreau et al., 2005), a marker typically expressed by cells with potential to form hematopoietic, endothelial, or mesoangioblastic cells (Loges et al., 2004). In vitro and in vivo studies have demonstrated the differentiation potential of PB CFU-F in osteogenic, adipogenic and chondrogenic lineages (Kuznetsov et al., 2001; Wu et al., 2003; Tondreau et al., 2005). In response to tissue damage or stress conditions, it has been shown that PBMSC cells undergo a significant increase in number by a yet unknown mechanism. Some authors suggest that these cells are, in normal conditions, accumulated at the level of the bone marrow (Kucia et al., 2005) or in locations of peripheral blood vessels (perivascular stem cell niche) (Figure 4) (Songtao Shi and Stan Gronthos , 2003). In particular physiological conditions or in response to tissue damage, they get activated, migrating into the blood stream and stimulate the process of regeneration of the injured tissue. Some scholars speculate that the PB CFU-F cells are released into the circulation from the bone marrow and bone marrow stem cells that are derived from pluripotent primitive type or tissue-specific precursors, which accumulate in the bone during ontogeny (Figure 4).

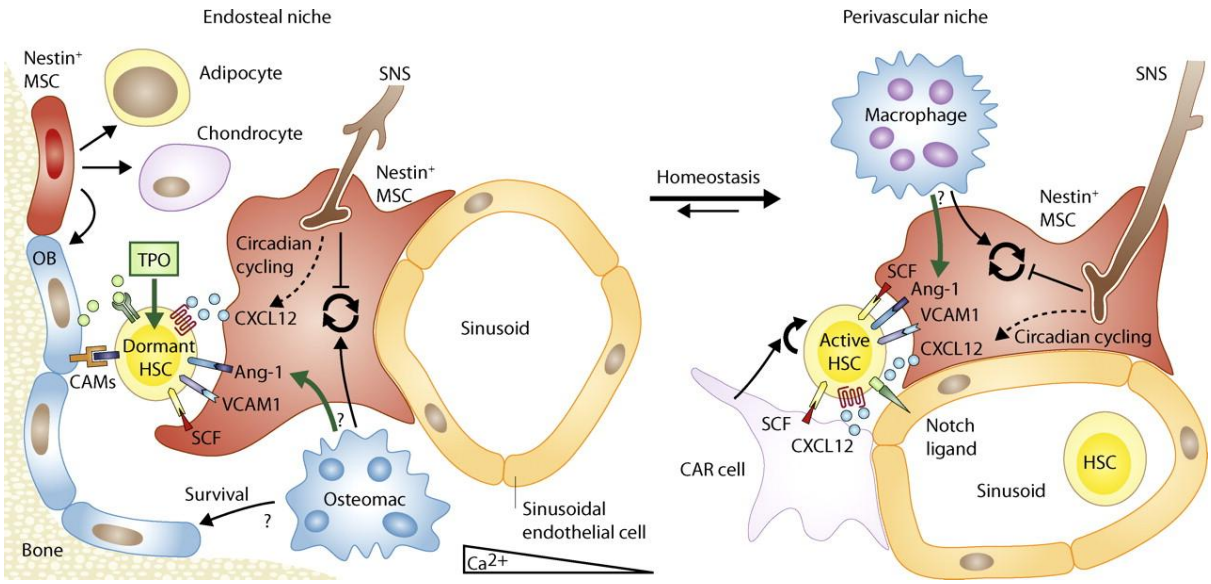


Figure 4. Stem Cell Niches

Kucia and colleagues (2005) suggest that during ontogeny and the rapid body growth and/or expansion of tissue-specific stem cells (e.g. for muscle, neurons, liver, heart, pancreas, endocrine, renal tubular epithelium). Pluripotent Stem cells (PSCs) and hematopoietic stem cells circulate at high levels in peripheral blood and gradually accumulate in the bone marrow, where they find the ideal microenvironment for their survival (Ratajczak et al., 2004). These cells, similar to the HSC express CXCR4 and respond to a gradient of SDF-1 (Ratajczak et al., 2003). Kucia and co-workers have also observed that the number of stem cells non-hematopoietic origin in peripheral blood can be increased by the administration of agents similar to those used for the mobilization of HSC (e.g. G-CSF) (Ratajczak et al. , 2004) or stimulated by stress factors associated with tissue damage (e.g. stroke) (Kucia et al., 2005). An increase of these cells in peripheral blood following damage supports the hypothesis that these populations may play an important role in tissue regeneration. Thus, it is likely that the processes of regeneration involves, not only the local recruitment of progenitors present in the region, but also the stem cells specific to the damaged organ, which reside in the bone marrow or perivascular niche.

3. Therapeutic significance of stem cell lineage shift

There has been considerable debate about the trans-differentiation of adult stem cells into other tissue types. If stem cells can repopulate a variety of tissue types (that is, be multipotential), or if tissue repair can be induced by the mobilisation or delivery of harvested stem cells, the clinical options for repairing damaged tissues are increased

(Trounson, 2004). Observations made in the last few years support the existence of pathways, in adult humans and rodents, which allow adult stem cells to be surprisingly flexible in their differentiation repertoires. Termed plasticity, this property allows adult stem cells, assumed, until now, to be committed to generating a fixed range of progeny, to switch, when they have been relocated, to make other specialized sets of cells appropriate to their new niche (Poulsom et al., 2002). The potential use of stem cells to replace functional tissue loss in degenerative disorders may depend on their capacity to derive tissue-specific cells without any detrimental in vivo side effects. By manipulating the culture conditions in which stem cells differentiate, it has been possible to control and restrict the differentiation pathways and thereby generate cultures enriched in lineage-specific cells in vitro (Trounson, 2004). Stem cell plasticity may occur through engraftment in another organ and assumption of some or all of the phenotypic traits of that organ – trans-differentiation, the acquisition of a new phenotype – or the engrafted cell could become a local stem cell in its new niche. The latter mechanism would ideally require the isolation and transplantation of single cells that self-renew and produce a family of descendants that eventually become fully functional. Some commentators have claimed that this phenomenon should be shown to occur 'naturally' in organs not forced to undergo degeneration before accepting that stem cells jump a lineage boundary (Anderson et al., 2001). Clearly, it is difficult to track cells without intervention and most of the studies to date involve damage consequent upon ablation of bone marrow by irradiation or chemical means, or the traumas of surgery and rejection, where organs have been transplanted and then studied some time later. A counter-argument is that a degree of organ damage is essential to allow trans-differentiation or stem cell plasticity to take place at recognizable levels. It may be that migration of bone marrow stem cells throughout the body acts essentially as a back-up system, able in extremis to augment an organ's intrinsic regenerative capacity (Poulsom et al., 2002).

4. Stem cell therapy for the treatment of neurological diseases

Human neurological disorders such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, multiple sclerosis (MS), stroke, and spinal cord injury are caused by a loss of neurons and glial cells in the brain or spinal cord. Cell replacement therapy and gene transfer to the diseased or injured brain have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases. However, the paucity of

suitable cell types for cell replacement therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach. In recent years, neurons and glial cells have successfully been generated from stem cells such as embryonic stem cells, mesenchymal stem cells, and neural stem cells, and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out (Kim SU and de Vellis J, 2009).

4.1 Parkinson's disease

Parkinson's disease (PD) is characterized by an extensive loss of dopamine neurons (DA) in the substantia nigra pars compacta and their terminals in the striatum (Kish et al., 1988; Agid, 1991). In the last twenty years, advanced regenerative medicine strategies such as stem cell therapy, have been investigated as a possible cure (Figure 5).

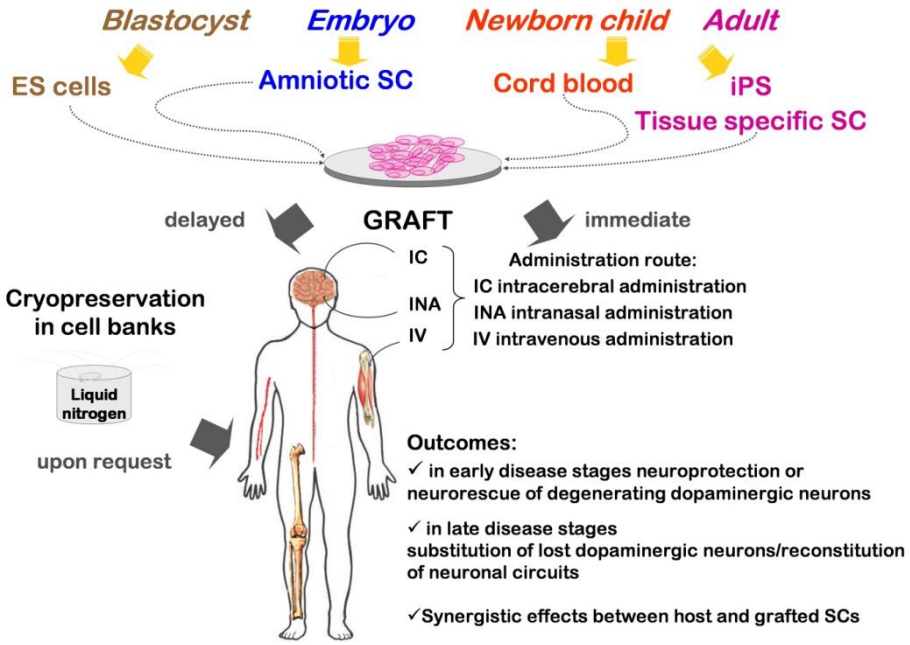


Figure 5. Possible stem cell sources for the treatment of Parkinson disease

Since the late 1980s, transplantation of human fetal ventral mesencephalic tissues into the striatum of PD patients has been adopted as a successful therapy for patients with advanced disease (Lindvall et al., 1990; Olanow et al., 1996; Kordower et al., 1997a; Dunnett and Bjorklund, 1999).

DA neurons were generated from mouse ESCs or mouse NSCs after treatment with fibroblast growth factor 8 (FGF8) and sonic hedgehog (Lee et al., 2000; Hagell and Brundin, 2002; JH Kim et al., 2002; TE Kim et al., 2003), overexpression of Nurr1

(Wagner et al., 1999; Chung et al., 2002; Kim et al., 2003), Bcl-xL (Shim et al., 2004), or co-culture with a mouse bone marrow stromal cell line (Kawasaki et al., 2000). Neurons with a DA phenotype have been generated from monkey ESCs by co-culturing with mouse bone marrow stromal cells (Takagi et al., 2005) and also from human NSCs derived from fetal brain (Redmond et al., 2007), and behavioral improvement was seen in MPTP lesioned monkeys following intrastriatal transplantation of these cells (Takagi et al., 2005; Redmond et al., 2007). DA neurons were also generated from fetal murine mesencephalic progenitor cells and induced functional recovery following brain transplantation in parkinsonian rats (Studer et al., 1998).

4.2 Alzheimer's disease

AD is characterized by degeneration and loss of neurons and synapses throughout the brain, particularly in the basal forebrain, amygdala, hippocampus, and cortical area. Recent studies have shown that intracerebral injection of a lentivirus vector expressing human neprilysin in transgenic mouse models of amyloidosis reduced Ab deposits in the brain and blocked neurodegeneration in the fronal cortex and hippocampus (Marr et al., 2003), and intracerebrally injected fibroblasts overexpressing the human neprilysin gene were found to significantly reduce the amyloid plaque burden in the brain of Ab transgenic mice with advanced plaque deposits (Hemming et al., 2007). These studies support the use of Ab-degrading proteases as a tool to therapeutically lower Ab levels and encourage further investigation of ex vivo delivery of protease genes using human NSCs for the treatment of AD.

4.3 Multiple sclerosis

In Multiple sclerosis, oligodendrocytes (OLs) and myelin are destroyed by an inflammation-mediated mechanism (McFarlin and McFarland; 1982; Ebers, 1988). Previous studies have reported that OLs or OL progenitor cells isolated from mouse or rat brain that were transplanted into the brain of dysmyelination mutants or chemically induced demyelination lesions in rats induced remyeliation in previously dysmyelinated or demyelinated lesion sites (Franklin and Blakemore, 1997; Espinosa de los Monteros et al., 1997; 2001; Learish et al., 1999; Zhang et al., 1999; Ben-Hur et al., 2003). Recent studies have reported that OLs could be generated from mouse and human ES cells (Brustle et al., 1999; Liu et al., 2000; Glaser et al., 2005), bone marrow mesenchymal stem cells (Akiyama et al., 2002), or mouse NSCs (Yandava et al., 1999). OLs could also

be generated from stable, established cell lines of human NSCs and used as cell source of transplantation.

4.4 Stroke

There are two major types of stroke: ischemia (infarct) and intracerebral hemorrhage (ICH). Ischemic stroke caused by abrupt and near-total interruption of cerebral blood flow produces ischemic changes in the striatum and cortex, leading to a long-term sensorimotor deficit. Once damage from a stroke occurs, little can be done to restore premorbid functions, and, although numerous neuroprotective agents have been clinically tried, no specific agents replaced the lost neurons, improved the deteriorated functions, and reduced the long-term sequelae (Marshall and Thomas, 1988). There have previously been several reports of cell transplantation in the brain in ischemia animal models (Savitz et al., 2002), and various cellular sources such as rodent bone marrow mesenchymal stem cells (Sinden et al., 1997; Chen et al., 2001; Chen et al., 2003; Zhao et al., 2002; Modo et al., 2002), human umbilical cord blood cells (Chen et al., 2001), immortalized mouse neural precursor cells (Veizovic et al., 2001), and human teratocarcinoma NT2-derived neurons (Borlongan et al., 1998; Saporta et al., 1999) were grafted into the ischemic brain, reducing the neurological deficits induced by experimental brain ischemia. A recent study has reported that, in humans with ischemic infarct, intracerebral implantation of human teratocarcinoma NT2-derived neurons has resulted in functional improvement (Kondziolka et al., 2000).

4.5 Spinal cord injury

Recent advances in stem cell biology have opened up an avenue to therapeutic strategies to replace lost neural cells by transplantation of stem cells in various disorders in the CNS. For spinal cord injury (SCI), various cell types such as genetically modified fibroblasts, olfactory ensheathing cells, Schwann cells, and stem cells have been used to promote axonal regeneration (Tuszynski et al., 1994; Xu et al., 1995; Li et al., 1997; Liu et al., 1999; Teng et al., 2002). Since an earlier study showing that transplantation of ESCs promotes functional recovery (McDonald et al., 1999), several studies have reported that various stem or progenitor cells types, including ESCs, bone marrow MSCs, neural stem cells, and glia restricted precursor cells, induce functional improvement following transplantation into the injured spinal cord (Teng et al., 2002; McDonald et al., 1999; Hofstetter et al., 2002; Ogawa et al., 2002; Cao et al., 2005; Cummings et al., 2005;

Keirstead et al., 2005; Iwanami et al., 2005; Karimi-Abdolrezaee et al., 2006; Xu et al., 2006). However, there are still many obstacles to be overcome before stem cell-based therapy can be adopted for SCI. One such problem is a massive death of stem cells transplanted into the injured spinal cord tissue. In the case of bone marrow MSCs, they barely differentiate into neurons or the glial lineage following transplantation, calling into question the therapeutic potential of bone marrow derived MSCs (Hofstetter et al., 2002).

5. Stem cell therapy for the treatment of muscular dystrophies

Muscular dystrophy comprises a group of genetic diseases that cause progressive weakness and degeneration of skeletal muscle resulting from defective proteins critical to muscle structure and function (Figure 6). This leads to premature exhaustion of the muscle stem cell pool that maintains muscle integrity during normal use and exercise.

Muscle wasting diseases affect millions of people worldwide. Among these, the various types of muscular dystrophy (MD) caused by mutations in structural proteins are characterized by loss of functional muscle due to muscle fiber damage, inflammation, and deposition of fibrotic tissue (Emery et al., 2002). With Duchenne muscular dystrophy (DMD) in particular, muscle tissue begins to deteriorate early in childhood, pushing the resident muscle stem cell pool to its limit, leading to the exhaustion of normal muscle repair mechanisms (Matsumura et al., 1994; Decary et al., 2000).

