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Department of Histology and Medical Embriology

PhD in
Cell Science and Morphogenesis

XVII CICLO

PHARMACOLOGICAL ACTIVATION AND REPROGRAMMING OF MUSCLE SATELLITE CELLS TO ACQUIRE AN EARLIER, PLURIPOTENT, CIRCULATING "STEM-LIKE" BEHAVIOUR, SUITABLE FOR THERAPEUTICAL APPLICATIONS

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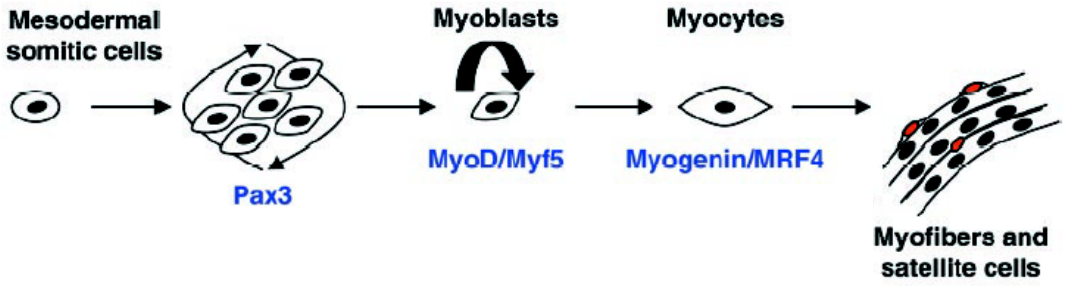
INTRODUCTION

Embryonic Myogenesis

All vertebrate skeletal muscles (apart from head muscles) derive from mesodermal precursor cells originating from the somites (Pourquie, 2001;Summerbell and Rigby, 2000). During embryonic development, specification of mesodermal precursor cells to the myogenic lineage is regulated by positive and negative signals from surrounding tissues. Specification to the myogenic lineage requires the upregulation of MyoD and Myf5, basic helix-loop-helix transcriptional activators of the myogenic regulatory factor family (MRF). Proliferating MyoD and/or Myf5 positive myogenic cells are termed myoblasts. These cells withdraw from the cell cycle to become terminally differentiated myocytes expressing the "late" MRFs, Myogenin and MRF4, followed by the expression of muscle-specific genes, such as myosin heavy chain (MHC) and muscle creatine kinase (MCK). Finally, mononucleated myocytes specifically fuse to each other to form multinucleated syncytium, which eventually mature into contracting muscle fibres (Fig. 1). The existence of this regulatory network mostly comes from the phenotype observed in mutant mice. In fact, MyoD/Myf5 double knockout mice totally lack skeletal muscle, and putative muscle progenitor cells remain multipotential contributing to non muscle tissues in the trunk and the limbs of these mice (Rudnicki et al., 1993;Kablar et al., 1999). By contrast, in myogenin-deficient mice MyoD and/or Myf5 positive cells are present, but the embryos die peri-natally due to a lack of myoblast differentiation (Nabeshima et al., 1993;Hasty et al., 1993). Similarly, MRF4-deficient mice display a range of phenotypes consistent with a late role for MRF4 in the myogenic pathway (Braun and Arnold, 1995;Patapoutian et al., 1995). During the course of muscle development, a distinct subpopulation of myoblasts fails to differentiate, but remains associated with the surface of the developing myofiber as quiescent muscle satellite cells, contributing to the post-natal skeletal muscle growth and remodelling.



(Chargè and Rudnicki ,2004 modified)

Fig.1 *Pax3 expression in precursor cells contributes to myogenic cell expansion. Upon Myf5 and/or MyoD induction, mesodermal somitic cells are committed to the myogenic lineage (myoblasts). Following upregulation of the late MRFs (myogenin and MRF4) myoblasts undergo differentiation into myocytes. Finally myocytes fusion gives rise to terminally differentiated multinucleated myofibres.*

Adult Myogenesis

Skeletal muscle contains a latticework of connective tissue surrounding bundles, or fascicle, of multi-nucleated myofibres (Seale et al., 2000c). Most of these myofibres are formed during postnatal development from the interaction of committed myogenic cells that fuse together to form multi-nucleate myofibres. The primary source of these committed myogenic precursors, often called myoblasts, is a population of resident progenitors termed satellite cells. These satellite cells maintain their own cellular structure and typically reside between the plasma membrane and basal lamina surrounding the myofibres (Mauro, 1961); they do not actively participate in generating force, rather, they remain quiescent until external stimuli trigger their activation and re-entering into the cell cycle. Satellite cell specification requires the expression of the *pair-ruled* transcription factor Pax7; in fact, almost no satellite cells are detectable in Pax7 null mice, where muscle regeneration is severely impaired (Seale et al., 2000b). Upon activation, the progeny of satellite cells, the myoblasts, fuse to form new myofibres or to augment existing fibres (Bischoff and Heintz, 1994;Cossu et al., 1980;Cornelison and Wold, 1997d;Yablonka-Reuveni and Rivera, 1994). In addition to satellite cells and myoblasts, much recent evidence suggests the maintenance, in the adult, of a separate and distinct progenitor cell population within skeletal muscle: the muscle derived stem cells (MDSC).

Isolation and identification of cell populations from various tissues is dependent upon the expression of specific markers, either exclusive or as a characteristic combination of common surface proteins. Figure 3 shows a proposed mechanism of skeletal muscle precursor cell differentiation within the myogenic lineage, including MDSC, and the consensus of markers distinguishing various stages along the lineage. To date Sca-1, Bcl-2 and CD34 have been consistently identified as characteristic markers of MDSC. Among MDSC, a subset population, the "side population" (SP), has been identified by the Hoechst exclusion technique, due to the high expression of ABC transporters (Jackson et al., 1999;Gussoni et al., 1999a). Pax7 up-regulation in MDSC induces satellite cell specification by restricting alternative developmental programs. Cell surface markers associated with the satellite cell phenotype, either in the quiescent or activated state also include: M-cadherin, c-Met and CD34 (Cornelison and Wold, 1997c;Beauchamp et al., 2000;Miller et al., 1999).

Quiescent satellite cells express no detectable levels of MRFs (Cornelison and Wold, 1997b). Upon activation, Pax7 rapidly induces MyoD expression, prior to the expression of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation; the subsequent differentiation events follow a program similar to that occurring during embryonic myogenesis. Myogenin and MRF4 are expressed last, to trigger terminal differentiation.