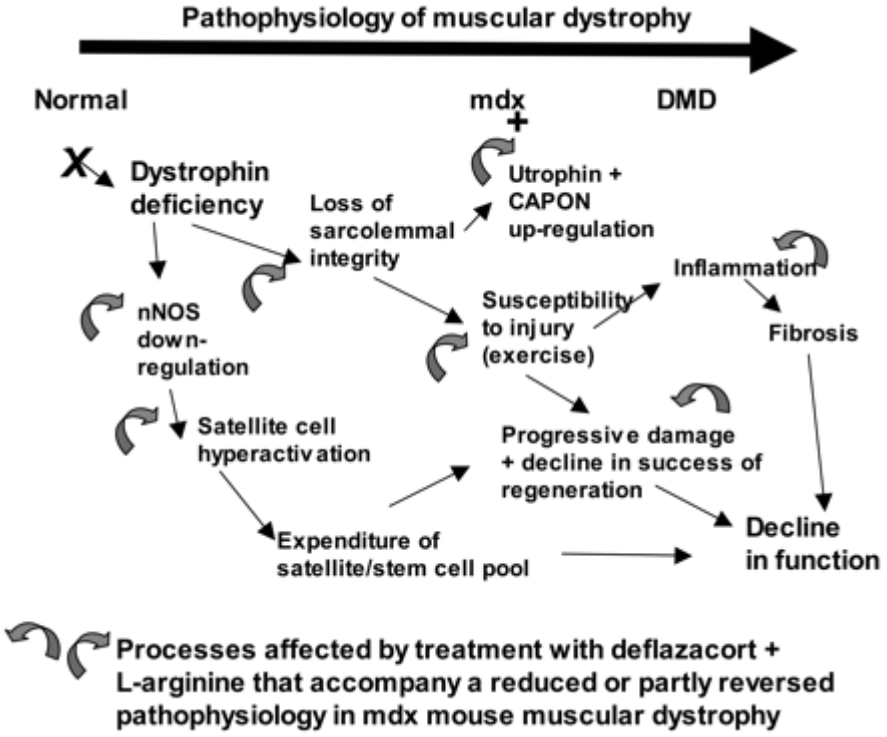


Figure 6. Mechanism of muscular dystrophy

DMD is caused by mutations in the *dystrophin* gene (Hoffman et al., 1987), which encodes a cytoskeletal protein found in skeletal muscle, smooth muscle, cardiac myofibers, and brain (Durbeej et al., 2002). Dystrophin deficiency primarily leads to the pathologic perturbation of myofibers; however, the disease also is associated with absence of several glycoproteins that interact with dystrophin. Although the precise sequence of the events is incompletely understood, the mechanical weakness leading to sarcolemmal lesions causes abnormal calcium influx and inflammation, which in turn alter the composition of structural glycoproteins in the extracellular matrix (ECM). This disruption of the ECM causes resident muscle stem cells to undergo fibrogenesis, rather than myogenesis, leading to abnormal collagen deposition and subsequent necrosis; multiple cycles of fibrosis and necrosis result in exhaustion of the stem cell pool (Heslop et al., 2000; Grounds et al., 2008). Progressive telomere shortening also has been associated with exhaustion of the muscle stem cell pool. Shorter telomeres have been reported in muscle cells from DMD patients compared with those of healthy individuals (Mouly et al., 2005; Sacco et al., 2010). Interestingly, human telomeres are shorter then mouse telomeres, which may explain why the X-linked muscular dystrophy (mdx) mouse model of DMD exhibits a less severe degenerative phenotype compared with the human disease. To test this, Sacco et al. (2010) engineered the *mdx/mTR* mouse strain, which lacks the RNA component of telomerase as well as dystrophin, and showed that

muscle wasting and a decline in muscle stem cells parallels human DMD when telomerase function is disturbed in the mouse.

5.1 Role of resident myogenic progenitors

The limitations encountered with myoblast transplantation have led many groups to pursue the identification of other populations of stem-like cells with myogenic properties for potential therapeutic application (Figure 7). These various progenitor cells differ in anatomical location, self-renewal, and differentiation potential, as well as cell surface marker expression (Zheng et al., 2007).

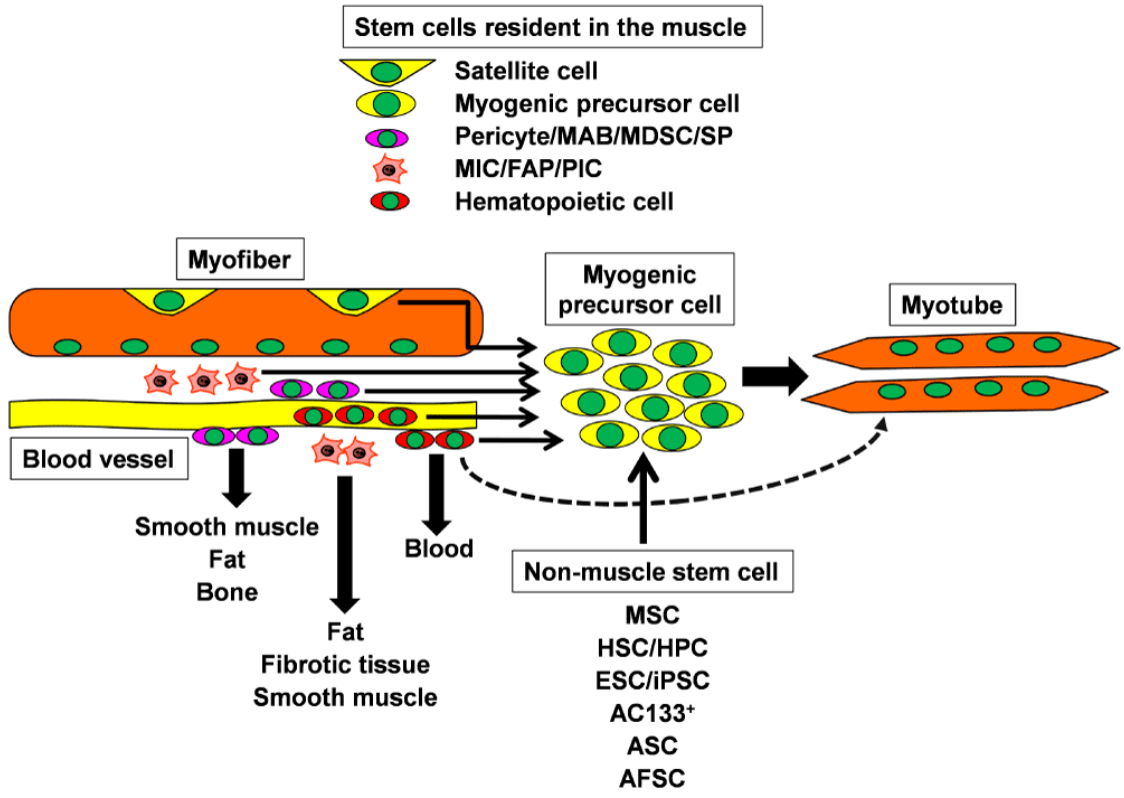


Figure 7. Stem Cells resident in the muscle

Whether these are derived from muscle resident satellite cells or other remnants of primary myogenesis is unclear. Some progenitor cells are associated with the circulatory system, suggesting that they may have the advantage of easy access to the vascular network throughout the muscle tissue, whereas others are in closer contact with myofibers. The phenotypes of these various resident stem cells have suggested specific modes of delivery to which they might be best suited. A subset of satellite cells in adult muscle that co-express markers associated with the vascular system have been

identified as myoendothelial cells (Zheng et al., 2007; Crisan et al., 2008). These are capable of long-term expansion in vitro and appear to support muscle regeneration at rates superior to myoblasts (Tamaki et al., 2002). Myoendothelial cells represent 0.4% of resident muscle stem cells and, on the basis of cell surface marker expression, share myogenic as well as endothelial features. The myoendothelial cell population ($CD56^+$ $CD34^+$ $CD144^+$) can be purified from myogenic ($CD56^+$ $CD34^-$ $CD144^+$) and endothelial ($CD56^-$ $CD34^+$ $CD144^+$) cell populations on the basis of surface marker expression (Zheng et al., 2007). Other blood vessel-associated cells called pericytes, located beneath the basal lamina of small vessels (Crisan et al., 2008; Dellavalle et al., 2007), lack endothelial markers but express NG2 proteoglycan, platelet-derived growth factor receptor (PDGFR), and CD146. They can be derived by outgrowth from tissue explants and purified by sorting for alkaline phosphatase expression in the absence of CD56 expression. Although pericytes lack expression of myogenic markers (Pax7, Myf5, MyoD), they differentiate into multinucleated myotubes when exposed to myogenic differentiation medium. Pericytes injected intra-arterially into immunodeficient mdx mice after in vitro expansion have led to formation of large numbers of new dystrophin-expressing muscle fibers (Dellavalle et al., 2007).

5.2 Directed differentiation of stem cells for muscle regeneration

Several groups have reported the derivation of myogenic progenitor cells from human ESCs (hESCs). The exposure of hESC-derived embryoid bodies to serum in the presence of epithelial growth factor directed their differentiation toward myogenic precursors (Zheng et al., 2006). Darabi et al. (2008) used inducible Pax7- and Pax3-overexpressing mouse ESC (mESC) lines to direct myogenic commitment. Another approach taken to direct the differentiation of mESCs toward paraxial mesoderm has been the selection of $PDGFR^+$ $VEGFR2^-$ cell populations from cultured mESC monolayers. VEGFR2 expression was excluded from paraxial mesoderm, whereas $PDGFR^+$ $VEGFR2^+$ cells were committed to lateral mesoderm (Sakurai et al., 2008). Barberi et al. (2007) enriched for a myogenic progenitor cell fraction from hESC-derived mesenchymal precursors. Transplantation of the $CD73^+$ Neural Cell Adhesion Molecule $(NCAM)^+$ cells to hind limb muscle of immunodeficient mice resulted in long-term survival and myofiber commitment. Other groups have taken advantage of known molecular cues to guide the differentiation of myogenic cells from hESCs. Activation of the transforming growth factor (TGF)/activin/nodal pathway through activin A and serum enhances endodermal

specification of hESCs (D'Amour et al., 2005). Mahmood et al. (2010) blocked the TGF pathway with SB431542 to obtain hESC-derived mesenchymal progenitors.

AIM OF THE WORK

The most exciting of all applications of stem cells could be their use in cell replacement therapies and regenerative medicine. The chronic shortage of organ transplants in conjunction with the limitation of artificial implants (prostheses) has intensified research in cell and tissue based therapies. The key advantage of cell and tissue therapy over pharmacological therapies to treating debilitating diseases and abnormalities is that the former offers "living biological replacements" while the latter merely provides a palliative solution. However, before stem cell based therapies could be transferred from the "bench to the bedside", many fundamental biological and engineering challenges need to be overcome that include: controlling the self renewal of stem cells, directing the lineage/tissue-specific stem cell differentiation, *in vivo* delivery, and integration to the host milieu. Adult multipotent stem cells from peripheral blood present a viable option for easy manipulation to control self renewal and to direct towards a tissue specific differentiation. In this thesis, human peripheral blood multipotent cells (hPBMCs), which are isolated by standard protocol and characterized for an unique immunophenotype, tested for stability in long term culture and plasticity towards mesenchymal lineages in agreement with the guidelines defined by the International Society for Cellular Therapy (Dominici et al., 2006), have been tested for lineage switching capacity based on:

- Analysis on directed differentiation of hPBMCs towards neuronal lineages by flow cytometry, RT-PCR, western blot and Immunofluorescence.
- Evaluation of hPBMCs for their ability to acquire a functional neuronal phenotype by HPLC and spectrofluorimetry.
- Validation of myogenic lineage shift responses of hPBMCs by flow cytometry, RT-PCR, western blot and immunofluorescence.
- Suitability of hPBMCs to integrate in a foreign milieu by in vivo responses in an induced muscle damage model.

MATERIALS AND METHODS

1. Characterization of human Peripheral Blood Multipotent Cells (hPBMCs)

Before testing shift lineage responses of multipotent cells from peripheral blood for their potential use in degenerative neural and muscle disorder treatment, a detailed characterization of cell population of interest was performed to assess their distinctive stem properties.

Multipotent cells were isolated from peripheral blood using Ficoll-Hystopaque (Sigma-Aldrich) for density gradient separation, and adherent cells were grown in proliferative culture medium prepared with Alpha-Modified Eagle Medium (α-MEM) Without Nucleosides (Life Technologies), 16.5% Fetal Bovine Serum (FBS) (Life Technologies), 1% glutamax (Life Technologies), 1% antibiotic solution of penicillin (100mg/ml) and streptomycin (100mg/ml) (Sigma-Aldrich). After isolation and stabilization of cell culture, all characterization studies were carried out on three different hPBMC populations, each analysed at three different generations (VII, XV, XXXI). First of all, morphologic analysis was performed, using optical and scanning electron microscopy and cell proliferation rate was defined by investigating doubling time and population doubling level. After that, hPBMC immunophenotype was identified by flow cytometry, labeling cells with monoclonal antibodies against CD14, CD45, CD34, CD44, CD13, NG2, CD73, CD90, CD105, CXCR4 and HLA-DR. Cell karyotype stability was assessed by cytogenetic analysis, while Pluripotency marker (NANOG, OCT4, SOX2, REX1, klf4, c-Myc, NOTCH, STAT3) gene expression was quantified by Real-Time PCR. hPBMCs were finally tested for their capacity to differentiate towards mesenchymal lineage, by treating them with specific adipogenic and osteogenic factors.

2. Lineage shift responses of hPBMCs

2.1 Neuronal shift

For the study of neurogenic differentiation, subcultures of hPBMCs (generations VIII-XV-XXXI) were seeded on 6-well multiwell plates (BD Falcon) at a density of 10^4 cells/cm^2. The cells were grown in specific inductive medium composed of Neurobasal Medium (Life technologies), 2% $B27^-$ (Life technologies), Epidermal Growth Factor (20 ng/ml) (EGF) (Sigma-Aldrich), basic Fibroblast Growth Factor (10 ng/ml) (bFGF) (Sigma-

Aldrich), 1% glutamax, 1% penicillin and streptomycin solution. To optimize the treatment, inductive cultures were set up on surfaces conditioned with gelatin (Sigma-Aldrich), prepared in MilliQ water at 2%.

After coating obtained by incubation of 2 h at 37°C, the plates were washed with PBS and subsequently used for cell seeding. The change of medium was performed after 3 days at which 50% of the total volume was replaced and on the seventh day, the culture medium was completely replaced with specific inductive medium composed of Neurobasal medium, 2% B27$^-$, 0.5 mM retinoic acid (RA) (Sigma-Aldrich), 20 ng/ml Nerve Growth Factor (NGF) (Sigma-Aldrich), 1% glutamax, 1% penicillin/streptomycin. The culture was maintained for another 7 days. Control samples were prepared by growing the cells in standard hPBMCs medium composed of Neurobasal Medium, 2% B27$^-$, 1% glutamax, 1% penicillin/streptomycin.

2.2 Myogenic shift

For the study of myogenic differentiation, subcultures of hPBMCs (7th generation) were seeded in 6-well plates at a density of 10^4 cells/cm^2. The hPBMCs were grown in standard medium composed of α-MEM, 16.5% FBS, 1% glutamax, 1% penicillin/streptomycin. On reaching a confluence of 90%, myogenic differentiation was performed using a myogenic induction medium, composed of proliferation medium supplemented with 100 ng/ml IGF and 200 µM Vitamin C. The medium was changed every 3 days. As a control, cells were cultured in proliferation medium.

2.3 Assessment of neurogenic and myogenic shift responses

hPBMCs were assessed at time intervals of 7 and 14 days for neurogenic differentiation response and 3, 7 and 14 days, in case of myogenic differentiation, by optical and scanning electron microscopy, flow cytometry, RT-PCR, immunofluorescence and Western blotting.

3. Morphologic analysis by optical microscopy

Cell cultures treated with neurogenic and myogenic factors were daily analysed by a DM/IL optical microscope and, at 7 and 14 days of treatment, images were captured by a Nikon Digital Sight DS-SMC camera (Nikon Corporation).

4. Scanning Electron Microscopy (SEM) study

A better morphologic analysis was performed by SEM (Scanning Electron Microscopy): differentiated hPBMC cultures were seeded on sterile glass slides (Falcon) and grown in proliferation medium. After 24 hours, samples were fixed with 0.1 M cacodylate buffer solution, pH 7.2 (Sigma) in 3% glutaraldehyde (Sigma) and stored at 4°C until the time of dehydration, which was performed by immersion in increasing concentrated alcohols (70%, 90%, 95%) (5 min/alcool). Cells were then kept in absolute alcohol until analysis, and subsequently subjected to Critical Point Drying and metallized with gold. The images were acquired using Jeol JSM 6490 scanning electron microscope of CUGAS, Interdepartmental Service Centre at University of Padua.

5. Flow cytometry evaluation

The identification of specific neural and muscle membrane markers on differentiated hPBMC populations was performed by flow cytometry.

5.1 Flow cytometry

Flow cytometry (FCM) is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in both research and clinical practice.

A flow cytometer is made up of three main systems: fluidics, optics, and electronics (Figure 8):

- ✓ the fluidics system transports particles in a stream to the laser beam for interrogation;
- ✓ the optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors;
- ✓ the electronics system converts the detected light signals into electronic signals that can be processed by the computer.

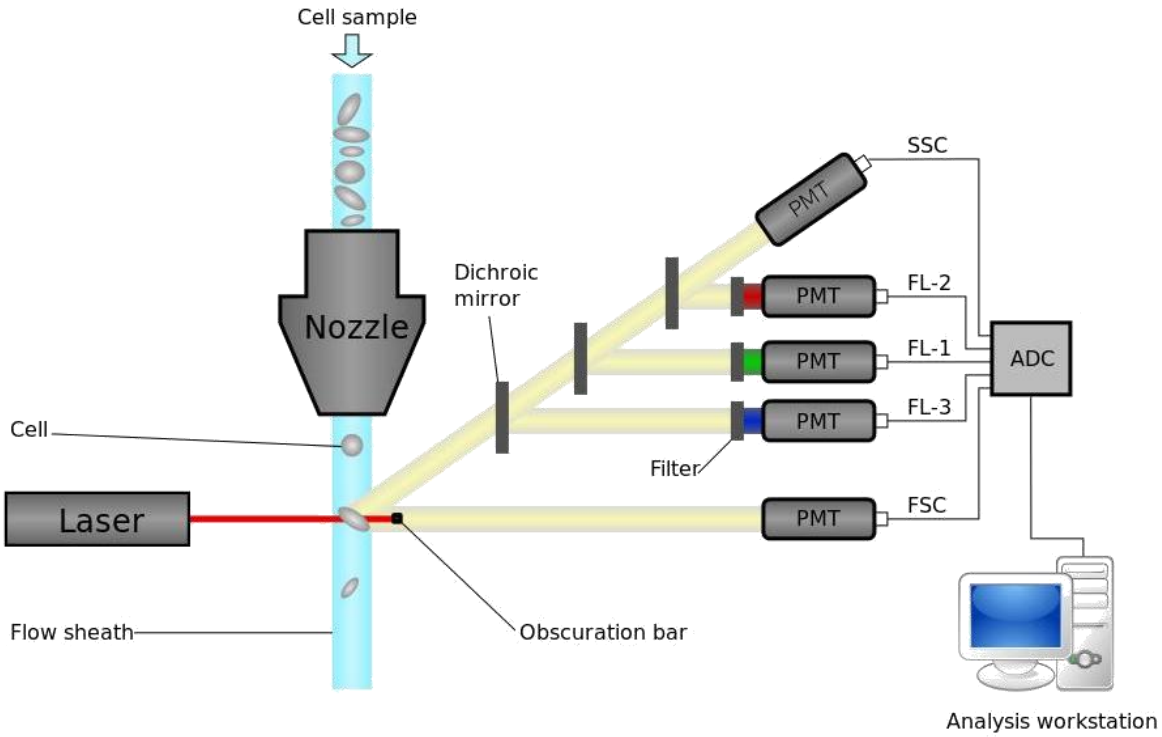


Figura 8. Flow cytometer components

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis. The portion of the fluid stream where particles are located is called the sample core. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them (Figure 9).