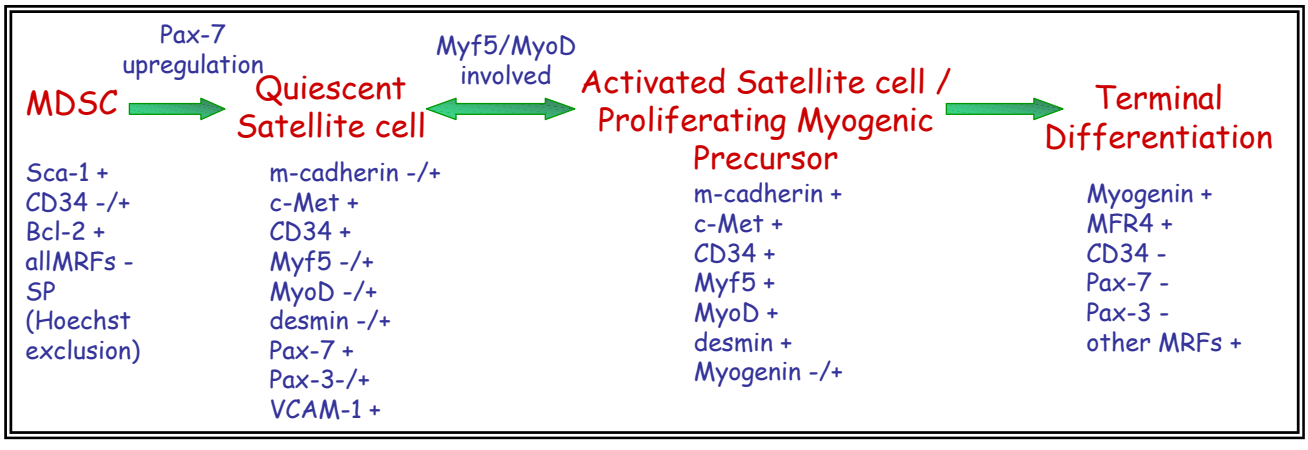
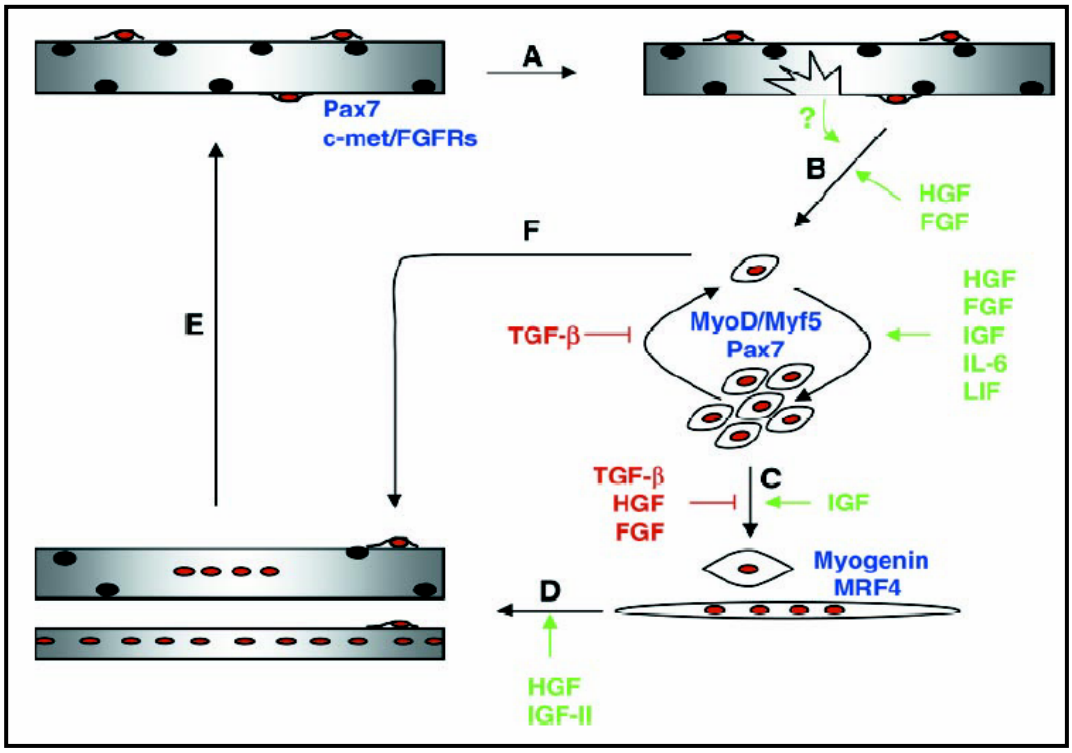


Fig. 3 *Proposed mechanism of myogenic differentiation in the adult and the consensus of markers that distinguish various stages along the lineage.*

Skeletal Muscle Regeneration

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei, but has the ability to complete a rapid and extensive regeneration in response to even severe damage. Whether muscle injury is a result of a direct trauma or of genetic defects, muscle regeneration is characterised by two phases: a degenerative phase followed by a regenerative phase. The initial event of muscle degeneration is muscle fiber necrosis. This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability. The early phase of muscle injury is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and myogenic cells. Current reports suggest that factors released by the injured muscle activate inflammatory cells residing within the muscle, which in turn provide the chemotactic signals to circulating inflammatory cells: neutrophilis and macrophages (Tidball, 1995).

Muscle degeneration is followed by the activation of the muscle repair process (Fig. 2). Satellite cells first exit their normal quiescent state to start proliferating. Cell proliferation is an important event required for muscle regeneration. The expansion of myogenic cells provides a sufficient source of new myonuclei for muscle repair (Grounds et al., 2002). After several rounds of proliferation, the majority of satellite cells, often referred to as myogenic precursor cells (mpc), differentiate and fuse to existing damaged fibres for repair or to one another for new myofiber formation. On muscle sections, the fundamental morphological characteristics of regenerating muscle are the presence of newly formed myofibres of small caliber with centrally located myonuclei (Fig. 2).



(Chargè and Rudnicki, 2004)

Fig. 2 *Schematic rapresentation of the molecular events regulating muscle satellite cell activation during skeletal muscle regeneration. Quiescent satellite cells are activated to enter the cell cycle and proliferate (A), allowing for expansion of the myogenic cell population (B). The proliferative phase is followed by terminal differentiation (C) and fusion of myoblasts to damaged myofibres for repair or to each other for new myofiber formation (D). Myoblast terminal differentiation is characterised by the upregulation of the MRFs Myogenin and MRF4. Finally, repaired or new myofibres grow to resemble original myofibres (E). During the course of muscle regeneration, a subset of myoblasts re-enters the quiescent state to replenish the satellite cell pool for subsequent muscle repair (F).*
The possible role of several growth factors is also highlighted, among which positive factors are in green while negative factors are in red. HGF, hepatocyte growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL-6, interleukin-6; LIF, leukemia inhibitory factor; TGFβ, transforming growth factor β family.

As mentioned before, the expression program followed during satellite cell activation, proliferation, and differentiation is similar to that occuring during embryonic myogenesis. The first phase is characterised by the rapid up-regulation of the two MRFs required for myogenic specification, MyoD and Myf5 (Cooper et al., 1999a;Cornelison et al., 2000a;Zammit et al., 2002). Proliferating myoblasts withdraw from the cell cycle to become differentiated myocytes expressing the two "late" MRFs, Myogenin and MRF4 and then fuse to damaged myofibres for repair or to each other for new myofiber formation.

In vitro and in vivo experiments have highlighted the possible role of several growth factors, acting through a paracrine or autocrine mechanism to regulate specific events of muscle regeneration. Among them FGF, HGF, IGF, IL6 and LIF may act as positive or negative regulators depending on the specific phase (Fig. 2).

Adult Stem Cells

Adult stem cells (SC) are defined by two major functions: self renewal and multilineage differentiation potentiality. Stem cells are functionally responsible for the development and the regeneration of tissues and organs. Developmental signals, both biochemical and biomechanical, trigger proliferation of stem cells in early and late development. Adult stem cells have been found to reside in a variety of tissues including skin (Watt, 1998), the central nervous system (Gage et al., 1995), muscle (Schultz and McCormick, 1994), bone marrow (Weissman, 2000) the liver (Alison and Sarraf, 1998), and many others. By definition, adult stem cells can self-renew and replenish multiple cell types within the tissue in which they reside.

A central tenet of developmental biology is that, during embryogenesis, all cells become committed to specific lineages, first via germ layer specification, and later through additional levels of differentiation and specialisation. Stem cells that replenish adult tissues are also set aside during development, and these cells were thought to be committed by default to generate only a restricted lineage of cells. However, in the last few years, several reports have suggested that the differentiation capability of adult stem cells might not be restricted to exclusively producing cells specific to the tissue in which they reside, but it may exert a much wider spectrum of potentiality, resulting in the so called “stem cell plasticity”. It is now well known that Hematopoietic Stem Cells (HSC) and Mesenchimal Stem Cells (MSC) are capable of metaplasia into various cell types (Priller et al., 2001b;Priller et al., 2001a). Upon bone marrow transplantation, several laboratories have observed the recruitment of donor-derived cells in several non-hematopoietic tissues, through the expression of reporter genes such as LacZ or green fluorescent protein (GFP) (Ferrari et al., 1998a;LaBarge and Blau, 2002c;Orlic et al., 2001). Similarly, transplantation of cells derived

from brain, muscle, skin and fat, has resulted in the detectable presence of these cells in several lineages distinct from the tissue of origin (Jackson et al., 1999;Bjornson et al., 1999). This behaviour was immediately attributed to a previously unexplored developmental plasticity of stem cells residing in these tissues. It was thus postulated that, given a new microenvironment, adult stem cells can trans-differentiate to produce cells according to the new resident tissue. The notion of adult stem cell plasticity thus challenged the long-held concepts of developmental biology.

Muscle Diseases

The term "myophaty" has come to apply to any disorder which can be attributed to pathological, biochemical or electrical changes occurring in the muscle fibres or in the interstitial tissues of the musculature; such disorders include many which are inflammatory, metabolic or endocrine in nature. The term "muscular dystrophy" should be reserved for cases of progressive, genetically determined, degenerative myopathy.

Muscular dystrophies (MDs) are a heterogeneous group of disorders caused by the mutation of any one of a large number of genes. They are usually characterised by progressive muscle weakness, which, depending on its severity, may result in immobilization and death. Most of these diseases depend on mutation of gene coding for any protein involved in the dystrophin-glycoprotein complex. This complex includes dystrophin, sarcoglycans and dystroglycans, and interconnects cytoskeletal actin with the membrane and extracellular matrix components. It maintains the mechanical stability of the muscle cell membrane by anchoring and supporting the sarcolemma (Ervasti and Campbell, 1993) (Fig. 3). The most common form of MD, Duchenne muscular dystrophy (DMD), is due to the lack of dystrophin expression (Hoffman et al., 1992). Morphologically, dystrophic muscle appears to be in a constant state of spontaneous necrosis and degeneration, with a concomitant gradual replacement of muscle fibres by fibrous tissue. Thus, satellite cell-driven muscle regeneration is not as efficient in balancing muscle degeneration.

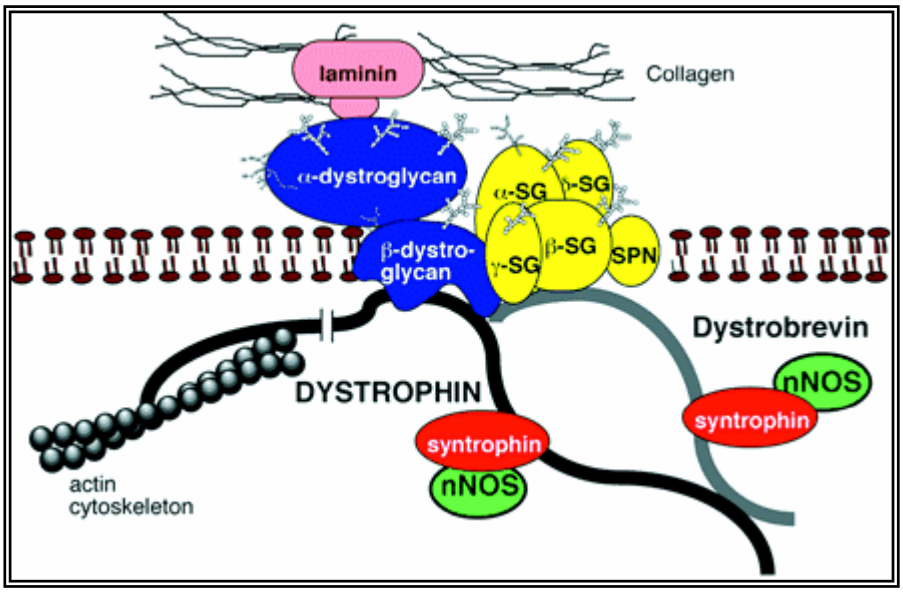


Fig.3 *Dystrophin and the dystrophin-glicoprotein complex in skeletal muscle.*

Although various approaches to deliver dystrophin in dystrophic muscle have been investigated extensively (e.g. cell and gene therapy), there is still no treatment that alleviates the muscle weakness in this common inherited disease.

Cell therapy

There are currently three main experimental approaches to setting therapy for muscular dystrophies. 1) Gene therapy focuses on the development of new vectors capable of efficiently delivering the missing gene to the postmitotic nuclei of the muscle fibres *in vivo*. 2) The pharmacological approach aims to restore the protein complex that is altered in many forms of muscular dystrophy through different strategies. 3) The cell therapy approach aims to functionally rescue the tissue by delivering "sane" cells, capable of reconstituting the tissue; these may be satellite cells or pluripotent stem cells.

Third approach appears to be the more promising one, although its use still presents many limitations. Re-appearance of dystrophin expression may result from the conversion of native myofibres through donor-host fusion and from the formation of new myofibres from both donor-donor and donor-host cell fusion. This process has been investigated extensively

in both animal and human clinical trials. Restoration of dystrophin expression can be achieved through transplantation of donor-derived dystrophin-expressing myogenic cells into dystrophic host skeletal muscle; however, only a minority of the grafted cells are responsible for new muscle formation, while the majority of them quickly die (Beauchamp et al., 1999).

The recent identification of stem cells suitable for protocols of organ regeneration has opened new perspectives in cell-mediated therapy. Some examples include muscle-derived stem cells (MDSCs) (Torrente et al., 2001b) and the mesoangioblasts (Sampaolesi et al., 2003a). Intra-muscular and intra-arterial injection of MDSCs into a dystrophic skeletal muscle have demonstrated the ability of these muscle-derived stem cells to increase muscle regeneration and improve the delivery of dystrophin (Torrente et al., 2001a;Lee et al., 2000), although the efficiency of muscle engraftment was only 12%. A hallmark of any stem cell therapy should be morphological and functional restoration of the target tissue. The recent identification of a vessel-associated stem cell population, the mesoangioblasts, has opened new perspectives. Under culture conditions, these clonally derived cells express CD34, c-Kit, and Flk-1. It has recently been shown that transplanted donor-derived mesangioblasts into α–sarcoglycan$^{-/-}$ (SCGα$^{-/-}$) dystrophic mice were capable of restoring SCGα expression, muscle architecture and function (Sampaolesi et al., 2003b). These results showed a significant functional improvement in the pathology of this mouse strain using a wide variety of physiological tests (Sampaolesi *et al*.2003). However, as these cells are derived from foetal tissue, it may be difficult to identify and purify them on the basis of expression of specific markers for use in adult humans.

In recent years cell therapy has even been taken into consideration for the treatment of heart disease. Cellular cardiomyoplasty (cellular transplantation for cardiac repair into injured cardiac regions) has recently become an area of intense research interest in the treatment of cardiovascular disease. Stem or progenitor cells can be delivered into the site of cardiac injury, restore blood flow and contractility to a previously infarcted, scarred or dysfunctional heart (Taylor, 2004a). Preclinically, many types of cells have been transplanted into injured myocardium, including cardiomyocytes, autologous myoblasts, smooth muscle cells, fibroblasts and bone marrow-derived stem cells. Although several of these cell types may hold future promise as a therapeutic option, to date only skeletal myoblasts, and bone marrow-derived stem cells have been used in safety studies as a first step towards myocardial repair (Carvalho et al., 2004;Taylor, 2004c;Horackova et al., 2004). The transplantation of

these two types of cells is still ongoing. Clinical studies show that skeletal myoblasts can be engrafted into infarcted regions of myocardium, increase myocardiac wall thickening and contractility; however, it is not clear if and how myoblasts electrically integrate into surrounding myocardium and what impact integration may have on either function or rhythmicity. In fact, early clinical data suggest that myoblast transplantation may be associated with a transient period of electrical instability (Taylor, 2004b). The advantage of using bone marrow-derived stem cells is that they contribute to the increase of cardiac angiogenesis. The use of these cells in clinical trials shows an enhancement in left-ventricular function and improvement in infarct tissue perfusion. Thus, transplantation of stem cells is considered the most promising therapeutic approach to the damage caused by cardiovascular, myogenic, neuro degenerative diseases and aging. However, the use of stem cells in cell therapy applications remains problematic for a number of reasons: the difficulty in obtaining, expanding and culturing ex vivo adult stem cells, the insufficient number of cells obtained from a biopsy, the lack of robust methods for their propagation and efficient differentiation, as well as host rejection to allogenic cells (Deasy et al., 2002); de Hann et al. 2003).

A desirable goal in cell therapy is to obtain a population of primary cells retaining genetic and phenotypic flexibility to commit toward distinct lineages (pluripotent stem cells), suitable for efficient in vitro expansion, for genetic engineering, and for successful reintroduction in vivo as a gene-cell therapy protocol.

Reprogramming of committed cells

Terminally differentiated mammalian cells are thought to be incapable of undergoing reversion from the differentiated state. Muscle cells represent a very suitable model for terminal differentiation. These cells have permanently exited the cell cycle through the expression of cyclin-dependent kinase (cdk) inhibitors, and are driven “irreversibly” to their final morphology and function through the accumulation of proteins critical for cellular specialisation. However, a number of recent studies indicate that ectopic expression of certain genes can induce established cell lines and even terminally differentiated cells, to dedifferentiate and/or transdifferentiate, indicating that some plasticity can be induced in already committed/differentiated cells. This adds to the well established capability of both

HSC and MSC to transdifferentiate into various cell types (Ferrari et al., 1998b;Gussoni et al., 1999b;LaBarge and Blau, 2002b;Camargo et al., 2003). In fact, several groups have recently demonstrated that somatic cells can be induced to functionally reprogram. As an example, fibroblasts can be induced to express T-cell specific genes and functions, when cultured in the presence of nuclear and cytoplasmic extract derived from lymphocytes (Hakelien et al., 2002d); myoblasts can also be converted to physiologically active neuronal phenotype through the activation of genes of neuronal differentiation (Watanabe et al., 2004b); surprisingly, even terminally differentiated myotubes can be induced to dedifferentiate when stimulated with appropriate signals such as the expression of msx1 (Hu et al., 1995c;Hakelien et al., 2002c;Odelberg et al., 2000;Kondo and Raff, 2000b).