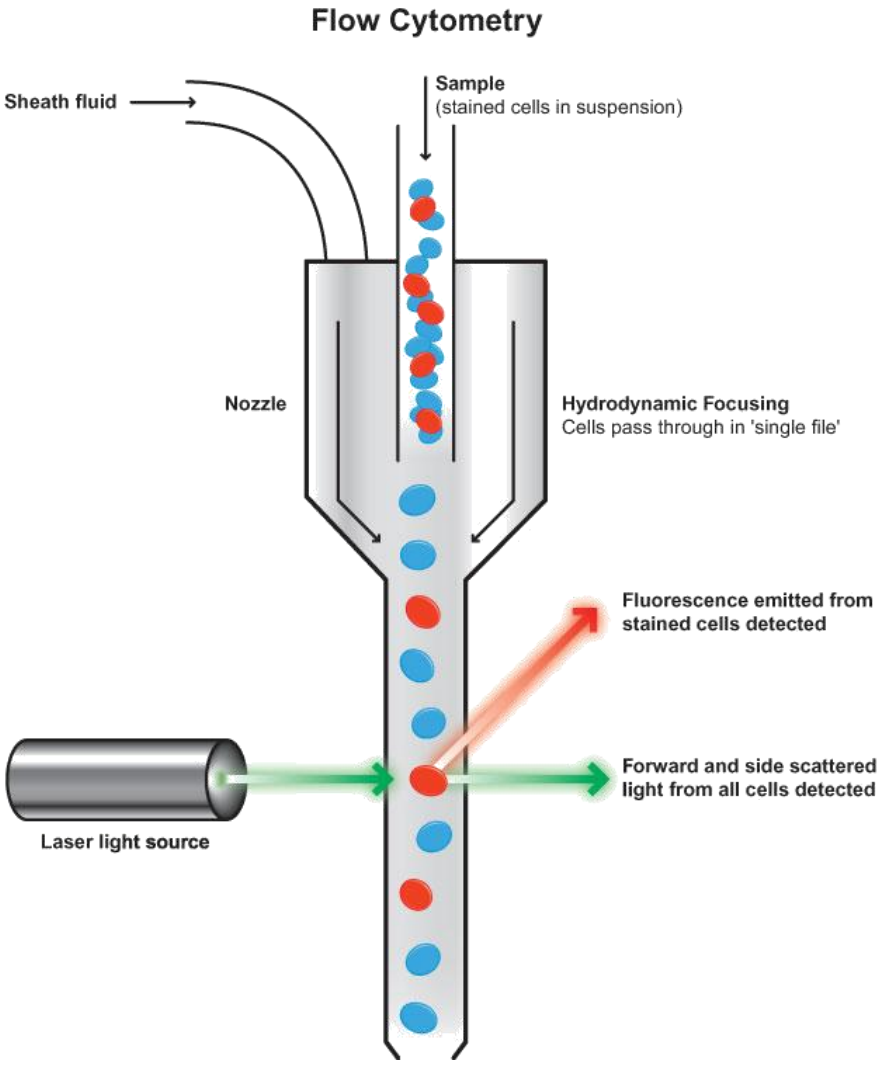


Figure 9. Cell analysis on the flow cytometer

In FCM, the identification of specific cell markers is performed by using fluorochrome-conjugated antibodies. Fluorochromes are fluorescent compounds which absorb light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence. The range over which a fluorescent compound can be excited is termed its absorption spectrum, while the range of emitted wavelengths for a particular compound is termed its emission spectrum. The absorption and emission peaks are 15-40 nm shifted (Stokes shift) (Figure 10).

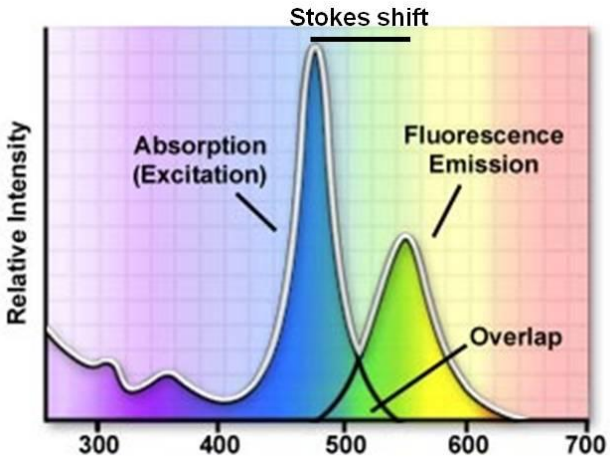


Figure 10. Typical fluorochrome absorption-emission spectral diagram

The argon ion laser is commonly used in flow cytometry because the 488-nm light that it emits excites more than one fluorochrome. One of these fluorochromes is fluorescein isothiocyanate (FITC). In the absorption spectrum of FITC, the 488-nm line is close to the FITC absorption maximum. Excitation with this wavelength will result in a high FITC emission. More than one fluorochrome can be used simultaneously if each is excited at 488 nm and if the peak emission wavelengths are not extremely close to each other. The combination of FITC and phycoerythrin (PE) satisfies these criteria. The excitation and emission spectra of each of these fluorochromes is shown in Figure 11.

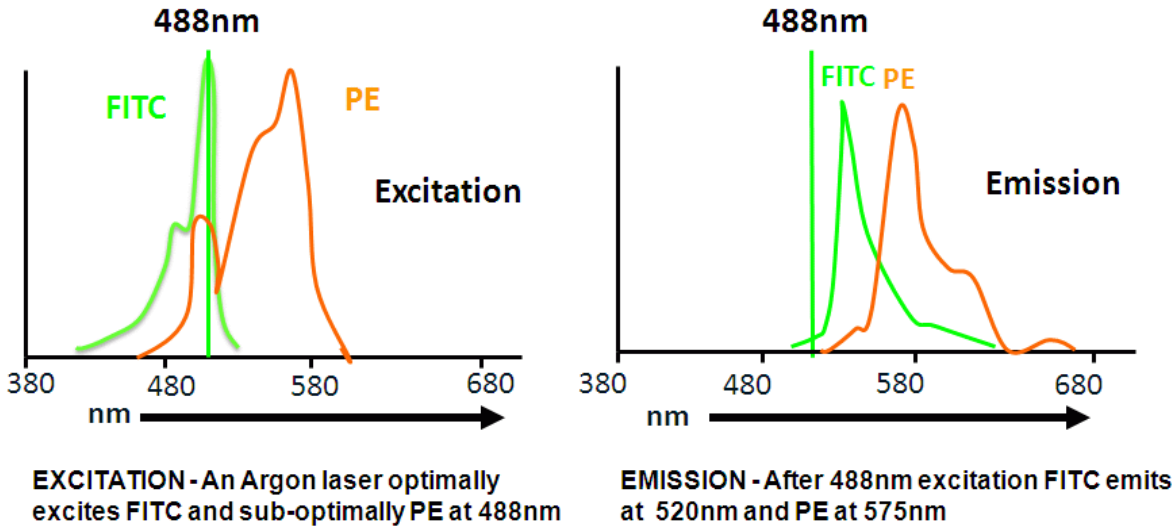


Figure 11. FITC and PE emission spectra

5.2 Immunophenotype shift of differentiated hPBMCs

For flow cytometry analysis, differentiated hPBMC subcultures were detached from the culture dish by EDTA/trypsin treatment, centrifuged at 1200 rpm for 5 min and finally resuspended in PBS, 0.2% bovine serum albumin (BSA) (Sigma-Aldrich) (PBS-BSA) . Each sample was prepared by treating, at room temperature (RT) in the dark for 15 min, $2x10^5$ cells/100µl PBS-BSA with 5 µl of the following primary monoclonal antibodies: mouse anti-human Tyrosine Hydroxylase (TH), mouse anti-human Microtubule Associated Protein 2 (MAP2), mouse anti-human βIII Tubulin (TBB3) (Millipore), mouse anti-human Tropomyosin (TPM), mouse anti-human, mouse anti-human CD73, mouse anti-human CD105, mouse anti-human Myogenic factor 5 (Myf5), mouse anti-human Myoblast determination protein 1 (MYOD) (Santa Cruz). After incubation with the unconjugated primary antibody, samples were rinsed in PBS-BSA and treated at room temperature (RT), in the dark for 15 min with the secondary antibody FITC- or PE-conjugated. In parallel, indirect labeling controls have been set up as cells labeled only with the secondary antibody. At the end of the procedure, all samples were rinsed with PBS-BSA and centrifuged at 1200 rpm for 5 min. Analysis was performed on FACS Canto II (Becton Dickinson) resuspending samples in 200 µl of FacsFlow buffer. Data relative to 10^4 total cells were acquired using FacsDIVA software, in the wavelength range from 530 ± 40 nm (FITC) and 580 ± 30 nm (PE). Results have been expressed as percentage of positive cells compared to the control sample and were obtained by applying the statistical function Substraction of Summit 4.3 software.

6. RT-PCR

RT-PCR technique allows to identify the presence of a specific messenger RNA (mRNA) by reverse transcription of the same and the amplification of the cDNA complementary to it.

a) Extraction of total RNA

The procedure of extraction of mRNA was done using Trizol® Reagent (Sigma-Aldrich), a monophasic solution of phenol and guanidine isothiocyanate. Induced cells were detached, rinsed in PBS, fixed in RNA later R0901 (Sigma-Aldrich), kept at 4°C for one night and then frozen at -80°C until extraction of RNA. At the time of extraction, each sample was treated first with 1 ml of Trizol for 5min, at RT and then with 200 µl of chloroform. To promote greater separation of RNA, the samples were shaken by hand

for 15 sec and then incubated at RT for 3 min. After centrifugation at 12000 rpm for 15min at 4°C, the samples were resolved as a lower phase containing proteins, white interphase containing DNA and finally an upper aqueous and colorless phase, containing RNA. After collection of the aqueous phase, the precipitation of RNA was performed by adding 500 µl of isopropanol (Fluka) (0.5 ml per 1 ml of Trizol used) and mechanically shaking the sample. After incubation for 10 min at RT, the sample was centrifuged at 12000 rpm for 10 min at 4°C. After removal of the supernatant, the pellet was washed with 1 ml of cold 75% ethanol and then centrifuged at 8600 rpm for 5 min, at 4°C. The supernatant was removed and the pellet was allowed to air dry for 5-10min and then resuspended in 10 µl of RNase-free water (Life technologies). After quantification by spectrophotometric analysis, RNA samples were stored at -80°C.

b) Spectrophotometric quantitation of extracted RNA

The quantification of RNA was performed by the spectrophotometer NanoDrop 2000 (Thermo Scientific) measuring absorbance of 1 µl of sample at the wavelength of 260 nm. In parallel, purity of the sample was evaluated by identification of absorbance at 280 and 230 nm, corresponding respectively to absorbance wavelength of proteins and carbohydrates. RNA samples, whose 260/280 ratio were in the range of 1.8 - 2.0, were further used for gene expression studies.

c) One Step RT -PCR

The study was conducted using the Qiagen® One Step RT-PCR Kit, which allows to run in a single tube, the reverse transcription of RNA into cDNA and then its amplification, using a mixture of enzymes specially formulated containing a) Sensiscript and Omniscript Reverse Transcriptases (reverse transcription)and b) HotStarTaq DNA Polymerase (amplification). Specific action and efficiency of the system is dependent on temperature. During reverse transcription, the HotStarTaq DNA Polymerase is completely inactive. Instead, during amplification, simultaneously with the deactivation of the reverse transcriptase, DNA polymerase is activated at a temperature of 95°C. The reaction mixture (25 µl) was prepared in ice using 1 µl of RNA at a concentration of 30 ng/µl, 3 µl forward primer (5 µM), 3 µl reverse primer (5µM), 1 µl dNTP mix (10mM), 1 µl Qiagen® One Step RT-PCR Enzyme Mix, 1 µl RNase inhibitor (125 U), 5µl 5X buffer and RNAse-free water. One Step RT-PCR was performed with the thermal cycler iCycler iQ™ (Bio-Rad) and the conditions reported in Table 1.

Reaction Step	Time	Temperature
Reverse Transcription:	30 min	50°C
Initial PCR activation step:	15 min	95°C
3 step cycling		
Denaturation:	1 min	95°C
Annealing:	1 min	50-68°C
Extension:	1 min	72°C
Number of cycles	40	
Final extension:	10 min	72°C

Table 1. Thermal cycler conditions

The primer pairs used for RT-PCR analysis are shown in Table 2. In parallel, the expression of the housekeeping gene GAPDH was also evaluated.

Primers	Abbreviation	Sequence (5'-3')	Reference	Lenght
Microtubule-associated protein 2	MAP-2	F- GAGGTTGCCAGGAGGAAATCAGT	NM_002374.3	703 bp
		R- GCCCTGAAGCCATCTGTCCAAA		
Synaptophysin	SYP	F- TGTGAAGGTGCTGCAATGGGTC	NM_003179.2	337 bp
		R- GGGCCCTTTGTTATTCTCTCGGT		
Glutamate aspartate transporter	GLAST	F- ATCGCCTGCCTGATCTGTGGAAA	U01824.1	249 bp
		R- AACGAAAGGTGACAGGCAAAGT		
Neurofilament, medium polypeptide	NEFM	F- AATATGCACCAGGCCGAAGAGT	NM_005382.2	296 bp
		R- AAATGACGAGCCATTTCCCACT		
Neurogenin 1	NEUROG1	F- GCGCTTCGCCTACAACTACATCT	NM_006161.2	301 bp
		R- TGAAACAGGGCGTTGTGTGGAG		
Nestin	NES	F- GACACCTGTGCCAGCCTTTCTTA	NM_006617.1	469 bp
		R- TGCTGCAAGCTGCTTACCACTTT		
βIII tubulin	TBB3	F- CAACGAGGCCTCTTCTCACAAGT	NM_006086.3	325 bp
		R- TACTCCTCACGCACCTTGCTGAT		
Nerve Growth Factor	NGF	F- GCCCACTGGACTAAACTTCAGCA	NM_002506.2	356 bp
		R- GATGTCTGTGGCGGTGGTCTTA		
Brain-derived neurotrophic factor	BDNF	F- GCAAACATCCGAGGACAAGGTG	NM_170735.5	244 bp
		R- GCTCCAAAGGCACTTGACTACT		
Glial-derived neurotrophic factor	GDNF	F- GCGCTGAGCAGTGACTCAAATA	NM_000514.3	275 bp
		R- GTTTCATAGCCCAGACCCAAGT		

Neuronal Nuclei Antigen	NeuN	F- ACCAACGGCTGGAAGCTAAATC	NM_001082575.1	216 bp
		R- ATCCATCCTGATACACGACCGCT		
Myogenic factor 5	Myf5	F- ACCCTCAAGAGGTGTACCACGA	NM_005593.2	213 bp
		R- ACAGGACTGTTACATTCGGGCA		
Myoblast determination protein 1	MYOD1	F- GCCACAACGGACGACTTCTATGA	NM_002478.4	316 bp
		R- GGCCTCATTTACTTTGCTCAGGC		
Tropomyosin	TPM	F- AGCACATTGCTGAAGATGCCGAC	NM_001018005.1	244 bp
		R- AGCTTGTCGGAAAGGACCTTGA		
Skeletal α-actin	ACTA1	F- TCACGAGACCACCTACAACAGCA	NM_001100.3	263 bp
		R- CTCCTGCTTGGTGATCCACATCT		
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	F- GGTCGGAGTCAACGGATTTGGT	NM_002046.3	887 bp
		R- AAAGTGGTCGTTGAGGGCAATG		

Table 2. Primer pairs for RT-PCR

d) Agarose gel Electrophoresis

The electrophoretic analysis of PCR reaction products was performed by running samples on 2% agarose gel (Sigma-Aldrich) prepared in 1X TBE buffer (tris, 0.04mM Borate, 0.001M EDTA, pH 8) (Sigma-Aldrich). For loading, 6 µl of amplified product were mixed with 2 µl of loading dye (Sigma-Aldrich). As a reference marker to the molecular weights between 100 and 1000 bp, the PCR 100 bp Low Ladder (Sigma-Aldrich) was used. The bands of amplified samples were visualized by staining with Gel Red (0.1µl/ml) (Biotium) and exposure to UV light. Images were acquired with Gel Doc 2000 (Bio-Rad).