These observations suggest that reprogramming of gene expression in committed cells in order to obtain the reversion into an earlier pluri/multipotent "stem-like" cell, might be a feasible option to develop innovative cell therapy strategies.

To this end, at first the differentiation program in committed cells should be reversibly inhibited. In muscle cells it is well known that the expression of many oncogenes (such as c-Myc or v-Src) reversibly inhibits differentiation *in vitro* due to reduced expression and/or abnormal functioning of MRFs (Falcone et al., 2003;La Rocca et al., 1994). Although these studies demonstrate that myogenic differentiation can be reversibly inhibited, the use of oncogene expressing cells or retroviruses infection, even as a transient expression, cannot be proposed for therapeutic strategies.

The possibility of pharmacologically inducing intracellular signalling pathways in order to inhibit differentiation while maintaining cell proliferation, could be a reasonable alternative; reversibility should be achieved just by removal of the drug. It is well known that NFkB and AP1 transcriptional activity are quickly and strongly down-regulated when muscle cells are transferred into differentiation medium (Lehtinen et al., 1996). Thus, the activity of these factors is not compatible to the differentiation program. In fact, forced activity NF-kB and/or AP-1 inhibits myogenesis through regulation of Cyclin D1, and/or Myogenin and MyoD (Guttridge et al., 1999d;Guttridge et al., 2000). Thus, the use of drugs which activate these pathways may represent a possible approach in reversibly inhibiting muscle differentiation.

NFkB

The nuclear factor-κB (NF-κB) proteins are a small group of closely related transcription factors, which in mammals consists of five members: Rel (also known as c-Rel), RelA (also known as p65 or NF-κB3), RelB, NF-κB1 (also known as p50) and NF-κB2 (also known as p52). All five proteins have a Rel homology domain (RHD), which serves as the principal regulatory domain for dimerization and DNA-binding (Fig. 4). The C terminus of RHD contains the nuclear-localization sequence (NLS) which is masked in non stimulated cells through the binding of specific NF-κB inhibitors, known as the IκB proteins (Boone et al., 2002b).

Nuclear transcription factor-kB plays a pivotal role in expression of various inducible target genes related to immune and inflammatory responses, including the type I human immunodeficiency virus. In non-stimulated cells, the heterodimer NF-kB complexes are associated with its inhibitor, one of the IkB family members, thus retained in the cytoplasm. In mammalian species, six structural homologs of IkB have been identified, but only one of them, the IkBα form, has been extensively studied. In response to stimulation by various agents, such as phorbol esters (*e.g.* phorbol 12-myristate 13-acetate), tumor necrosis factor (TNF), interleukin-1a (IL-1a), γ-radiation, and lipopolysaccharide, IkBα undergoes degradation, allowing the heterodimers to translocate to the nucleus (Fig.4). Upon stimulation by TNF or IL-1, a protein kinase complex, containing the IkB kinase subunits α, β and γ (also known as NEMO), phosphorylates IkBα in Ser-32 and Ser-36 residues; these phosphorylation events are required for its degradation and consequent NF-kB nuclear import. Before being degraded by 26 S proteasome, serine-phosphorylated IkBα is polyubiquitinated at the Lys-21 and Lys-22 positions. Recently, it was also shown that hypoxia, reoxygenation, and the PTPase inhibitor pervanadate (PV) induce IkBa Tyr42 phosphorylation and results in NF-kB activation. Accordingly, protein tyrosine kinases (PTKs) and phosphatases (PTPase) inhibitors suppress NF-kB activation. Thus, IkBα Tyr42 phosphorylation also triggers NF-kB activation, but the PTK that phosphorylates IkBα is not known nor is the molecular mechanism fully understood. Whether Tyr42 phosphorylation leads to IkBα degradation and whether degradation is required for NF-kB activation is not known either.

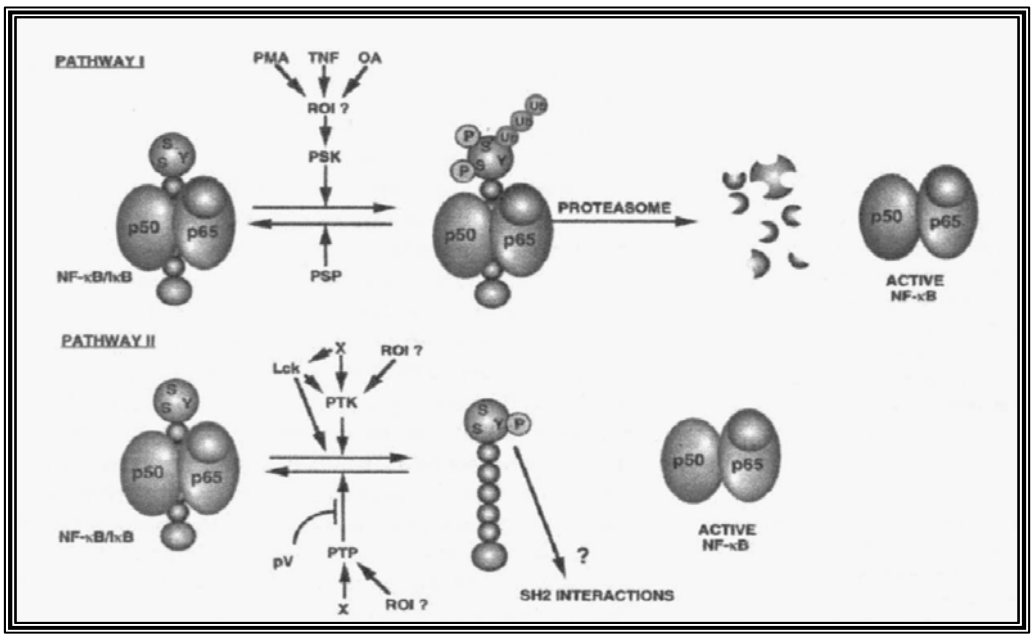


Fig. 4 *Model of NFkB activation. Pathway I: activation of NFkB through degradation of IkBα. Pathway II: NFkB activation through tyrosine phosphorylation of IkBα.*

AP-1

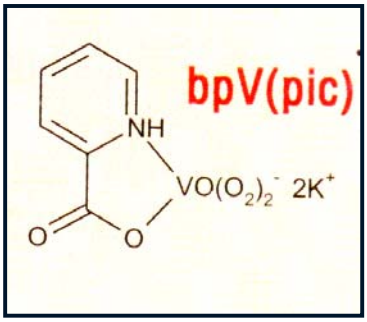
Activation Protein 1 (AP-1) consists of several basic region leucine zipper (bZIP) domain proteins, Jun, Fos, and ATF subfamilies; all of them have to dimerize before binding to the specific DNA target sites. AP-1 trancription factor regulates many aspects of cell physiology in response to environmental changes such as stress, radiation or in response to growth factor signals (Zhou et al., 2001).

Recent studies, using cells and mice deficient in individual AP-1 proteins, have begun to clarify their physiological functions in the control of cell proliferation, neoplastic transformation and apoptosis. Above all such studies have identified some of the target genes that mediate the effects of AP-1 proteins on cell proliferation and death. There are evidences that AP-1 proteins, mostly those belonging to the Jun group of proteins, control cell life and death through their ability to regulate the expression and the function of cell cycle regulators such as Cyclin D1, p53, p21, p19 and p16. Among the Jun proteins, c-Jun is unique in its ability to positively regulate cell proliferation through the repression of the expression and function of tumor suppressor genes, and the induction of cyclin D1 transcription. These

actions are antagonized by JunB, which upregulates tumor suppressor genes and represses cyclin D1 (Shaulian and Karin, 2002).