7. Real Time PCR (qPCR)

Real-Time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real-Time PCR is based on the detection of the fluorescence signal produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase (Lekanne Deprez et al., 2002). In this work, SYBR® Green was used as dye reporter for fluorescence signal generation. SYBR® Green I binds to the minor groove of the DNA double helix. In the solution, the unbound dye

exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double strand DNA. SYBR® Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. SYBR Green fluorescence upon binding double strand DNA is detected by the qPCR machine's detector so, as the amount of target sequence increases in the reaction mix, there will be a corresponding increase in the fluorescent signal. It allows to measure the number of cycles required for detection of a fluorescent signal (threshold cycle, Ct) and use it to quantify the level of expression of the target sequence, defined by comparison with that of a housekeeping gene (reference). In fact, the greater the number of starting copies of the target gene, the lower the amplification cycle in which a significant increase of the fluorescence will be observed. Since SYBR® Green technology does not allow to distinguish between specific and unspecific amplification products, it is important to perform melting point analysis after each qPCR run. The utility of this analysis derives from the observation that the temperature at which a DNA duplex will denature is dependent upon length and nucleotide composition. Fluorescence measurements are made while slowly increasing the temperature of the reaction products (in this case, from 55° to 95°C). At the low temperature, the amplicons are all double stranded and thus bind the SYBR Green dye, producing a strong fluorescence signal. As the temperature increases, the PCR products are denatured, resulting in a decrease in fluorescence. This fluorescence is measured continuously and when the Tm (melting temperature) of a particular target gene is reached, there will be a rapid decrease in the fluorescence over a short temperature range. This is detected by the instrument and plotted as the first negative differential of the fluorescence signal with respect to temperature. In case of specific amplification, the plot will appear as one peak centered on the specific Tm of the target gene.

7.1 RNA reverse transcription with random hexamers

The total RNA extracted from hPBMCs was reverse transcribed into cDNA with reverse transcriptase enzyme using as a primer a mixture of hexanucleotides. To performed this reaction, the kit "Thermoscript™ RT-PCR System" (Life technologies) was used. The reaction was carried out in a 0.2-ml tube by adding 1 µl of random hexamers (10 ng/µl), 2 µl of dNTP mix (10 mM), a volume of mRNA containing 1 µg of nucleic acid, and finally water RNase-free up to a total volume of 12 µl. After incubation at 65°C for 5 min to promote RNA denaturation, 4 µl of cDNA Synthesis 5X buffer, 1 µl of DDT (0.1 M), 1 µl of

RNAse OUT™, 1 µl of Thermoscript™ and 1 µl of water were added to the tube. The reaction was performed in the iCycler iQ™ thermocycler (Bio-Rad) under the following conditions: 25°C 10 min (binding of RNA hexamers), 55°C 45 min (reverse transcription) and 85°C 5 min (transcriptase enzyme inactivation). Finally 1 µl of RNasiH was added and samples were incubated at 37°C for 20 min: this is useful to degrade the RNA left. After reverse transcription, the cDNA was stored at -20°C.

7.2 qPCR reaction

The cDNA samples obtained by reverse transcription were amplified using the oligonucleotides listed in Table 3 and HPRT (hypoxanthine phosphoribosyltransferase) as the reference gene for data quantitation.

For cDNA amplification, the kit Platinum® SYBR® Green qPCR SuperMix - UDG (Life Technologies) was used. It consists of a SuperMix containing Platinum® Taq DNA polymerase, SYBR® Green I, Tris-HCl, KCl, 6 mM $MgCl_2$, 400 mM dGTP, 400 mM dATP, 400 mM dCTP, 800 mM dUTP, the enzyme uracil DNA glycosylase (UDG) , and other reaction stabilizers. The reaction was carried out in a 0.2-ml tube by adding 12.5 ml of the described mix, 0.5 µl of forward primer (10 µM) and reverse primer (10 µM), 2 µl of cDNA and 9.5 µl of RNAse-free water. The amplification conditions were the following: 50°C for 2 min (to allow the action of the enzyme UDG), 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min. At the end of the amplification, the melting curves were obtained in a range of temperatures between 55 and 95°C, increasing one degree per second.

Real-Time PCR was performed using the DNA Engine Opticon® Real-Time Thermal Cycler (MJ Research), a 96-well plate thermocycler with a fluorescence detector, a light source (LED Lamp) and the Opticon Monitor 3 software for data extraction. This detection system allows to monitore qPCR products: during each amplification cycle, fluorescence emissions are collected from each tube and at the end of PCR reaction data are processed by the software.

Primers	Abbreviation	Sequence (5'-3')	Reference	Lengh
Nanog homeobox	NANOG	F- CGGACAAAGAGTTGGCTGTGCAAT	NM_024865.2	106 pb
		R- AGCTGGGTGGAAGAGAACACAGTT		
POU class 5 homeobox	OCT4	F- TATGCAAAGCAGAAACCCTCGTGC	NM_002701.4	102 pb
		R- TTCGGGCACTGCAGGAACAAATTC		
Zinc finger protein, omolog 42	REX1	F- TGGAGGAATACCTGGCATTGACCT	NM_174900.3	105 pb
		R- AGCGATTGCGCTCAGACTGTCATA		
Sex determining region Y box 2	SOX2	F- CACATGAAGGAGCACCCGGATTAT	NM_003106.3	191 pb
		R- GTTCATGTGCGCGTAACTGTCCAT		
Kruppel-like factor 4	KLF4	F- TGAACTGACCAGGCACTACCGTAA	NM_004235.4	106 pb
		R- TCTTCATGTGTAAGGCGAGGTGGT		
Gene coding for p67 myc protein	c-Myc	F- ACAGCATACATCCTGTCCGTCCAA	D10493.1	79 pb
		R- TGTTCTCGTCGTTTCCGCAACAAG		
Signal transducer and activator of transcription 3	STAT3	F- ATGGAAGAATCCAACAACGGCAGC	NM_213662.1	175 pb
		R- GGTCAATCTTGAGGCCTTGGTGA		
Hypoxanthine phosphoribosyltransferase 1	HPRT1	F- ATGGACAGGACTGAACGTCTTGCT	NM_000194.2	79 pb
		R- TTGAGCACACAGAGGGCTACAATG		

Table 3. Primers for Real-Time PCR

8. Western Blotting

Western blotting or immunofixation is a biochemical technique that allows to identify a specific protein in a protein extract, through the binding with specific antibodies. In general, proteins are first separated on a polyacrylamide gel based on the molecular weight and then transferred to a membrane nitrocellulose or polyvinyldifluoride (PVDF). The target protein is then recognized through the use of a specific primary antibody and a secondary conjugated to an enzyme or a fluorophore.

a) Extraction of proteins

After treatment with Trizol (Sigma-Aldrich), proteins in the phenol-chorloform fraction were collected, treated with 300 µl of absolute ethanol and then incubated for 2 -3 min at RT. After centrifugation at 4°C for 5 min, at a speed of 4600 rpm, the supernatant was collected and treated for 10 min with 1.5 ml of isopropanol (Carlo Erba). The samples were then centrifuged for 10 min at 12000 rpm and the pellet was collected, treated

with 2 ml of a solution 0.3 M guanidinium chloride for 20 min and finally centrifuged at 9000 rpm for 5min. The samples were further purified by washing for 20 min, at room temperature with 2 ml of absolute ethanol. After centrifugation at 9000 rpm for 5min at 4°C, the pellet was dried for 5-10min and then resuspended in 100 µl of 1X SDS and then stored at -20°C until the time of protein quantification.

b) Protein Quantification

The quantification of proteins extracted included the use of the BCA Protein Assay Reagent Kit (Pierce). This method combines the reduction of copper ($Cu^{+2} \rightarrow Cu^{+1}$) carried out by the target protein in an alkaline medium (the biuret reaction) with the colorimetric determination of the cation Cu^{+1} by bicinchoninic acid (BCA). In particular, the colorimetric reaction is given by the chelation of two molecules of BCA with a copper ion. The absorbance of this complex is water-soluble at 562 nm with corresponding increase of protein concentration. For the assay quantification, a set of 8 dilutions of the stock standard BSA (2 mg/ml; Pierce) and a blank solution (Table 4) were prepared. Subsequently, 25 µl of each dilution solution and the samples to be quantified were placed in 96-well plates and 200 µl of Working Reagent was added to all the wells. The plate was then incubated for 30 min at 37°C. The Working Reagent solution was prepared by mixing 50 parts of Reagent A (sodium carbonate, sodium Bicarbonate reagent for the detention of BSA and sodium tartrate in 0.2 N NaOH), with a part of reagent B (solution of copper sulphate to 4%).

	Diluent (µl)	**BSA (µl)**	**[BSA] (mg/ml)**
A	0	300 µl from stock solution	2
B	125	375 µl from stock solution	1,5
C	325	325 µl from stock solution	1
D	175	175 µl from standard B	0,75
E	325	325 µl from standard C	0,5
F	325	325 µl from standard E	0,25
G	325	325 µl from standard F	0,125
H	400	100 µl from standard G	0,025
I	400	0	0 mg/ml = blank

Table 4. BSA standard dilutions

The absorbance was then measured at a wavelength of 562 nm using the Microplate EL 13 autoreader tool (Bio-Tek Instruments). The calibration curve was prepared using the concentrations of standard. Using linear regression analysis, performed on the computer (Prism, Graph Pad), standard equation was calculated to extrapolate the protein concentration (mg/ml) of the individual samples.

c) Polyacrylamide gel electrophoresis

This procedure allows to analyze and separate the proteins, exploiting their size and their charge (Figure 12). The gel is prepared by copolymerization of acrylamide (monofunctional monomer) (CH2 = CH-CO-NH2) and an agent that form cross-links to form a three-dimensional lattice (N,N'-methylene bisacrylamide) (bifunctional monomer) (CH2 = CH-CO-NH-CH2-NH-CO-CH = CH2). The polymerization takes place by means of a chain reaction due to the formation in a series of free radicals by addition of ammonium persulfate and the base N, N, N', N'-tetraetilendiammina (TEMED). TEMED catalyzes the decomposition of the ion persulfate with the production of the corresponding free radical. In this way long chains of acrylamide are formed. These are held together by bonds arising from the occasional intersection within the chain of molecules bis-acrylamide.

Figure 12. Protein gel electrophoresis equipment

The polyacrylamide gel is composed of two parts: the stacking gel and the running gel. The stacking gel is to concentrate the sample protein loaded into the appropriate wells, so that all samples are at a same level, when they begin to separate. The running gel allows to separate proteins according to their molecular weight. Stacking gel and

running gel have same composition but have a different concentration of acrylamide, usually lower in the stacking gel (Table 5). In particular, the composition of the running gel is decided according to the size of the protein to be detected. A higher concentration allows to separate the small proteins from those of larger. For the analysis of TH protein, NSE, β-III tubulin in the treated samples in case of neuronally induced cells and Myogenin and Myosin Heavy chain (MHC), in case of myogenic differentiation, it is was used a 4% stacking gel and a 10% running gel. Acrylamide, ammonium persulfate (APS), the TEMED were purchased from Bio-Rad company while SDS and Tris-HCl from Sigma - Aldrich. 5 µg of total protein was loaded for each sample, supplemented with SDS and loading buffer (Bio-Rad) in a total volume of 30 µl. In parallel, 5 µl ProteinTM, the Precision Plus Dual Color (Bio-Rad) for the definition of the molecular weight reference, was loaded. After loading, electrophoresis apparatus was filled with 1M running buffer, with the lid closed, and after connecting the electrodes, electrophoretic run was performed at 140 Volt for 2 h.

4% Stacking gel	**12% Running gel**
Acrylamide and bis-Acrylamide solutions (37:1) 0.5 M Tris-HCl, pH 6.8 MilliQ water	*Acrylamide and bis-Acrylamide solutions (37:1)* 1.5 M Tris-HCL, pH 8.8 MilliQ water
10% SDS 10% APS	10% SDS 10% APS
TEMED	TEMED

Table 5. Stacking and Running gel composition

d) Transfer

At the end of the run, the gel was transferred into the apparatus, in contact with the PVDF membrane and the electrophoretic run was performed at 25 Volt overnight, at 4°C (Figure 13). The transfer of the proteins from the gel to the membrane was verified by staining with Ponceau Red (Sigma-Aldrich): the membrane was immersed for a few minutes in the dye solution and then rinsed with distilled water.

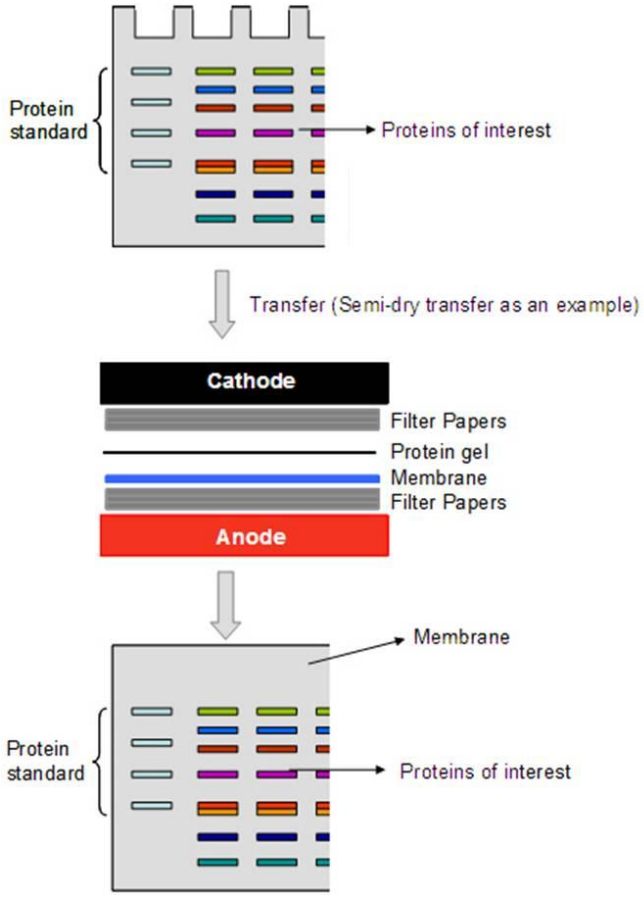


Figure 13. Protein transfer on PVDF membrane

e) Immunoblotting

To prevent any non-specific interactions with the antibody, the membranes were saturated in 5% milk (Sigma-Aldrich) in PBS, for 2 h, under stirring. Subsequently, the membranes were treated for one night at 4°C with rabbit polyclonal antibody anti-human Tyrosine Hydroxylase (TH) (Millipore), monoclonal mouse anti-human β-III tubulin (Millipore) diluted 1:1000 in 1% milk in PBS, monoclonal mouse anti-human myogenin, monoclonal mouse anti-human MHC and with the monoclonal antibody mouse anti-human Neuron Specific Enolase (NSE) (Millipore) diluted 1:500 in 1% milk in PBS. Mouse and rabbit anti-human GAPDH antibodies (Millipore; diluted 1:1000) were used as control protein. At the end of the incubation period, the antibody solution was aspirated and subsequently repeated washes with PBS + 0.25% Tween (Bio Rad). Subsequently, the membrane was incubated for 1 h with a solution of the secondary antibody anti-rabbit-HRP or anti-mouse HRP (Bio-Rad) diluted (1:5000) in 1% milk prepared in PBS.

f) Detection of signal

The signal detection was performed after repeated washings of the membranes with PBS + 0.25% Tween and incubation of the membrane with the Chemiluminescent Peroxidase Substrate (Sigma-Aldrich) for 1 min, prepared using the solutions A and B in the ratio 1:1. Once dried, the membrane was placed in a Deposit autoradiography, in the dark and in contact with an autoradiographic film (Sigma-Aldrich) for a variable time depending on the antibody (5-30 min). Subsequently, the impressed plate was developed by incubation in liquid development XOMAT EX II and fixing RP X-OMAT LO (Kodak).

9. Immunofluorescence

This technique allows to detect the presence of specific antigens in the sample of interest using antibodies conjugated directly or indirectly with fluorescent substances. In this work, the immunolocalization of the target of interest was performed by indirect staining with the following primary antibodies : polyclonal rabbit anti-human Tyrosine Hydroxylase (TH) (Millipore), monoclonal anti-human Neuronal Nuclei (NeuN) (Millipore), monoclonal mouse anti-human Neurofilament (NEFM) (Millipore), rabbit polyclonal anti-human Dopamine Transporter (DAT) (Santa Cruz), rabbit polyclonal anti-human Musashi (Millipore), monoclonal mouse anti-human Nestin (NES) (Millipore), monoclonal mouse antihuman Neural Cell Adhesion Molecule (NCAM) (Millipore). For myogenic differentiation, monoclonal mouse anti-human myogenin and monoclonal mouse anti-human MHC were used. For each marker, the primary antibody was subsequently localized with the secondary antibody FITC-conjugated donkey anti-rabbit/anti-mouse (Millipore). The samples were then fixed in BD CytofixTM Fixation Buffer (BD Biosciences) for 20min at 4°C and then rinse with cold PBS. The deactivation of nonspecific sites was performed treating the samples with donkey serum (DS) (Sigma-Aldrich) prepared at 10% in PBS. Each sample was then treated overnight at 4°C in a humidity chamber with 100 µL of the primary antibody solution (1:250) prepared in PBS + 10% HS (Sigma Aldrich). After three washes with PBS + 3% DS, each sample was incubated for 30 min, at room temperature, in humidity chamber, with secondary antibody (1:200) in PBS + 1.5% DS. After three washes with PBS, the samples were mounted with the mounting medium with DAPI (Vectastain) for nuclear counterstaining.

10. Evaluation of membrane properties: measurement of intracellular calcium

To perform the detection of intracellular calcium, Jasco FP-6500 spectrofluorometer was used. This analysis allows us to determine whether the cells, following neuronal induction, have acquired the ability to transport calcium in them and release from intracellular stores, a typical feature of excitable cells such as cells of the nervous system. Calcium plays, in fact, an important role as a mediator in the transduction of transmembrane signals. After 14 days of induction, the treated samples (T7 and T14) and controls (C14) were stimulated with 56 mM KCl at room temperature for 30 min. Each sample was then incubated for 30 min at 37°C with 5 µl of calcium fluorescent indicator Indo-1 AM (Life technologies), which is a membrane-permeable dye used for determining changes in calcium concentrations in the cell using fluorescence signal detection. Once Indo-1 enters the cell, esterases cleave the AM group yielding a membrane-impermeable dye. Upon binding calcium, there is a shift in the peak emission of Indo-1 (Figure 14). The analysis was carried out at 37°C in PBS, before and after permeabilization of the cells with 0.05% Triton X-100. The samples were excited at a wavelength of 355 nm and the fluorescence intensity was recorded in a range of wavelength 355-600 nm.

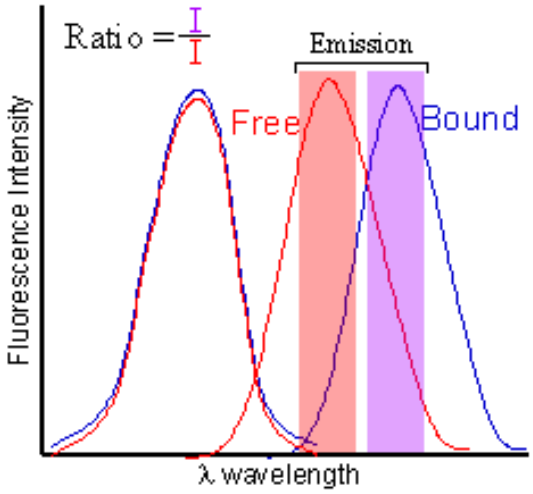


Figure 14. Free and bound INDO-1 AM fluorescence emission

11. HPLC analysis for neurotransmitter release

The inductive medium of hPBMCs were discarded after 7 and 14 days of neuronal differentiation and the cultures were placed in HBSS for 30 min at 37°C under stimulation with 56 mM KCl. The supernatant was collected and separation of the injected samples (20 µl) was achieved by isocratic elution on a Hewlett- Packard Series

1050 HPLC system with a reverse-phase C18 column (3 µm particle size, 80x4.6 mm dimension, ESA, Inc.) (Figure 15) in a commercially available MD-TM mobile phase (ESA Inc.). The flow rate was set at 1 ml per min, resulting in a working pressure of 100 bar and the results were validated by co-elution with nor adrenaline standards under varying buffer conditions and detector settings. The baseline elutions were detected.

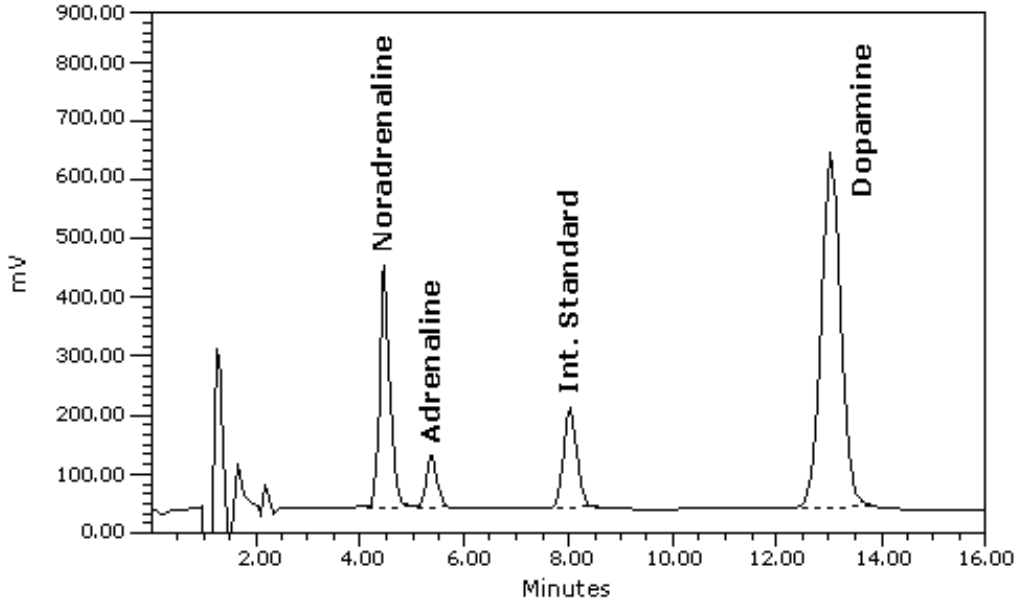


Figure 15. HPLC analysis of catecholamines

12. In vivo evaluation of hPBMCs in an induced muscle damage model

Regenerative potential of isolated hPBMCs was assessed in vivo by transplanting cells into a rat model of muscle damage (Figure 16). After Lewis rats (n=6) were anesthetized gaseously, an incision was made in the cutaneous followed by subcutaneous layer to reveal the tibialis anterior muscle. After That, 0.5 ml of 0.5% bupivacaine hydrochloride diluted in saline was injected both at the two ends and in the middle part of the muscle, as described by Hill and Goldspink (2003). After surgery, the animals were treated with antibiotic (Baytril 0.2 ml/kg for two days) and painkillers (Contramal 3-5 mg/kg 2-3 times daily intramuscularly for 2-3 days). hPBMCs were stained with 15 nM Qdot 800 (cell labeling kit, Life technologies) for 45 min at 37°C and then washed with PBS to remove excess staining material. After 48 hours of muscle damage, pre-stained hPBMCs ($1.5x10^5$ cells per animal, n=6) were injected intramuscularly. Animals (n=6) treated with physiological solution were considered as controls. The animals were treated with antibiotics and painkillers as described above and were sacrificed by overdose of

anesthesia after 7 and 14 days from the administration of the cell suspension. The muscle specimens were fixed using isopentane and liquid nitrogen vapours and sections were made using Thermo RTE-111 cryostat (Leica).

Immunofluorescence staining was done using rabbit anti human vimentin antibody (Cell signaling) and mounted with DAPI to stain the nuclei.

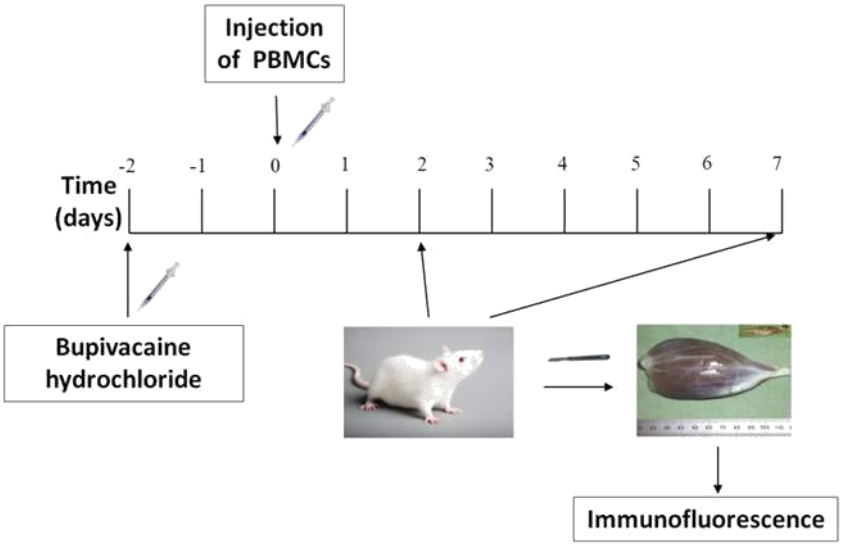


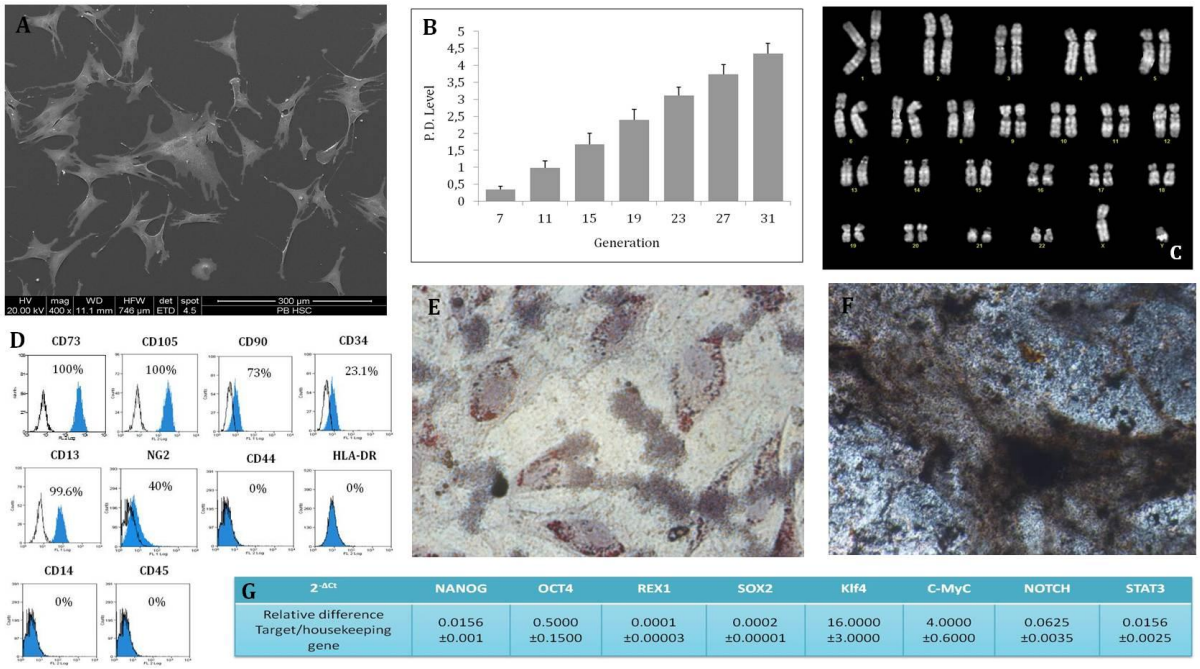
Figure 16. In vivo experimental plan

RESULTS AND DISCUSSION

1. hPBMC characterization study

Research studies on cell populations for tissue regeneration require to identify cellular and molecular properties to confirm their pluripotency and determine their regenerative potential for in vivo experiments.

The invitro model of hPBMCs, used for lineage shift studies, was found to possess distinct characteristics (Figure 17). They represented a unique population with a fibroblastic morphology (Figure 17 A). The population doubling time was calculated to be 48 hours and they had a constant population doubling level till 31 passages (Figure 17 B). They possessed a stable karyotype during long term culture (Figure 17 C). The flow cytometry characterization revealed an immunophenotype of $CD73^{+}/CD105^{+}/CD90^{+}/CD34^{+}/CD13^{+}/$ $NG2^{+}/$ $CD44^{-}/CD14^{-}/CD45^{-}/HLA\text{-}DR^{-}$ (Figure 17 D). hPBMCs showed differentiative potential towards adipogenic and osteogenic lineages (Figure 17 E, F) and were found to express various pluripotency genes such as Nanog, OCT4, Rex1, Sox2, Klf4, C-Myc, NOTCH and STAT3 (Figure 17 G).



$2^{-\Delta Ct}$	NANOG	OCT4	REX1	SOX2	Klf4	C-MyC	NOTCH	STAT3
Relative difference Target/housekeeping gene	0.0156 ±0.001	0.5000 ±0.1500	0.0001 ±0.00003	0.0002 ±0.00001	16.0000 ±3.0000	4.0000 ±0.6000	0.0625 ±0.0035	0.0156 ±0.0025

Figure 17. In vitro characterization model of hPBMCs. (A) Morphological analysis by SEM. (B) Population Doubling study. (C) Karyotype analysis. (D) Immunophenotype investigation by flow cytometry. (E) Adipogenic (Oil Red O) and (F) osteogenic (Von Kossa) differentiation responses. (G) Gene expression profile identification by qPCR

2. Neurogenic shift responses of hPBMCs

At specific time intervals, the lineage shift responses of hPBMCs towards neuronal phenotype were documented stage by stage. The morphological analysis of hPBMCs was the first indication that they were responding to the external stimuli as noted by the gradual change in their organization (Figure 18). The control cultures (C7) that were maintained only in Neurobasal medium tend to stay away from the differentiation pathway, as they proliferated to attain confluence and became elongated structures. Instead, the treated cultures at 7 days (T7) responded to the stimuli from EGF and FGF2 by random distribution and small spindles shaped structures. The initial preparative phase was efficient in producing a morphological change in hPBMCs. In the next 7 days, the control cultures, due to lack of space to proliferate, started to get detached from the culture plates. The treated cultures (T14), due to the effect of retinoic acid and nerve growth factor, were obtaining a drastic morphological change similar to dendrite like structures as evidenced clearly by SEM analysis (Figure 18). hPBMCs made a shift from their initial fibroblastic morphology to dendrite like processes. The inductive factors used in this differentiation process were effective enough to bring about a neuron like morphology in hPBMCs. The first indication that hPBMCs were entering into a differentiation pathway was the fact that the control cultures continued to proliferate and the treated cultures stopped proliferating. The immediate lineage shift capacity of hPBMCs to respond to initial phase of induction, where they respond to epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) by changing their morphological organisation, was evident that these cells enter into commitment state. The EGF and bFGF responsiveness of hPBMCs were highly correlated to many precursor cells of neural origin as reported in the literature (Ciccolini et al., 1998). After initial priming, hPBMCs were induced with Retinoic acid (RA) and Nerve Growth factor. RA is a developmentally regulated morphogen that has diverse roles that include controlling generation of primary neurons in Xenopus (Sharpe and Goldstone, 2000; Franco et al., 1999), patterning of the hindbrain (Begemann and Meyer, 2001), motor neuron specification (Novitch et al., 2003) and limb bud patterning (Thaller and Eichele, 1987). Consequently, RA has been widely used for differentiating embryonic carcinoma and embryonic stem cells since the pioneering studies of Strickland (Strickland and Mahdavi, 1978). Both RA and nerve growth factor have been implicated in the process of neuritogenesis (Scheibe et al., 1991).

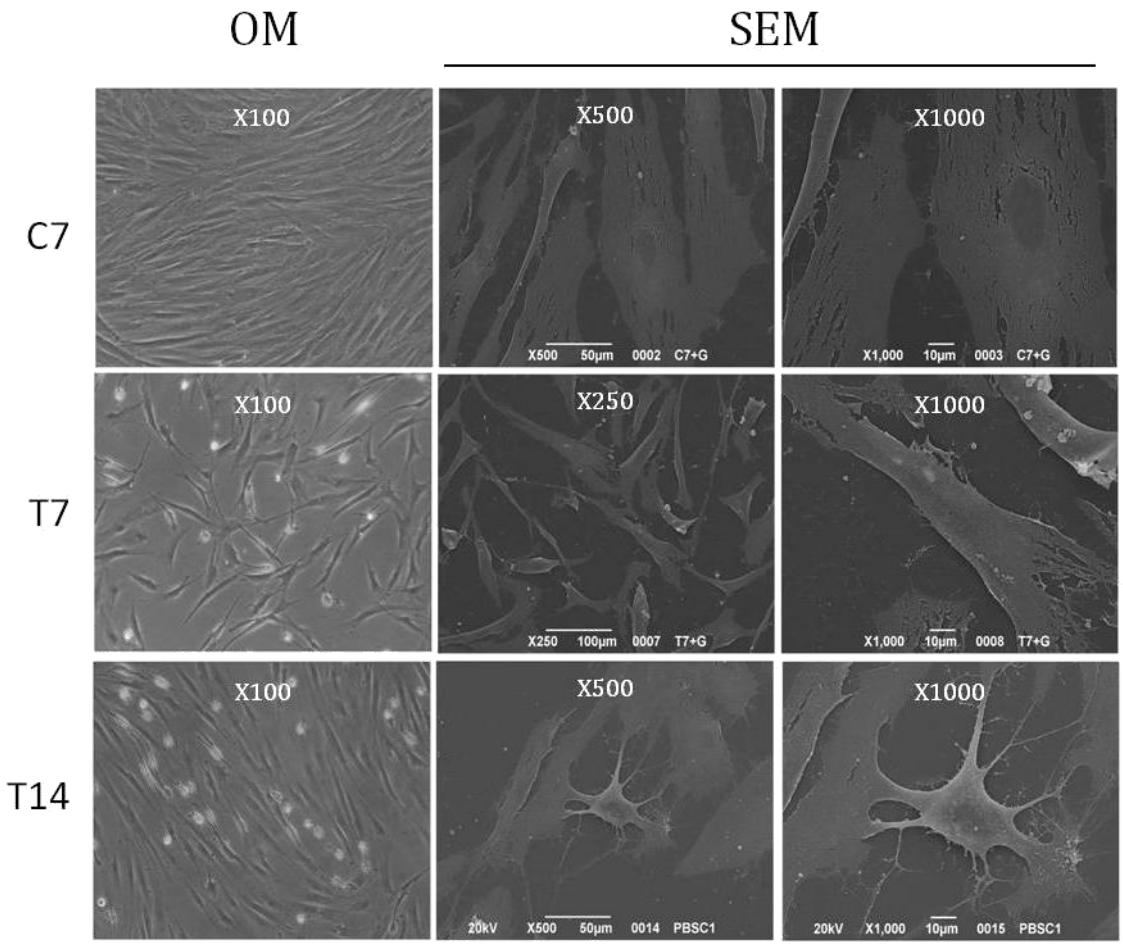


Figure 18. Morphological study by Optical Microscopy (OM) and SEM of hPBMCs cultured in NeuroBasal Medium (C7), treated with EGF (20 ng/ml) and bFGF (10 ng/ml) for 7 days (T7) and then with Retinoid Acid (RA) (0.5 µM) and NGF (20 ng/ml) up to 14 days (T14)

The next step in the process of confirmation of neuronal shift was the analysis of gene expression of early and late neuronal markers. Undifferentiated hPBMCs expressed many neurotrophins namely, BDNF (brain derived neurotrophic factors), GDNF (glial derived neurotrophic factors) and NGF (nerve growth factor) (Figure 19). The absence of nestin expression at mRNA level can be attributed to the sensitivity of the technique as the expression was confirmed by immunofluorescence even at the undifferentiated state (Figure 19). The expression of some neurotrophins were maintained in the control cultures (C7) as Neurobasal medium contained factors that could stimulate the expression of neurotrophins at mRNA level. The expression of typical neuronal markers such as TBB3 and MAP2 appeared at T7 days and their expression was maintained at T14 days (Figure 19). Some of the late neuronal markers such as Neurofilament medium (NEF M), Neuronal nuclear antigen (NeuN), GLAST (Glutamate-Aspartate transporter),

SYP (Synaptophysin), and neurogenin 1 (NEUROG 1) were detected only at the final phase of the induction (T14) (Figure 19).