Bis-peroxovanadium (BpV)

In order to pharmacologically activate NFkB and AP1 pathways in muscle cells, we treated myoblasts, cultured in differentiating conditions, with Bis-peroxovanadium (BpV), a protein tyrosine phosphatase (PTP) inhibitor, known to be a strong activator of both pathways. As expected, in these conditions, myogenesis is inhibited; moreover, the cells continue to proliferate and acquire a gene expression profile and a plasticity compatible with an "earlier" and/or "stem-like" phenotype. Removal of the drug rescues the muscle differentiation capability. This may thus represent a valuable approach to easily obtaining multi-potent cell populations from committed cells, suitable for gene-cell therapy applications; moreover, it may be instrumental to study the molecular mechanisms responsible for "stem cell" phenotype and genome reprogramming.



Phospho-Tyrosine phosphatases (PTP) inhibitor

AIM OF THE WORK

It is well established that both HSC and MSC are capable of transdifferention into cell types different from the tissue in which they originate (Ferrari et al., 1998c;Gussoni et al., 1999c;LaBarge and Blau, 2002a;Camargo et al., 2003). However, given the difficulty to obtain, expand and culture *ex vivo* adult stem cells, the physiological reservoir of precursors for various tissues throughout life, and considering the ethical problems associated with the use of human Embryonic Stem Cells, a desirable goal in cell therapy is to obtain a population of primary cells retaining genetic and phenotypic flexibility to commit toward distinct lineages (pluripotent stem cells), suitable for efficient *in vitro* expansion, genetic engineering, and successful reintroduction *in vivo* as a gene-cell therapy protocol.

Terminal differentiation of mammalian cells was thought to be an irreversible process, however, a number of recent results indicate that some "stem-like" plasticity can also be induced in already committed cells (Hu et al., 1995b;Hakelien et al., 2002b;Odelberg et al., 2000;Kondo and Raff, 2000a). These observations suggest that reprogramming of gene expression in committed cells, in order to obtain their reversion into an earlier pluri/multipotent "stem-like" cell, might be a feasible option to develop innovative cell therapy strategies.

The aim of this work is to pharmacologically activate intracellular pathway(s) which may induce reprogramming of gene expression in committed muscle satellite cells, and lead the cells toward an earlier phenotype characterised by the ability to modulate self-maintenance, activation and expansion. The pharmacological approach should give the advantage of being a reversible and less invasive treatment in respect to a genetic-based treatment. To this end, we tested and characterised the ability of Bis-peroxovanadium (BpV), an activator of both NFkB and AP1 pathways, to induce the reversion of C2C12 or primary satellite cells to an earlier, multipotent and circulating progenitor, suitable for therapeutical applications.

MATERIALS AND METHODS

Cell cultures

C2C12 cells, a subclone of the C2 mouse myoblast cell line, were plated at 25.000 cells/35mm Petri dish and maintained in Dulbecco's Modified Eagle medium (D-MEM) supplemented with 10% Foetal Calf Serum (FCS) (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. To induce differentiation the medium was replaced with differentiation medium, D-MEM supplemented with 2% Horse Serum (HS) (Gibco). BpV (10μM) or TNFα (20 ng/ml) were added at the time of medium replacement.

Primary satellite cells (MSC) were obtained by enzymatic digestion of 6 old days mice leg muscles. Muscles were minced and incubated in PBS containing 1mg/ml collagenase/dispase (Roche) for 20 minutes at 37°C. The enzyme activity was inactivated by five times dilution with PBS. Tissue fragments were removed by filtration through 70μm-pore-diameter nylon mesh, while the cells were collected by centrifugation at 1200 rpm for 15 minutes. The cell pellet was suspended in D-MEM containing 20% HS and 3% chick embryo extract (EE) and cultured at 37°C in 10% CO_2 for a pre-plating. After three hours, cell suspension was plated in collagen-coated dishes. To induce differentiation the medium was replaced with D-MEM containing 5% HS and 1,25% EE. BpV (10μM) or TNFα (20 ng/ml) were added at the time of medium replacement.

Neonatal cardiac myocytes were obtained by enzymatic digestion of 1-2 day old mice hearts. Atrial tissue was removed, and the ventricles were cut in four equal parts in a ADS buffer (NaCl 116mM, KCl 5,36mM, NaH_2PO_4 0,9mM, $MgSO_4$ 0,4M, Glucose 5,5mM, Hepes 18mM) prior to enzymatic digesion. The ventricular tissue was subjected to multiple rounds of enzymatic digestion of 12 minutes at 37°C using an Enzyme Solution (ES) containing 108 U/ml Collagenase Type 2 (Worthington) and 0,9mg/ml Pancreatin (Sigma); the digestion was blocked by adding 1-2 ml of HS. Cells were collected by centrifugation, suspended in plating medium (DMEM supplemented with 10% HS and 5% FCS) and plated for 2 hours in

uncoated cell culture dishes. The cardiomyocyte enriched suspension was then plated on collagen-coated Petri dishes.

Cell transfections and luciferase assay

For transient transfection assay 1 x 10^5 C2C12 cells were plated into 35 mm tissue culture dishes. After 24 hours, proliferating myoblasts were transfected with 1,5 μg of plasmid DNA/dish, using the lipid based Lipofectamine Reagent (Invitrogen), according to the manufacturer's instructions. After additional 32 hours the cells were incubated in DM containing or not 10μM BpV or 20ng/ml TNFα. For reporter luciferase assay the cells were harvested in Reporter Lysis Buffer 1x (Promega) and then centrifuged at 12.000xg for 15 seconds at room temperature. Supernatant was then analysed for luciferase assay reporter activity with the employment of the AutoCliniLumat LB 952T/16 (Berthold), by using Luciferase Assay Reagent containing the luciferin substrate (Promega).
The firefly luciferase, a monomeric 61 kDa protein, catalyses luciferin oxidation, a bioluminescent reaction, using ATP-Mg^{2+} as a substrate. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the molecular product oxyluciferin. Luciferase activity was expressed as the relative fold induction compared to the luciferase activity assayed in untreated cells.

Western Blot analysis

C2C12 cells were harvested and lysed in ice cold RIPA extraction buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 150 mM NaCl, 50mM NaF) supplemented with protease inhibitors. Supernatant was collected following high speed centrifugation for 20 minutes at 4°C. Equal amounts of protein extracts were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blocking was performed by using 5% dry milk in TBST (25mM Tris-HCl pH 8, 125mM NaCl, 1.1% Tween20). Primary and secondary antibodies were diluted in 5% dry milk in TBST, and the incubation proceeded for 30 minutes at room temperature. Washes

were performed in TBST for 5-10 minutes and repeated five minutes. Detection was performed with HRP-conjugated antibodies (Bio-Rad Laboratories) and membrane-bound immune complexes were visualised by CDP Star (PerkinElmer).
Western blots were probed with following antibodies: anti-p65 (Laboratories Tranduction), ph-Tyr, IkBα, Pc-jun, actin (Santa Cruz).

Immunofluorescence analysis

Collected muscle samples were embedded in OCT compound (Tissue freezing medium, Fluka) and frozen in liquid nitrogen cooled isopentane. Seven-micrometer cryosections were fixed in 4% paraformaldehyde. Cells were fixed in Ethanol/Acetone (1:1 ratio) at -20°C for 20 min.
Fixed cells or tissue sections were then washed with Ca and Mg Free PBS (CMF) (Sigma), incubated in CMF containing 1% BSA (bovine serum albumine) for 10 minutes at R.T. followed by an additional incubation in CMF containing 1% goat serum for 30 minutes. The cells or tissue sections were then incubated with primary antibody, at the appropriate dilutions, overnight at 4°C in humidified conditions. After an additional incubation with CMF/BSA 1% the cells were incubated for 1 hour with fluorochrome-conjugated secondary antibody (Sigma) and Hoechst 33342 (Fluka) for nuclear staining.
After extensive washing with CMF, cell monolayers or tissue sections were mounted in 10 mM Tris-HCl, pH 9, containing 60% glycerol and examined under a Zeiss Axioskop 2 Plus fluorescence microscope. Images were acquired by a digital camera using the Axiocam software and then exported into Adobe Photoshop for processing.
Antibodies used were: MF20 provide by Fishman (Bader et al., 1982), anti Cardiac Troponin I (Covance Research Products), anti GFP (Molecular Probes) and anti Laminin (Sigma).