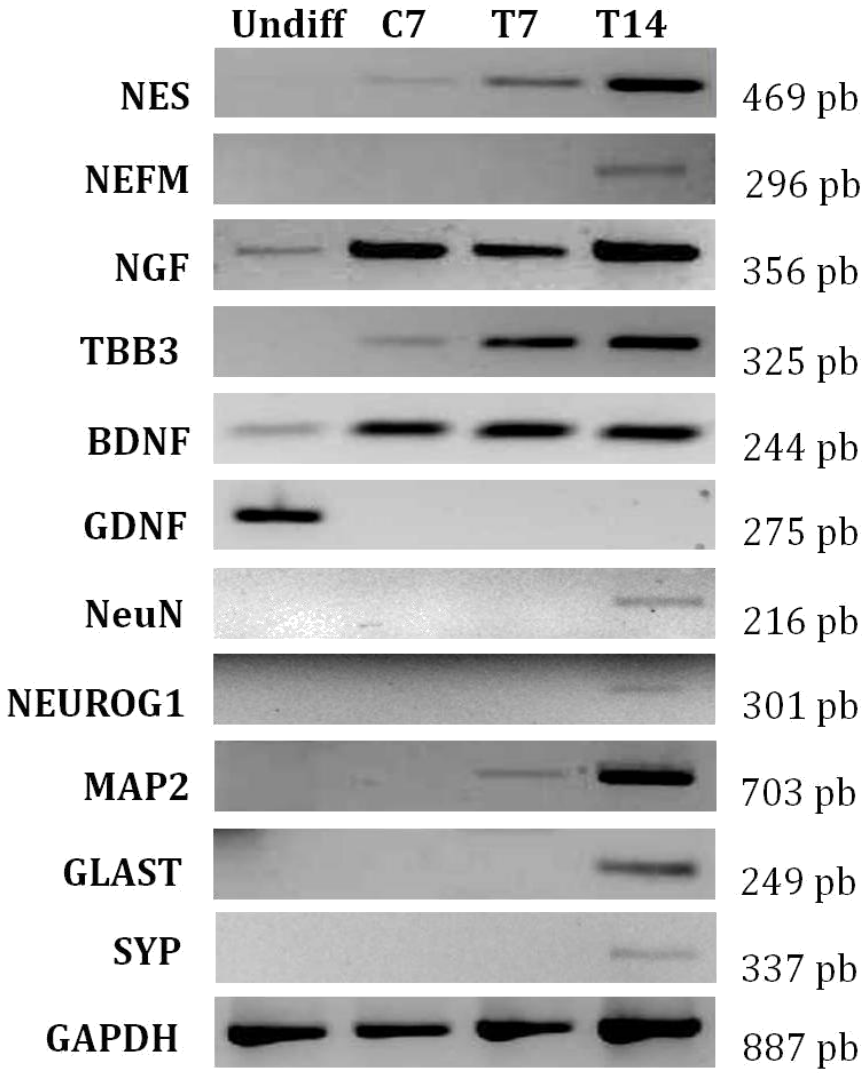


Figure 19. OneStep RT-PCR study of neuronal marker expression in hPBMCs grown in proliferation medium (Undiff), control cells (C7) and cells treated for 7 and 14 days (T7, T14) with inductive factors. In parallel, the expression of the housekeeping gene GAPDH was detected in all samples

After confirming the presence of mRNA towards a neuronal shift, the ability of hPBMCs to translate that into a neuronal phenotype was tested out using western blot, immunofluorescence and flow cytometry.

Flow cytometry analysis showed that- when hPBMCs were cultured for 14 days with αMEM containing ascorbic acid, a weak expression of MAP2 and TH was observed. NBM demonstrated to be effective to promote a significative increase of TBB3, MAP2 and TH and, after addition of neuronal factors, demonstrated to be suitable to differentiate terminally the cells (Figure 20).

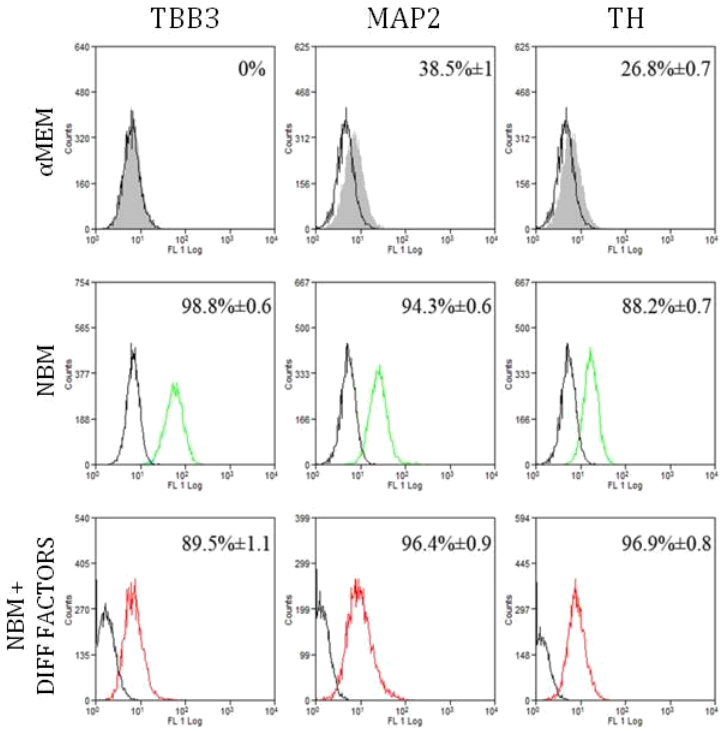


Figure 20. Flow cytometrical study of TBB3, MAP2, TH on induced (T14) hPBMCs (coloured profile) compared to control sample cultured in αMEM, NBM, and NBM added with neuronal factors for 14 days (black profile for all). Indirect staining with FITC-conjugated secondary antibodies was used. For each marker, data were expressed as % positives ± SD of T14 versus C14

As evidenced by Western Blot results, the expression of TBB3 was maintained at the protein level both in control and treated cultures (C7, T7, T14) (Figure 21) and the protein expression of TH (tyrosine hydroxylase) followed the same pattern. The evidence that retinoic acid and nerve growth factor directed hPBMCs towards a mature neuronal phenotype was highlighted from the protein expression of active form of Neuron specific enolase (NSE) 47 KDa only in T14 samples (Figure 21), whereas as the inactive Enolase form 66 KDa was detected in all other samples.

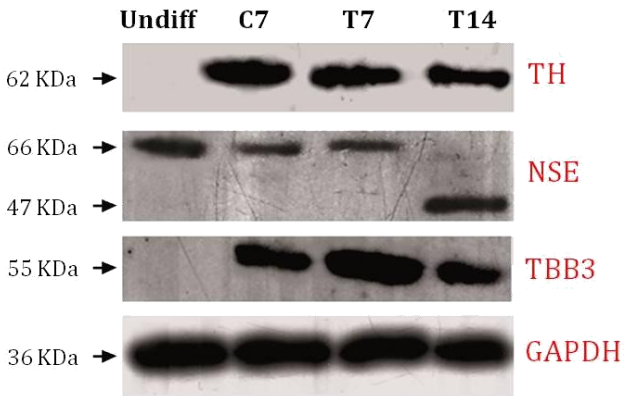


Figure 21. Western Blot analysis on undifferentiated (Undiff), C7, T7 and T14 hPBMCs using 10 μg protein extract and chemiluminescence detection

The immunofluorescence analysis revealed the expression on typical neuronal markers such as Musashi, neural cell adhesion molecule (NCAM), dopamine transporter (DAT), NEFM, NeuN and TH (Figure 22).

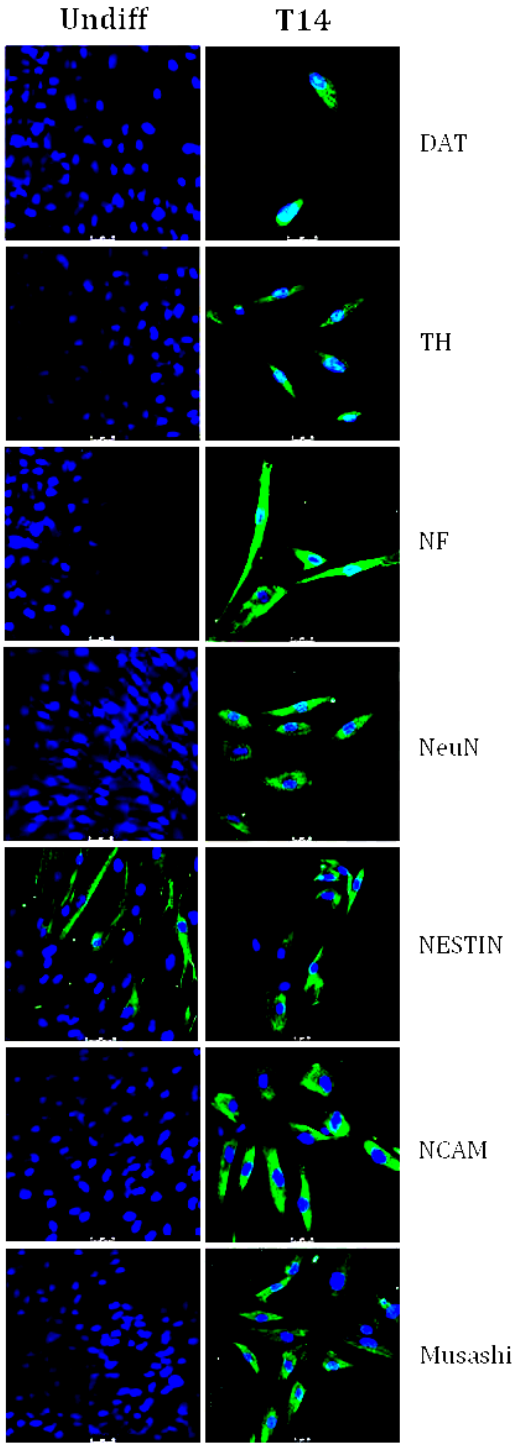


Figure 22. Assessment by immunofluorescence of neuronal lineage markers on induced hPBMCs (T14) compared to undifferentiated samples (Undiff). Cells were indirectly labeled using FITC-conjugated secondary antibodies and data were acquired with the Leica TCS SP5 confocal microscope

Both the RNA and protein expression studies on hPBMCs subjected to neuronal shift induction revealed some interesting results. The expression, even in the undifferentiated state, of mRNA for nestin, BDNF and NGF suggests a differentiative potential of hPBMCs towards neuronal lineage, besides highlighting the fact that, after specific induction, such markers continue to be expressed until achievement of a mature differentiated state. The expression of GDNF in the undifferentiated state and the loss of its expression during process of differentiation confirm that the inductive protocol is neural lineage specific. During the first preparative phase of the differentiation, hPBMCs expressed several typical markers of neuronal lineage. Nestin is a protein of intermediate filament type VI, which is expressed in the cytoskeleton of the nerve cells and it is involved in the radial growth of the axon (Yan et al., 2001) and expressed in dividing cells during the early stages of development of the nervous tissue, where it is progressively replaced in the nerve cells as tissue-specific intermediate filament proteins. It is uniquely expressed in multipotent stem cells and regarded as a characteristic marker for plasticity. GDNF is a dopaminergic neurotrophic factor secreted by glial cells. It promotes the survival, proliferation and differentiation of different types of neurons of the nervous system (Ernest et al., 1995; Luis et al., 2001). BDNF is a secreted protein, belonging to the family of neurotrophins, critical in the regulation of structural, synaptic and morphological plasticity (Thoenen, 2000). It is also involved in nerve regeneration, and in maintaining the structural integrity and neuronal plasticity in the adult brain, regulating the synthesis of neurotransmitters (Huang and Reichardt, 2001). There was a genomic shift in the identity of hPBMCs as they started to express some typical neuronal markers such as TBB3, constitutive element of microtubules in neurons of fetal and postnatal age (Seve et al., 2008; Katsetos et al., 2003), MAP2, the gene encoding the associated protein neurotubules type II, SYP, a integral membrane glycoprotein of presynaptic vesicles and present in the neuroendocrine cells and in almost all the neurons of the brain and spinal cord that participate in the synaptic transmission and GLAST, a protein of the inner mitochondrial membrane that mediates the transport of L-glutamate and L- and D-aspartate. In the central nervous system mammalian L-glutamate is the main transmitter for the majority of excitatory neurons, which are involved in complex physiological processes such as learning and memory (Jungblut et al., 2012; Storck et al., 1992). NEUROG1 is a protein belonging to the family of transcription factors, neuronal basic helix- loop- helix (bHLH), involved in the regulation of the process of differentiation (Cau et al., 2002; Gowan et al., 2001). Neural cell adhesion molecules

(NCAMs) of the immunoglobulin superfamily engage in multiple neuronal interactions that influence cell migration, axonal and dendritic projection, and synaptic targeting. Their downstream signal transduction events specify whether a cell moves or projects axons and dendrites to targets in the brain (Schmid et al., 2008). The fact that differentiated hPBMCs expressed NCAM, may attribute to their migratory capacities in vivo. The final phase of induction, where hPBMCs responded to external stimuli from retinoic acid and nerve growth factor, was a crucial one in the progression of the differentiation to the mature state. Withdrawal of FGF2 and the addition of retinoic acid lead to the expression of specific neuronal markers that characterise a mature neuronal phenotype in hPBMCs. The ability of hPBMCs to attain a mature neuronal phenotype was dependent on the addition of retinoic acid and NGF in the culture. The stimuli from RA and NGF were specific for neuronal lineage and it is confirmed by the expression of Tyrosine hydroxylase, a catalyzing enzyme in the rate-limiting step in the biosynthesis of Dopamine, and Dopamine transporter, a membrane-spanning protein that pumps the neurotransmitter dopamine out of the synapse back into cytosol, from which other transporters sequester Dopamine and Nor epinephrine into vesicles for later storage and release. The progression of hPBMCs towards neuronal differentiation pathway was completed by the verification of their functionality with HPLC and spectrofluorimetry. Noradrenaline, a neurotransmitter involved in the catecholamine synthesis pathway, was detected only in the T14 (Figure 23) samples at the final phase of the neuronal induction indicating the fact that hPBMCs, when evoked by KCl, acquired an excitable neuronal phenotype.