BrdU assay

BrdU labelling medium (Roche) was added to BpV-treated or untreated C2C12 cells for 1 hr at treatment different periods of time. BrdU incorporation was then detected following

manufacturer's instructions (5-Bromo-2'-deoxy-uridine Labelling and Detection kit I, Roche), and BrdU positive cells were visualised under an epifluorescence Zeiss Axioskop 2 Plus microscope.

Preparation of RNA and RT-PCR analysis

Total RNA was extracted from cell cultures using a High Pure RNA Isolation Kit (Roche), according to the manufacturer's instructions. Total RNA was retrotranscribed into cDNA by using the Superscript III system (Invitrogen), according to the supplier's instructions. PCR amplification was performed using Taq Polimerase (Taqara). To check for genomic contamination, parallel aliquots of RNA were incubated without reverse transcriptase (RT) and subjected to PCR amplification as well. The same reaction profile was used for all primer sets: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of: 95°C for 1 minute; primer specific annealing temperature for 1 minute; 72°C for 2 minutes; a final extension step of 7 minutes at 72°C. Amplification of a housekeeping transcript (beta-actin) was used for normalisation. PCR products were run on 1% agarose gel in a TAE buffer containing Etidium Bromide and digitised images were obtained using a CCD camera Detection System (Diana II, Raytest).
Sequences of the primers, FW and RV, used for PCR were as follows:

TARGET cDNA primer Sequences (5'-3')	Annealing Temp. (°C)	Product Size (bp)
β-actin	52	649
ATGCCTCTGGTCGTACCACAGGCATTG		
TTGCTGATCCACATCTGCTGGAAGGTG		
MyoD	59	330
GAGCAAAGTGAATGAGGCCTT		
CACTGTAGTAGGCGGTGTCGT		
Myogenin	59	450
AGTGAATGCAACTCCCACAG		
TCAGAAGAGGATGCTCTCTGC		
Myf-5	60	370
TGCCATCCGCTACATTGAGAG		

CCGGGGTAGCAGGCTGTGAGTTG		
Pax-3	60	450
AGGAGGCGGATCTAGAAAGGAAG		
TGTGGAATAGACGTGGGCTGGTA		
Pax-7	57	236
TACCAGGAGACCGGGTCCATC		
TCCGAACTTGATTCTGAGC		
PCNA	52	165
TCCTTGGTACAGCTTACT		
TGCTAAGGTGTCTGCATT		
Cyclin D1	52	362
GTGCCATCCATGCGGAA		
GGATGGTCTGCTTGTTCTCA		
Sca-1	56	200
GATTCTCAAACAAGGAAAGTA		
GACTGAGCTCAGGCTGAACAG		
Flt-1	57	300
CTCTGATGGTGATCGTGG		
CATGCGTCTGGCCACTTG		
c-Met FW	60	370
GAATGTCGTCCTACACGGCC		
CACTACACAGTCAGGACACTGC		
ALP	55	372
GCCCTCTCCAAGACATATA		
CCATGATCACGTCGATATCC		
Osteocalcin	60	292
AAGCAGGAGGGCAATAAGGT		
AGCTGCTGTGACATCCATAC		
Collagen 1A2	57	484
GCAATCGGGATCAGTACGAA		
CTTTCACGCCTTTGAAGCCA		
Cbfa 1	60	289
CCGCACGACAACCGCACCAT		
CGCTCCGGCCCACAAATCTC		
MITF	57	450
ACCATCAGCAACTCCTGTCC		
TTCTTGCTTGATGATCCGATTC		
MMP9	59	380
CCTGTGTGTTCCCGTTCATCT		
CGCTGGAATGATCTAAGCCCA		
IL3-Rα	60	428
TACCACATCCAGATGGAACC		

TACCACATCCAGATGGAACC		
GM-CSFRα	59	454
AACGTGACTGACAGGAAGG		
TGTGTGTGCTGGCTGTTAAGG		
Bcpr 1	57	330
CCATAGCCACAGGCCAAAGT		
GGGCCACATGATTCTTCCAC		
MDR 1a	55	500
CCCATCATTGCGATAGCTGG		
TCCAACATATTCGGCTTTAGGC		
MDR1b	55	430
TGCTTATGGATCCCAGAGTGAC		
TTGGTGAGGATCTCTCCGGCT		

Flow Cytometry Analysis

BpV-treated or untreated C2C12 or primary satellite cells were suspended by very rapid trypsin digestion, washed in CMF/BSA 1% solution at 4°C and then incubated on ice with 1μg /10^6 cells fluorochrome-conjugated specific antibody. An aliquot of the cell suspension was incubated with the corresponding labelled antibody isotype, to determine non-specific fluorescent emission. Cells were then analysed by using a FACS FacsStar Plus. The antibodies used were: Sca-1, CD45, c-kit, CD34, CD11b, Gr-1, Ter-119 and Mac-3 directly conjugated to phycoerythrin, fluorescein or cy-chrome (all from Pharmingen),

For cell cycle analysis, BpV-treated or untreated C2C12 cells were suspended by trypsin digestion and washed in 50% FCS in CMF; the cells were then suspended in the same solution to which 70%ethyl alcohol was added. After an overnight incubation at 4°C the cells were incubated in Propidium (Sigma) and the fluorescent DNA content was analysed by using the FacsStar Plus.

For dye exclusion technique, the protocol by Goodell et al. (1996) was substantially followed. In brief, BpV-treated and untreated C2C12 cells were suspended by trypsin digestion, collected by centrifugation and suspended in pre-warmed DMEM containing 2% FCS and 10mM Hepes (Gibco) at a working concentration of 10^6 cells/ml. Hoechst was then added to a final concentration of 10μg/ml and the cells were incubated in a 37°C water bath for exact 90

minutes. The cells were then collected by centrifugation at 4°C and suspended in ice cold PBS-BSA 2% containing 2μg/ml propidium iodide (Sigma), and run on the FACS Vantage SE cell sorter (BD) equipped with two lasers. Hoechst dye was excited at 475 nm and its fluorescence was splitted through a 640/LP filter and detected at two wave-lengths using a 424/44 (Hoechst blue) and a 660/20 (Hoechst far red) filters.

Trans-differentiation assays

C2C12 cells were cultured in DM containing or not 10μM BpV for 24 hrs; the cells were then cultured in the appropriate conditions in the presence or not of 2μM BpV for additional 5-7 days.

Hemopoietic conditions. BpV-treated or untreated cells (10^3 cells/35mm Petri dish) were plated in complete methylcellulose medium for colony assay of murine cells (StemCell Technologies). The appearance of clones was analysed after 5-7 days in methylcellulose culture; the cells were then collected and either cyto-centrifuged for morphological analysis or disrupted for RNA preparation.

To test their ability to acquire "osteoclast-like" phenotype BpV-treated or untreated C2C12 cells were cultured in DM supplemented with 100nM $1,25(OH)_2$ vitamin D3. After 5 days the cells were analysed histochemically for the expression of the osteoclest marker acid phosphatase (TRAP) or were disrupted for RNA preparation.

Osteoblast differentiation conditions. BpV-treated or untreated C2C12 cells were cultured in DM supplemented with 50ng/ml of Bone Morphogenetic Protein 2 (BMP2) for 5 days; the cells were then analysed histochemically for the expression of the osteoblast marker or disrupted for RNA preparation.

Cardiomyocyte differentiation conditions. BpV-treated and untreated GFP-transduced C2C12 cells were cultured on a monolayer of neonatal cardiomyocytes for 4 additional days in DM. The cells were then fixed in 5% paraphormaldehyde and GFP-expressing cells were analysed for the expression of cardiac specific markers by immunofluorescence.

Cell staining and histochemical analysis

Wright staining. The cells were washed with PBS (Sigma) and incubated in Blu Wright solution (Merck) for 5 minutes at RT. Two volumes of Wright buffer was then added. After an additional incubation of 7 minutes the cells were washed with PBS.

TRAP histochemical analysis. For tartrate-resistant acid phosphatase activity (TRAP) cells were washed with PBS, fixed with 3%Paraphormaldehyde diluted in 0,1M cacodilate tampon for 15 minutes and incubated in a buffer containing: 0.4 M sodium acetate buffer, 2 mM naphthol AS-BI phosphate, 100 mM sodium tartrate and 2 mM Fast Garnet. The incubation was carried out for 90 minutes at 37°C, the reaction was stopped in distilled water and the cells were observed with a light microscope.