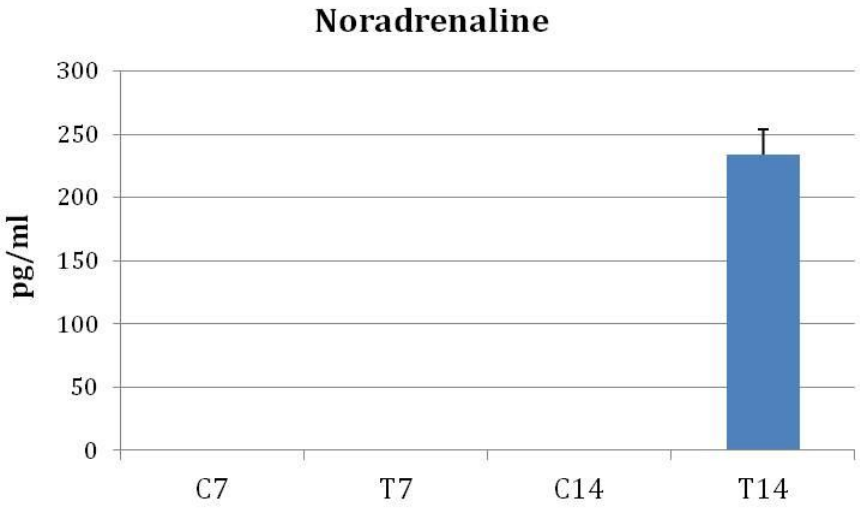


Figure 23. Noradrenaline release study by HPLC in hPBMC cultures after neurogenic induction

hPBMCs showed to respond to the external stimuli to acquire membrane excitability by conducting calcium flux, when evoked with KCl. The intracellular free calcium concentration subserves complex signaling roles in brain. Calcium cations (Ca^{2+}) regulate neuronal plasticity underlying learning and memory and neuronal survival. Homo- and heterocellular control of Ca^{2+} homeostasis supports brain physiology maintaining neural integrity. Ca^{2+} fluxes across the plasma membrane and between intracellular organelles and compartments integrate diverse cellular functions (Zündorf et al., 2011). Calcium flux, a major event in the synaptic signal transduction, was analyzed with the help of Indo-1, a fluorescent calcium indicator having absorbance at 355nm, after stimulation with KCl and permeabilizing with Triton X-100. The treated samples at 14 days showed a significant accumulation of both intracellular and extracellular calcium as evidenced by the increased fluorescent absorbance at 355nm when compared with control samples (Figure 24). hPBMCs were able to conduct calcium flux across their membranes, when stimulated by KCl, underlines the fact that induction protocol was efficient enough to promote the attainment of a functional phenotype. Neurotrophins are essential players in neuronal maturation during development. The synergistic action of retinoic acid and nerve growth factor switched hPBMCs towards mature neurotransmitter phenotypes. These results indicate that RA and NTs act sequentially and dependently during adult neurogenesis, with RA promoting the early events of neuronal differentiation and NTs acting later in the RA-stimulated cascade to promote neuronal maturation.

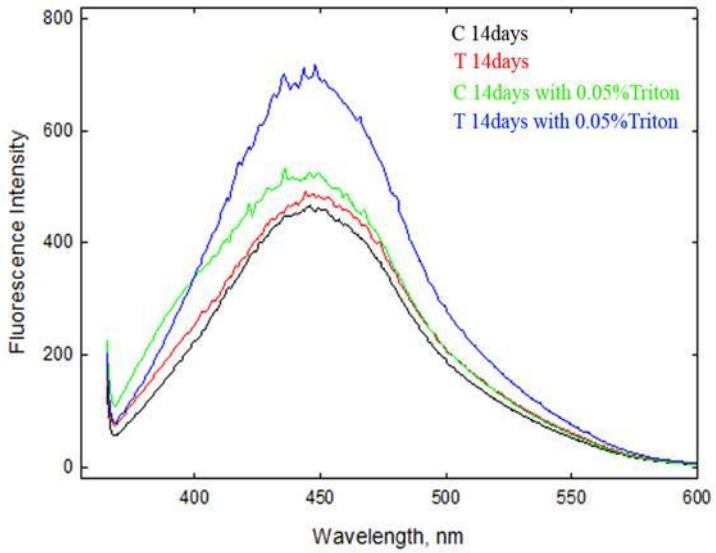


Figure 24. Emission spectrum of the fluorescent calcium indicator Indo1 AM (5µM) after incubation (30 min, 37°C) with differentiated hPBMC samples (C14, T14) previously treated with KCl 56 mM

3. Myogenic lineage shift responses of hPBMCs

To investigate the potential of hPBMCs to respond to specific stimuli to activate a myogenic cascade within them, control and treated cultures were examined morphologically both under optical microscopy (Figure 25) and SEM (Figure 26).

Control samples, starting from day 3, presented a random organization, whereas the treated samples (T3) acquire differentiation-related morphological changes and aligned parallelly to each other (Figures 25, 26). At 7 days, treated samples continued to respond by forming packed bundles as evidenced from the Figures 25 and 26; at 14 days, some multinucleated structures with partial stratification can be appreciated (Figure 26). This morphological re organization by hPBMCs was confirmed by the immunofluorescence staining for the characteristic mesodermic intermediate filament protein, Vimentin (Figure 27).

Cells switched from a fibroblast like phenotype to an appearance that resembled that of primary myotubes, ascertaining that hPBMCs were responsive to stimuli from IGF 1 and vitamin C. It is well known that insulin-like growth factor-I (IGF-I) plays multiple important roles during myogenesis by stimulating both growth and differentiation. Transgenic mice which over- express IGF-I in skeletal muscles display promoted adult muscle regeneration and hypertrophy via activation of muscle satellite cells (Musarò et al., 2001). Unlike most growth factors, the insulin-like growth factors, IGF-Iand IGF-II, are also capable of promoting muscle differentiation in cell culture(Engert et al., 1996), and their actions through the IGF-I receptor have been linked to the formation, maintenance, and regeneration of skeletal muscle in vivo (Coleman et al., 1995).

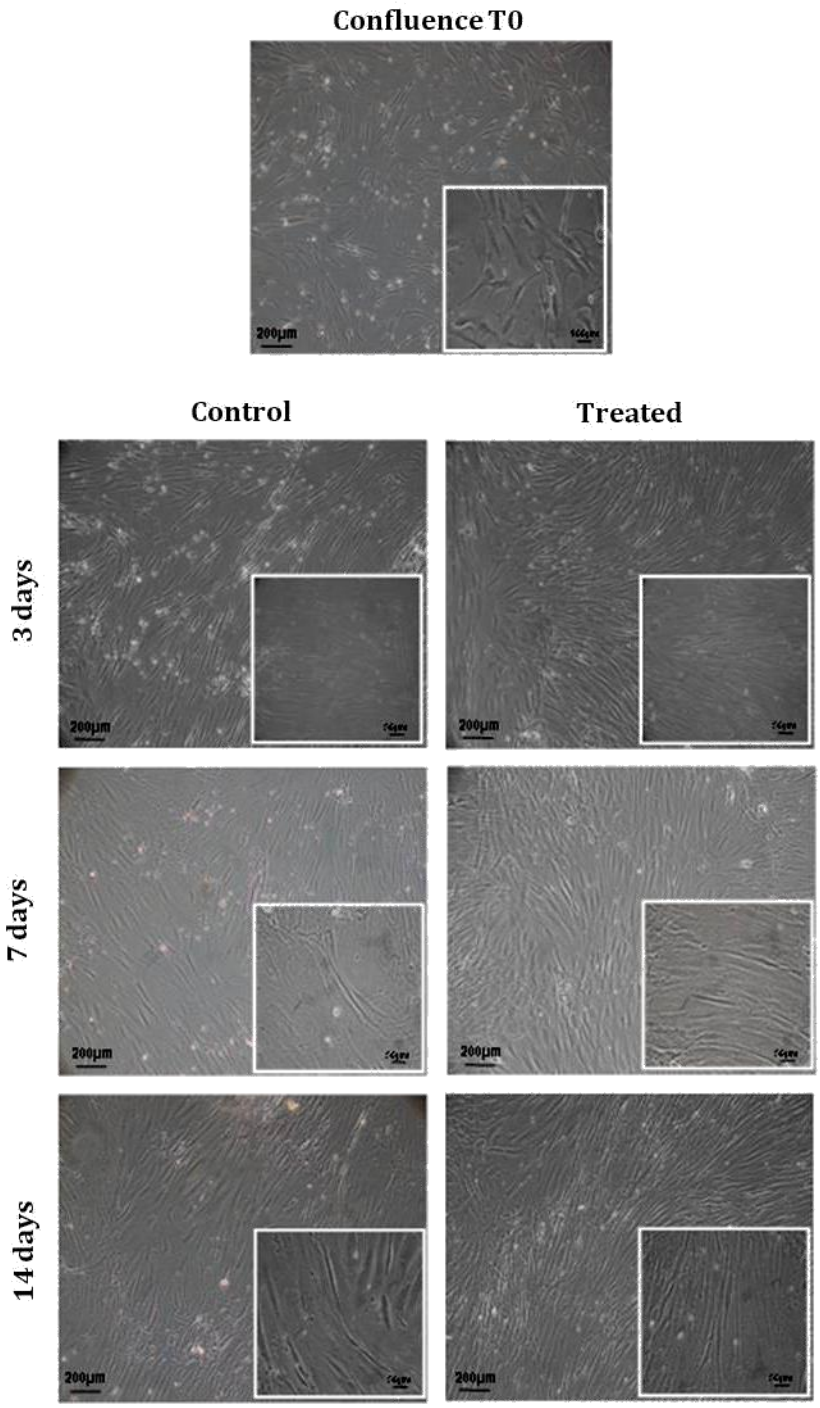


Figure 25. Morphological analysis by optical microscopy of hPBMC cultures at 100% confluence (T0) and after treatment with myogenic inductive medium for 3, 7 and 14 days

The stimulatory effect of various nutrients, especially ascorbic acid, on the extracellular matrix (ECM) production of cells in vitro has been extensively investigated. Ascorbic acid plays a key role as a cofactor in the post-translational modification of collagen molecules and increases collagen production (Sodek et al., 1982). An investigation of the ascorbic acid effect on procollagen synthesis in human skin fibroblast cultures revealed an increased production of Type I collagen (Chan et al., 1990).

Furthermore, it is well-known that ascorbic acid stimulates the proliferation of various mesenchyme-derived cell types including osteoblasts, adipocytes, chondrocytes, and odontoblasts in vitro (Alcain et al., 1994), and modulates cell proliferation in vitro (Chepda et al., 2001). As reported in the literature, IGF 1 and ascorbic acid acted synergistically on hPBMCs to bring about a change in morphological organisation with syncytium formation and as the differentiation proceeds, stratification can be observed due to excess extracellular matrix deposition.

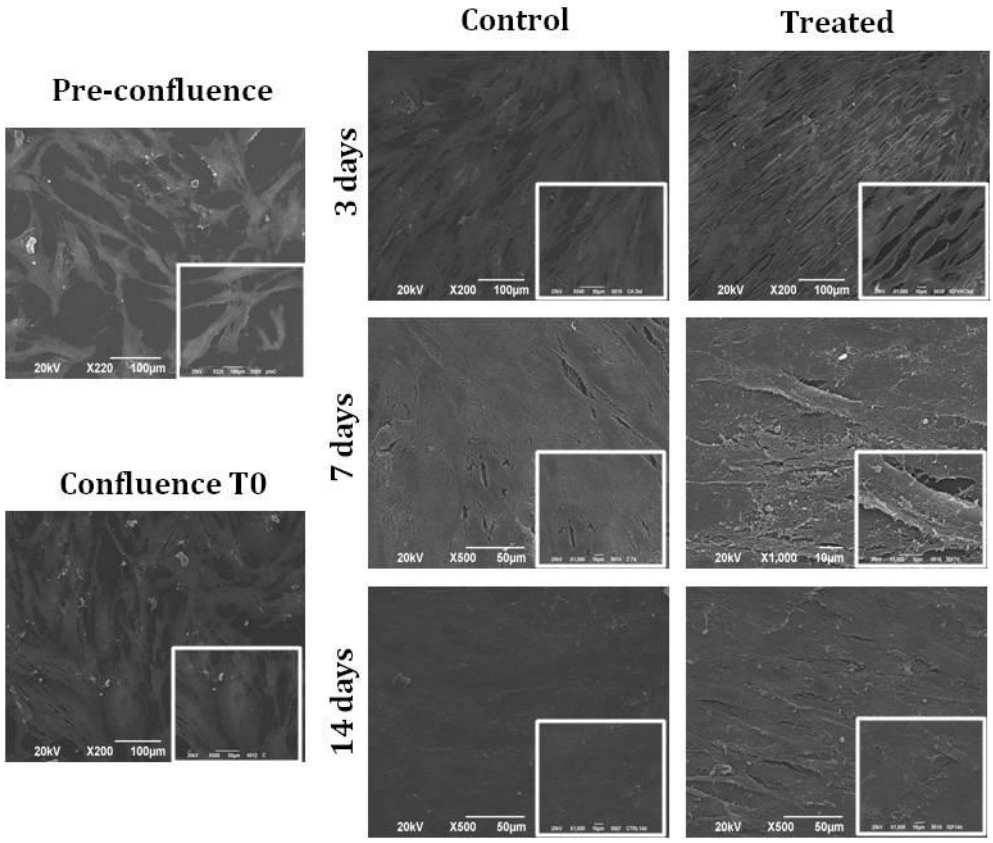


Figure 26. Morphological study by SEM of hPBMC cultures at pre-confluent state, at 100% confluence and after treatment with myogenic inductive medium for 3, 7 and 14 days

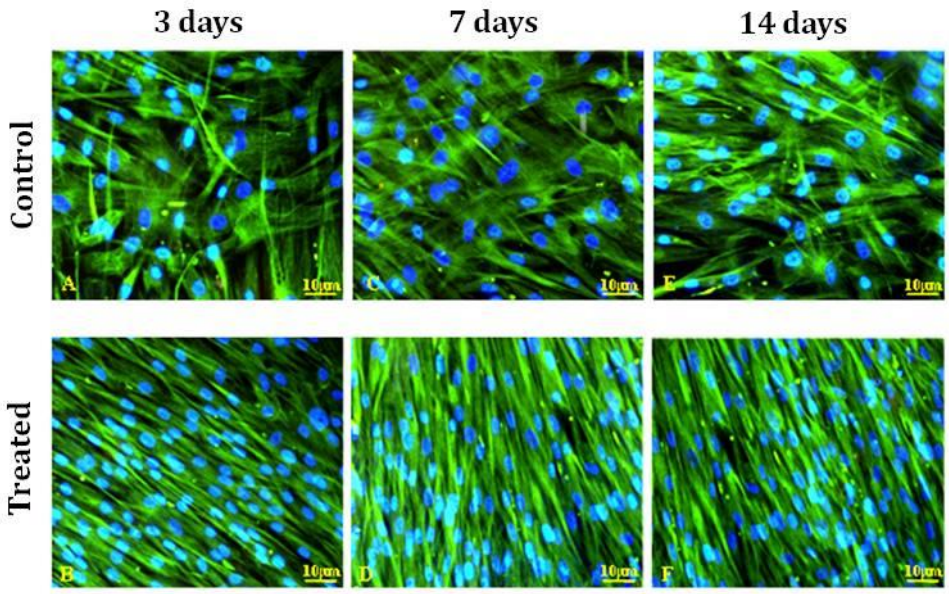


Figure 27. Immunofluorescence study of hPBMCs grown in proliferation (A, B, C) and differentiation (D, E, F) media and labeled with mouse anti-human Vimentin-FITC conjugated antibody (green). Nuclei were counterstained with DAPI (blue)

The FCM analysis (Figure 28) revealed a marked reduction (94-98%) in the expression of CD73 and CD105, known stem cell markers, starting from 3 days after induction confirming that hPBMCs have entered into the differentiation pathway.

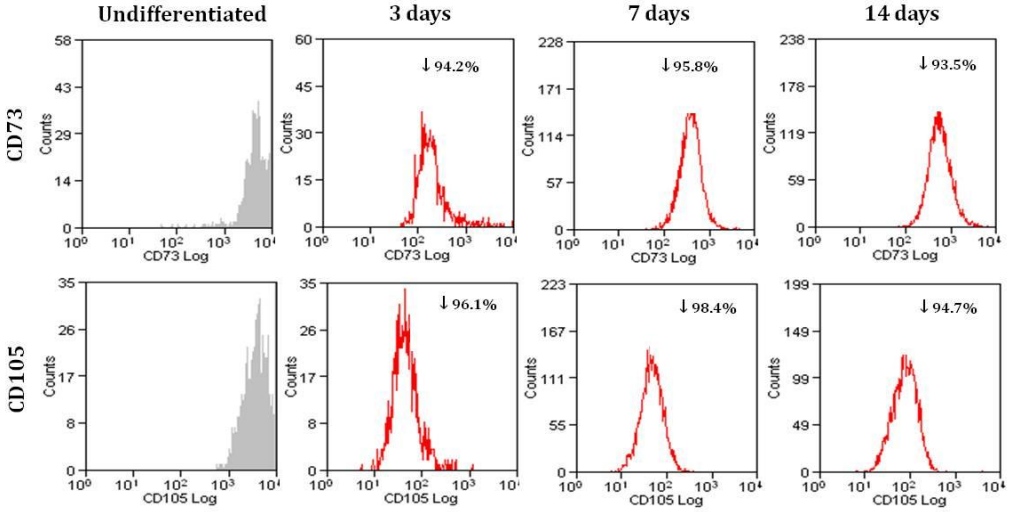


Figure 28. FCM study of stem cell markers in hPBMCs differentiated towards myogenic lineage. Data are expressed as % positive treated cells compared to undifferentiated control cells

To further analyze the initiation of the skeletal muscle program in hPBMCs, we examined the expression of the skeletal muscle-specific myogenic determination factors, namely Myf5, MyoD, myogenin, MHC (myosin heavy chain) and tropomyosin by RT-PCR

(Figure 29), flow cytometry (Figure 30), Western Blot (Figure 31A) and immunofluorescence (Figure 31B).

As shown by gene expression study, hPBMCs mimicked the in vivo myogenic differentiation process as noted from the expression pattern of MYF5 and MyoD.