ALP histochemical analysis. For the histochemical localization of alkaline phosphatase activity (ALP), cells were washed with PBS and fixed with 3%Paraphormaldehyde diluted in 0,1M cacodilate tampon for 15 minutes; the cells were then incubated at 37°C for 30 minutes in ALP substrate solution: 0,04 mg/ml naphthol AS-MX phosphate, 0,25 mg/ml Fast Violet B Salt (Sigma). After washing in distilled water, the cells were observed with a light microscope.

In vivo transplantation

BpV-treated and untreated GFP-transduced C2C12 cells were suspended by trypsin digestion, washed in PBS and suspended in 25µl of PBS; $4x10^5$ cells were injected into the femoral artery of $SCG^{-/-}$ dystrophic adult mice. The mice were then sacrificed at the appropriate time points after transplantation and analysed by immunofluorescence.

RESULTS

BpV treatment induces NF-kB and AP-1 trascriptional activities in C2C12

In order to pharmacologically activate signalling pathways known to inhibit myogenic differentiation, we treated C2C12 cells with BpV, a phosphotyrosine phosphatase inhibitor, known to activate both NFkB and AP-1 transcriptional activities in different cell types (Barat and Tremblay, 2003b). In fact, it is well known that myogenic differentiation requires down regulation of both these pathways (Guttridge et al., 1999c;Lehtinen et al., 1996).

As first, we analysed the kinetics of NF-kB activation in BpV-treated C2C12 cells using a gene reporter assay. C2C12 cells were transfected with a plasmid where Luciferase expression is driven by a basic promoter element (TATA box) joined to tandem repeat of NFkB binding elements. The medium was then replaced with DM and the cells were treated with 10 μM BpV or 20ng/ml TNFα, another well known NFkB activator. Luciferase activity was assayed at different periods of time, within the 24 hours of treatment. As shown in Figure 1, while BpV induces a slow 2-3 fold activation of luciferase activity, which is maintained through 24 hours of treatment, TNFα treatment induces a transient 2-3 fold induction, which decreases to below the basic activity within 12 hours.

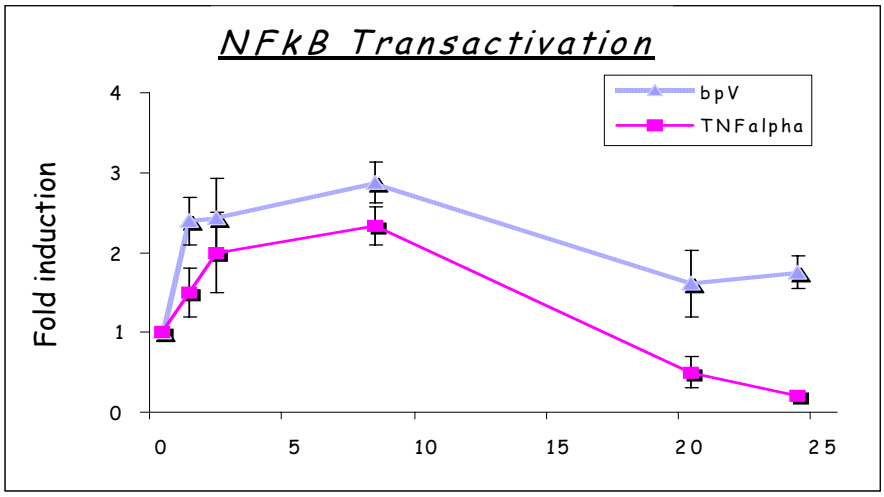


Fig.1 NFkB activation in BpV-treated and untreated C2C12 cells. *C2C12 cells were transfected with an NFkB-Luc reporter plasmid, treated with TNFα or BpV and analysed at different periods of time within 24h. Values were normalised to basal levels of promoter activity obtained by untreated NFkB-Luc plasmid transfected cells.*

Activation of NFkB is usually mediated by IkB kinase (IKK) complex-dependent Ser32,36 phosphorylation of the NFkB inhibitor IkBα; these events are then followed by ubiquitin-proteasome dependent degradation of IkBα. However, as shown in Figure 2a, Ser32,36 IkBα phosphorylation is not affected by BpV treatment, nor the IkBα degradation. Recently, an "alternative" NFkB activation pathway has been identified, which appears to be mediated by Tyr42 phosphorylation of IkBα, followed or not (depending on the cell system) by its proteolitic degradation. This pathway is activated by numerous stimuli including the PTPase inhibitor pervanadate. Since BpV is a pervanadate derivative we evaluated whether it might induce this "alternative" pathway in C2C12 cells. By immunoprecipitation and Western Blot analysis we found that BpV induces, within 5 hrs of treatment, Tyr42 IkBα phosphorylation Fig. 2a) followed by nuclear translocation (thus activation) of the NFkB p65 subunit (Fig.2b).

Taken together, these results demonstrate that BpV induces a "sustained" NFkB transcriptional activity through the IKK-independent Tyr42 IkBα phosphorylation.

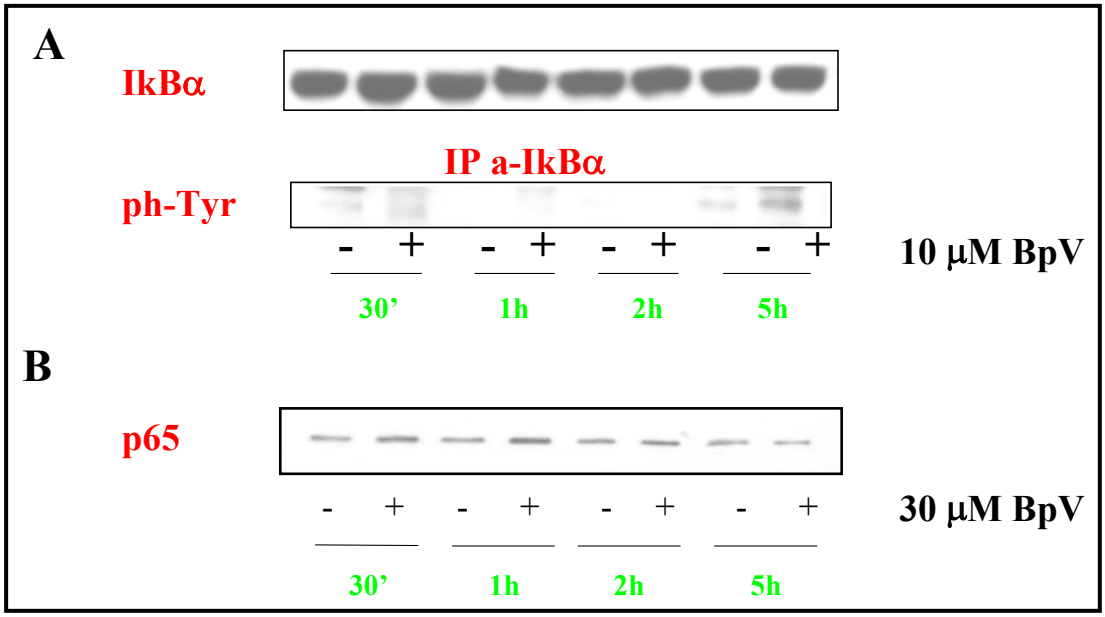


Fig.2 NFkB nuclear translocation through the IKK-independent Tyr42 IkBα phosphorylation induced by BpV treatment. A) Immunoprecipitation and Western Blot of Tyr42 IkBα phosphorylation. B) Western Blot analysis of p65 within 5 hrs of treatment.

Since, as mentioned before, BpV is known to also activate AP-1 pathway, we then analysed AP-1 transcriptional activation in BpV-treated C2C12 cells. BpV-treated and untreated cells were transfected with a plasmid were Luciferase gene was under control of two human collagenase III sites. The medium was then replaced with DM and the cells were treated with 10 μM BpV or 20ng/ml TNFα. Luciferase activity was assayed at different periods of time, within the 8 hours of treatment. As shown in Figure 3a, while BpV induces a transient fold activation of luciferase activity which peaks at 6 hours, TNFα does not exert any induction. Moreover, c-jun Ser63 phosphorilation was observed by Western Blot analysis, in BpV-treated C2C12 cells (Fig.3B).

Taken together, these results demonstrate that BpV activates both NFkB and AP-1 signalling pathways, making it a good candidate to pharmacologically inhibit myogenic differentiation.