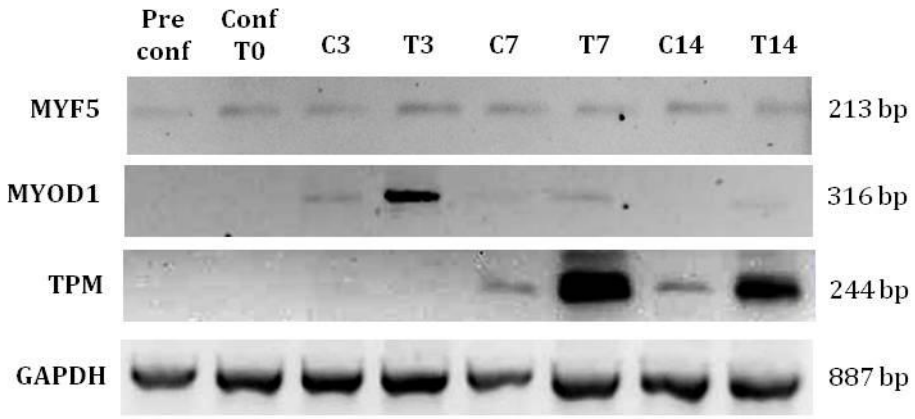


Figure 29. Gene expression analysis by One step RT-PCR of myogenic markers in specific differentiated hPBMC cultures. In parallel, the expression of housekeeping gene GAPDH has been detected in all samples

The mRNA and protein expression of both the transcription factors was elevated in treated cultures with respect to controls at 3 days of induction (Figure 29, 30) and as the differentiation proceeds, the expression level of MYF5 was greatly reduced at 7 and 14 days. Instead, MyoD expression was lower than the level at 3 days, but maintains a higher level than the control cultures (Figure 30). During the in vivo myogenic differentiation, MYF5 and MyoD controls the phase of cycling myoblasts and the expression of MYF5 fades away in the differentiation phase, whereas MyoD continues to be expressed in the myocytes. In the final phase of maturation, MyoD expression is replaced by the expression of Myogenin(Le Grand and Rudnicki, 2007). The temporal expression pattern of Myogenin, followed immediately after MyoD in vivo, was observed from 3 days and maintained at 7 days and faded away at 14 days (Figure 30). This data is consistent with the function of this protein that induces the formation of multinucleated cells, observable in treated cultures at 14 days.

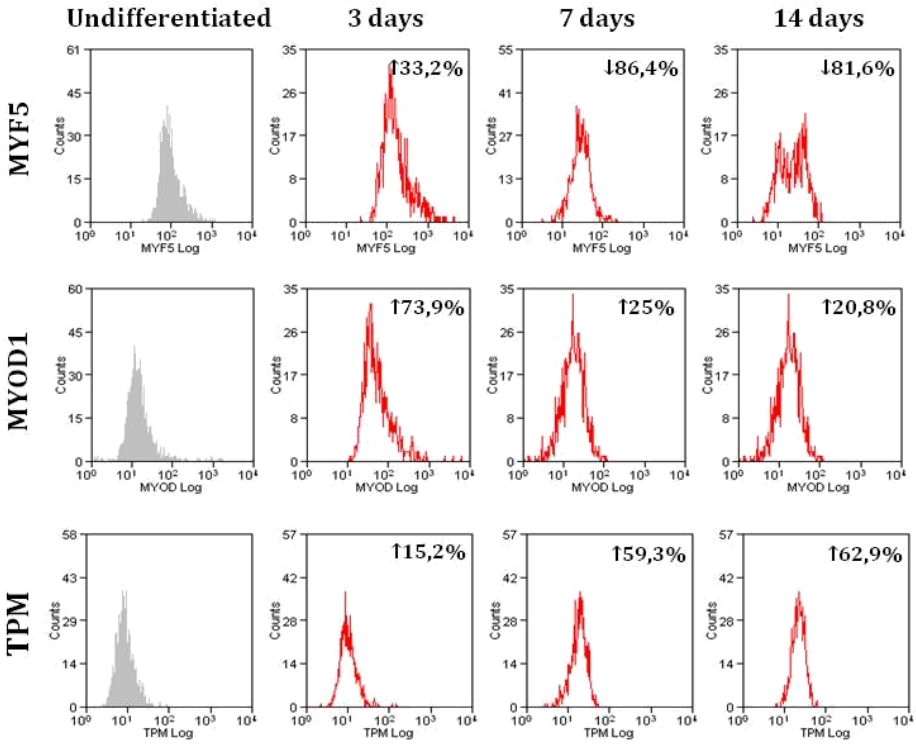


Figure 30. FCM study of specific markers in hPBMCs differentiated towards myogenic lineage. Data are expressed as % positive treated cells compared to undifferentiated control cells

The differentiation of hPBMCs towards a mature muscle phenotype was highlighted by the presence of proteins that constitute to form sarcomeres, such as myosin and tropomyosin. Myosin heavy chain, as protein, was not expressed in early days of induction and started to appear at 7 days and progressively increased with the time of differentiation (Figure 31). Moreover, at 7 and 14 days, as evidenced from the Figure 29 and 30, the expression of sarcomeric tropomyosin, both as mRNA and protein, was less appreciable at 3 days and during the final fusion stage of differentiation, there was a progressive increase.

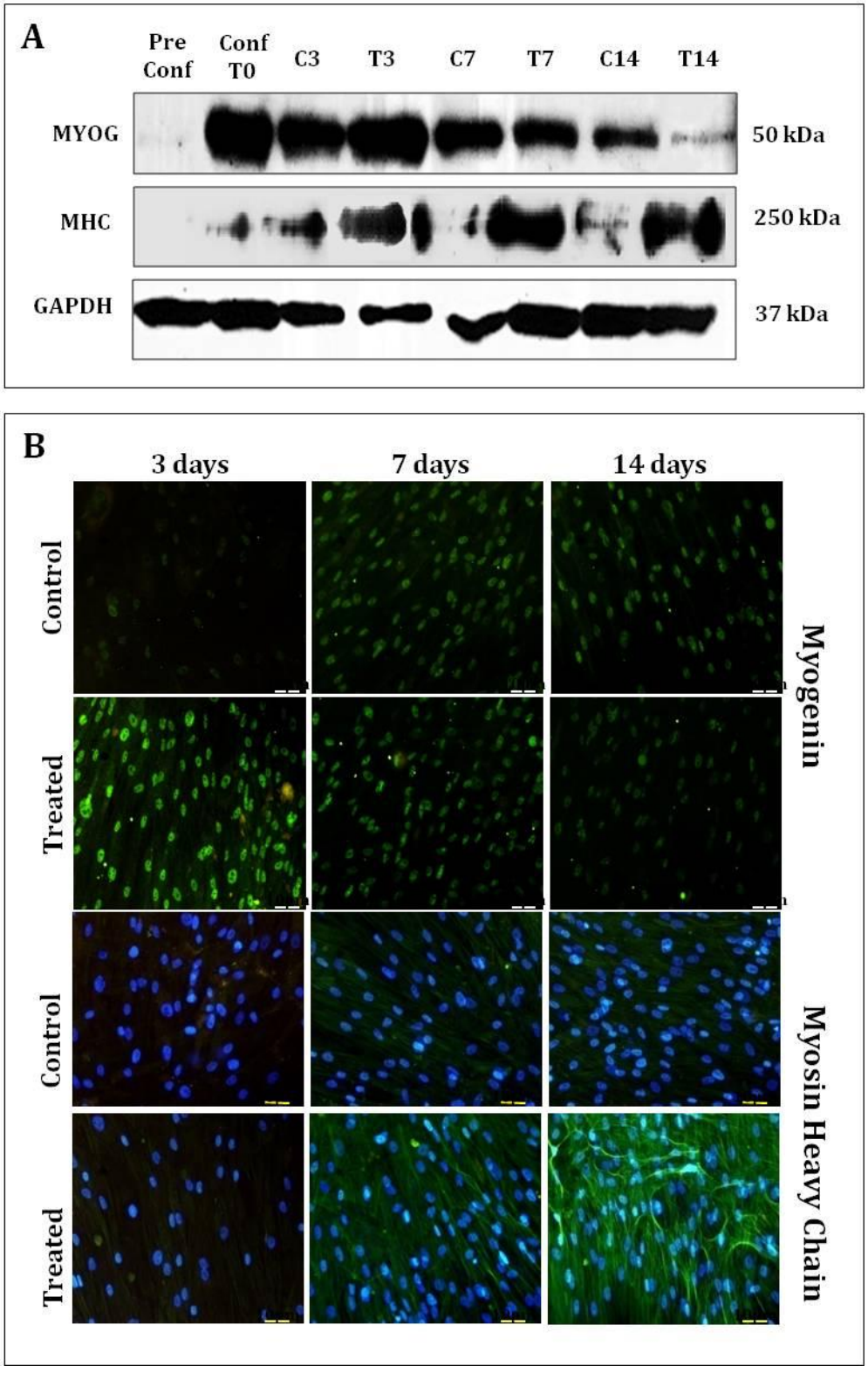


Figure 31. Protein expression analysis by Western Blot (A) and Immunofluorescence (B) of myogenic markers in hPBMCs treated with inductive medium

Skeletal myogenesis is a developmental cascade that involves the regulatory MyoD gene family that determines the progress of multipotential progenitors to myogenic lineage. The MyoD family is one of the basic helix loop helix transcription factors that directly

regulate myocyte cell specification and differentiation (Edmondson et al., 1993). In all the anatomical sites where skeletal muscle forms, determination and terminal differentiation of muscle cells are governed by a network of four MRFs: myogenic factor 5 (MYF5), muscle-specific regulatory factor 4 (MRF4; also known as MYF6), myoblast determination protein (MYOD) and myogenin. MRFs are transcription factors that activate many downstream genes to initiate muscle cell differentiation. MYOD and MYF5 are muscle-specific transcription factors and constitute a cross-regulatory transcriptional network that is at the core of muscle cell determination and differentiation; disruption of this network completely abrogates skeletal muscle formation. MYF5 and MYOD are generally thought to act as determination genes, whereas myogenin is essential for the terminal differentiation of committed myoblasts (Braun et al., 2011). In vitro differentiation of hPBMCs as a response to stimulation by IGF1 and vitamin C seem to be in line with the time specific expression pattern of skeletal muscle diiferentiation, evidenced by the initial expression phase of MYF5, immediately followed by MyoD and the expression of Myogenin during the terminal differentiation.

4. In vivo evaluation of hPBMCs in a muscle damage model

To evaluate the integration capacity of hPBMCs, a bupivacaine induced muscle damage model was used. After 2 days of inducing necrosis in the tibialis anterior muscle, Qdot 800 marked hPBMCs were injected on the site of injury and the cryostat sections revealed interesting results. As we can appreciate from Figure 32, co localization of hPBMCs (red) along with vimentin (green) were located along the muscle fibres of the host and hPBMCs took the parallel alignment of the host muscle organization from 7 days. At 14 days, hPBMCs seem to fuse with the host muscle fibres giving an appearance of an ex vivo repair at the site of damage. When transplanted into the tibialis anterior muscle, hPBMCs survived for up to two weeks in absence of immunosuppression, migrating into the muscle among muscle fibers. Moreover, we never observed cell masses suggestive of tumorigenesis. Those which remain close to the injection site show an immature phenotype, whereas those in the muscle have more elongated morphologies. When in close proximity to muscle cells, hPBMCs displayed an elongated morphology aligned with muscle fiber orientation. This is in accordance with existing reports, who showed that strings of peripheral MSC nuclei can be positioned along the length of pre existing fibers (Shabbir et al., 2009).

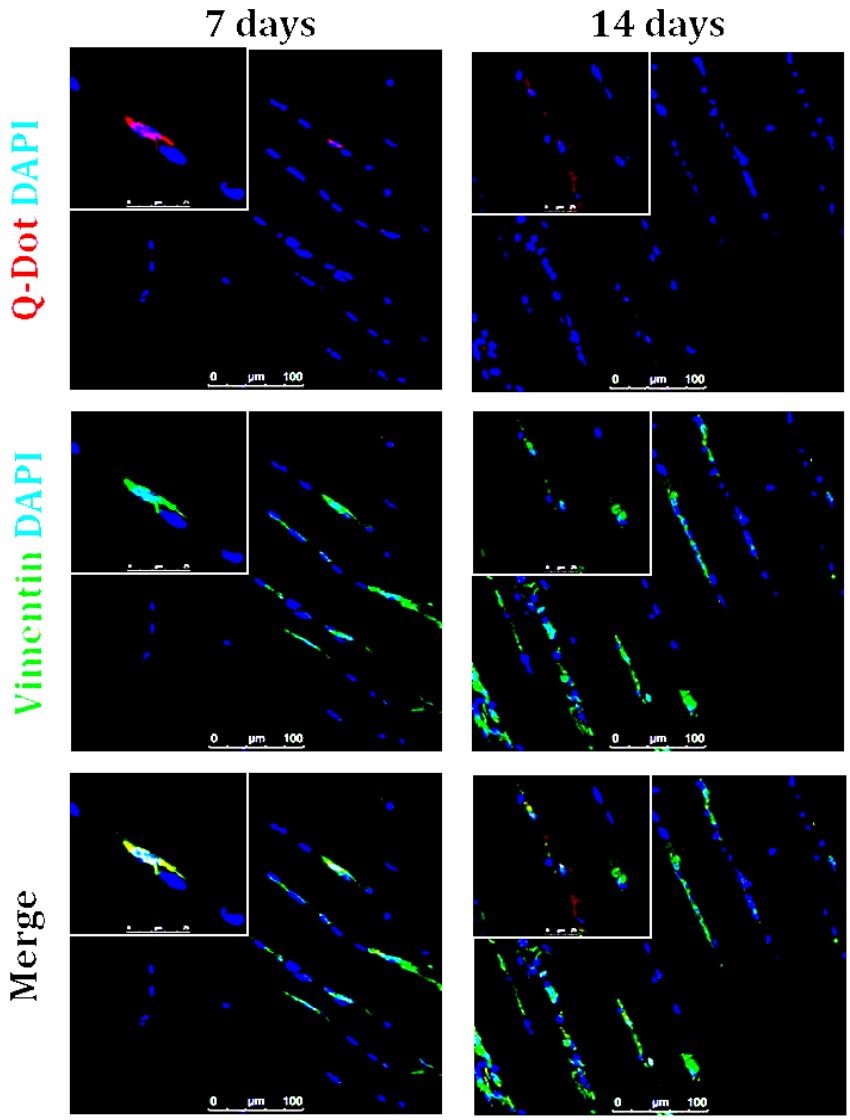


Figure 32. Immunofluorescence analysis of vimentin in rat tibialis anterior muscle after damage induction with bupivacaine hydrochloride and injected with Q Dot labeled hPBMCs for 7 and 14 days

CONCLUSIONS

'Stem cell plasticity' is a new term that has been used to describe the recent observations that greater potential might persist in post-natal adult stem cells than previously thought. Normally, stem cells are maintained in a quiescent state and need specific stimuli for renewal and differentiation. When stem cells differentiate, they have to suppress genes that are incompatible with the upcoming cell type (Belema et al., 2005). In this thesis, peripheral blood multipotent cells (hPBMCs), having an identity close to mesenchymal and non-hematopoietic, were isolated from normal healthy patients without any invasive mobilization techniques, cultured in a standard proliferation medium without any specific growth factors and were directed to switch phenotype towards neuronal and myogenic lineage. Peripheral blood, when compared with bone marrow, present an optimal source due to its easy and abundant availability and the multipotent cells (hPBMCs), which were isolated without any usual mobilization techniques, represent an ideal type of cells for regenerative medicine applications. hPBMCs were highly responsive to external stimuli and presented a stable karyotype with multidifferentiative potential, essential characteristics of adult stem cell plasticity. The absence of expression of HLA-DR on hPBMCs was evident that these cells have the least probability to elicit an immune response during transplantation. hPBMCs hide in them an innate ability to break the lineage barrier was evident by the findings that they expressed several specific markers for neuronal and myogenic lineages at an undifferentiated state. The neuronal lineage switch was evident from the late phase of differentiation, when Retinoic acid and Nerve growth factor acted synergistically to induce the expression of several specific neuronal markers in hPBMCs. Partial differentiation of adult stem cells had been widely explored, yet the functional side of it remains elusive. It was interesting to note that hPBMCs could overcome their lineage imprint and initiate a different lineage program to attain a functional neuronal phenotype as highlighted by the calcium flux activity and neurotransmitter release. Transcription factors involved in myogenic lineage progression are not strictly acting in a linear manner but are organized in complex feedback and feed-forward networks. The temporal expression pattern of Myogenic determination factors was successfully simulated in vitro due to the combined induction of IGF1 and Vitamin C. hPBMCs present a candidate population suitable for in vivo transplantation studies as they integrated

well within the host tissue and presented no risk of tumorigenesis. As highlighted by the abovementioned results, hPBMCs represent an ideal invitro model for synthetic substrate testing studies for neurogenic and myogenic differentiation and drug screening assays. hPBMCs can be subjected to high throughput expansions and can be stored for future translational studies. The potential to exploit adult stem cell plasticity for degenerative diseases has been met with disappointing results. It is yet to be well documented that single cells derived from adult tissue differentiate into multiple lineages characterized not only based on phenotype but also on function and support, sustained and functional multilineage engraftment in vivo. Nonetheless, future concerted effort, aimed at dissecting the rare phenomenon of lineage switching, rigorously identifying, purifying and potentially expanding the appropriate cell populations responsible for plasticity, characterizing the tissue-specific and injury-related signals that recruit, stimulate, or regulate plasticity, and determining the mechanisms underlying plasticity, enhance tissue regeneration to clinically useful levels and could yield fruitful results in animal models of degenerative diseases.

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