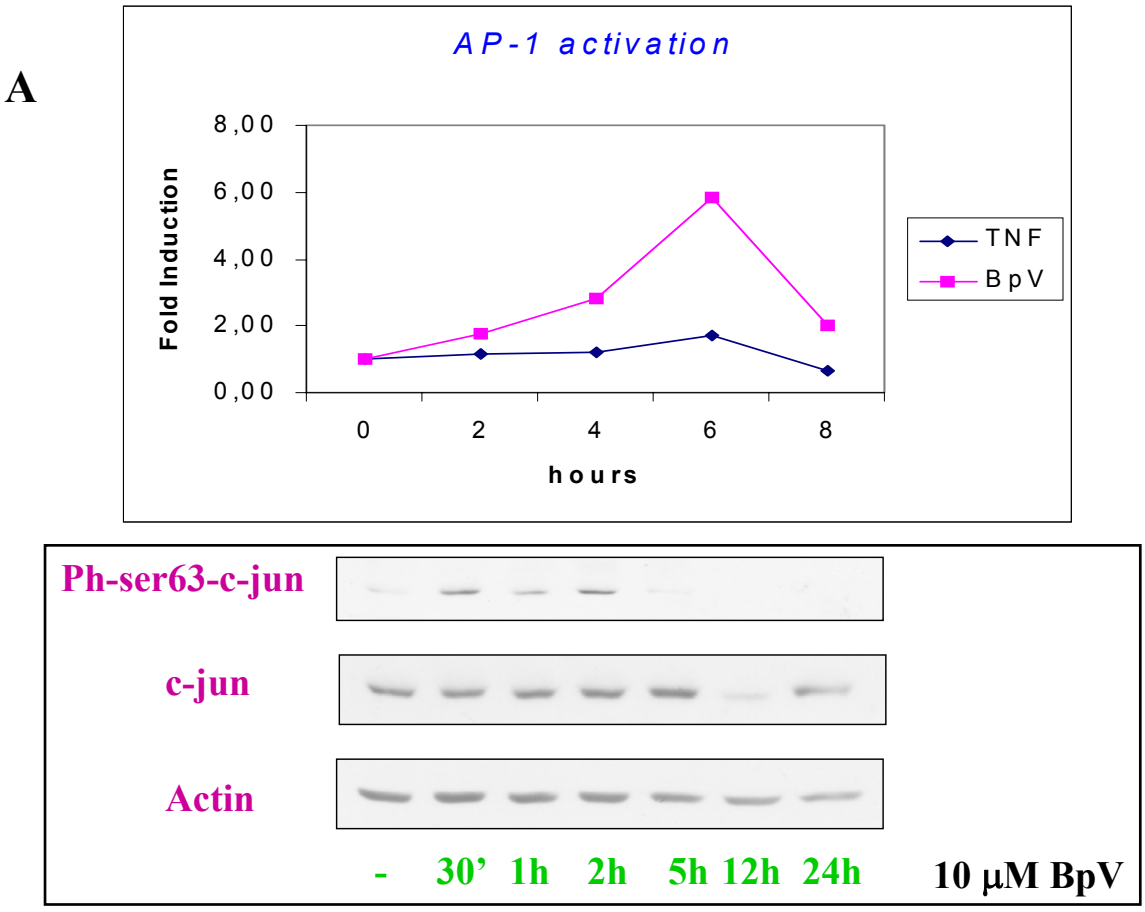


Fig.3 AP-1 activation in BpV-treated cells. *A) C2C12 cells were transfected with an AP-1 reporter plasmid, treated with TNFα or BpV and anslysed at different periods of time within 24 hours. Values were normalised to basal levels of promoter activity obtained by untreated NFkB-Luc plasmid trasfected cells. B) c-jun Ser63 phosphorilation analysed by Western Blot within 24 hours.*

BpV treatment induces morphological changes and reversibly inhibits myogenic differentiation.

The activation of NFkB and AP-1 signalling pathways is parallel to dramatic morphological changes in C2C12 cells. In fact, when the cells were cultured in BpV-containing DM, they promptly acquired a round shape morphology and many of them detached from the dish, without showing any features of degeneration or apoptosis (Fig. 4). This type of morphological change was evident even within 4-5 hours of treatment. Given the already round shape morphology of primary muscle satellite cells (MSC), when MSC were treated with BpV, as above, detachment from the dish was observed (not shown). As expected, long term (72-96 hrs) BpV treatment inhibits the formation of multinucleated myotubes (not shown). Treatment of C2C12 or MSC cells with TNFα did not induce the short-term phenotypic changes observed with BpV treatment, although it inhibits long-term myogenic differentiation (not shown).

However, when the drug was removed and BpV pre-treated cells (C2C12 or MSC cells) were cultured in DM for 3 additional days, the appearance of multi-nucleated myosin positive fibres was observed, with a fusion rate comparable to control cells (Fig.5). Taken together these results demonstrate that BpV-treated cells maintain their myogenic potentiality.

BpV treatment induces cell cycle progression

The morphological phenotype acquired by the BpV-treated cells prompted us to verify the proliferation capability of treated cells. BpV-treated, untreated and proliferating C2C12 cells were FACS analysed for cell cycle at different time points within 48 hours of treatment. As shown in Figure 6a C2C12 cells cultured in low serum containing medium in the presence of BpV display a slower accumulation in G1 phase by the time, when compared to untreated cells. The slower G1 accumulation is paralleled by a slower decrease in the fraction of cells still in the S phase. This slow progressive accumulation in G1 was comparable to that occurring when the cells were maintained in high serum containing medium due to cell confluence being reached. The results are summarised in Table 1.

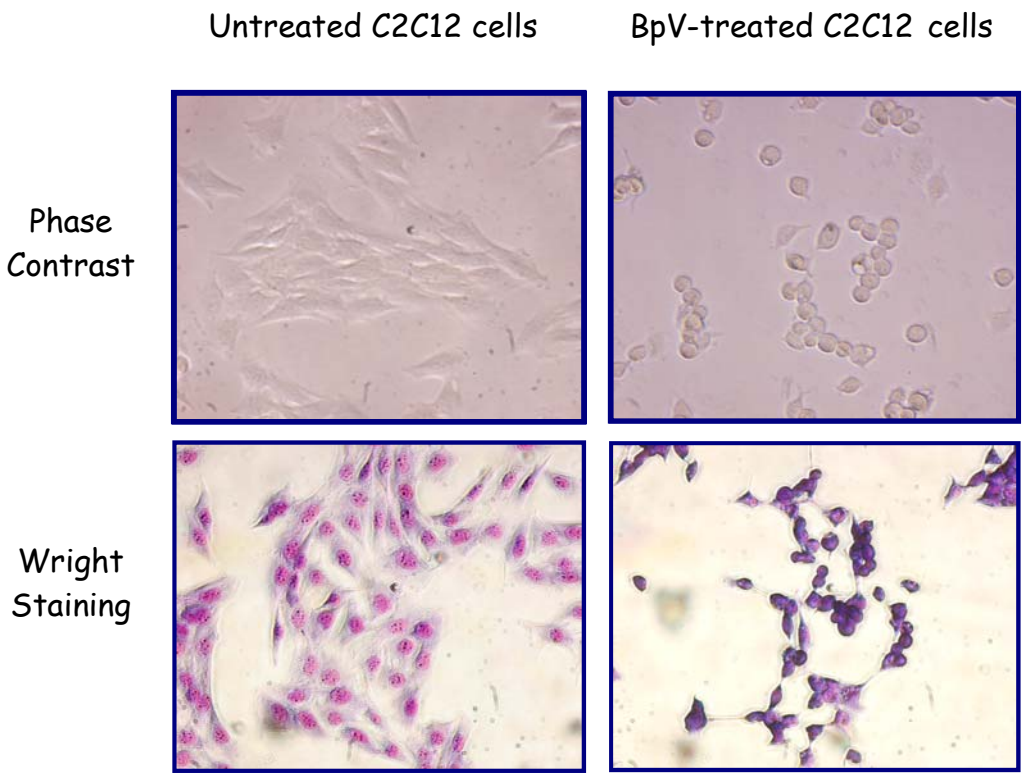


Fig.4 Morphology of BpV-treated and untreated C2C12 cells*. C2C12 cells were treated for 24h with BpV. Treated cells shortly acquire a rounded morphology and a large number of them detach from the dish when compared with control cells. The figure shows untreated and BpV-treated C2C12 cells both in phase contrast and Wright staining.*

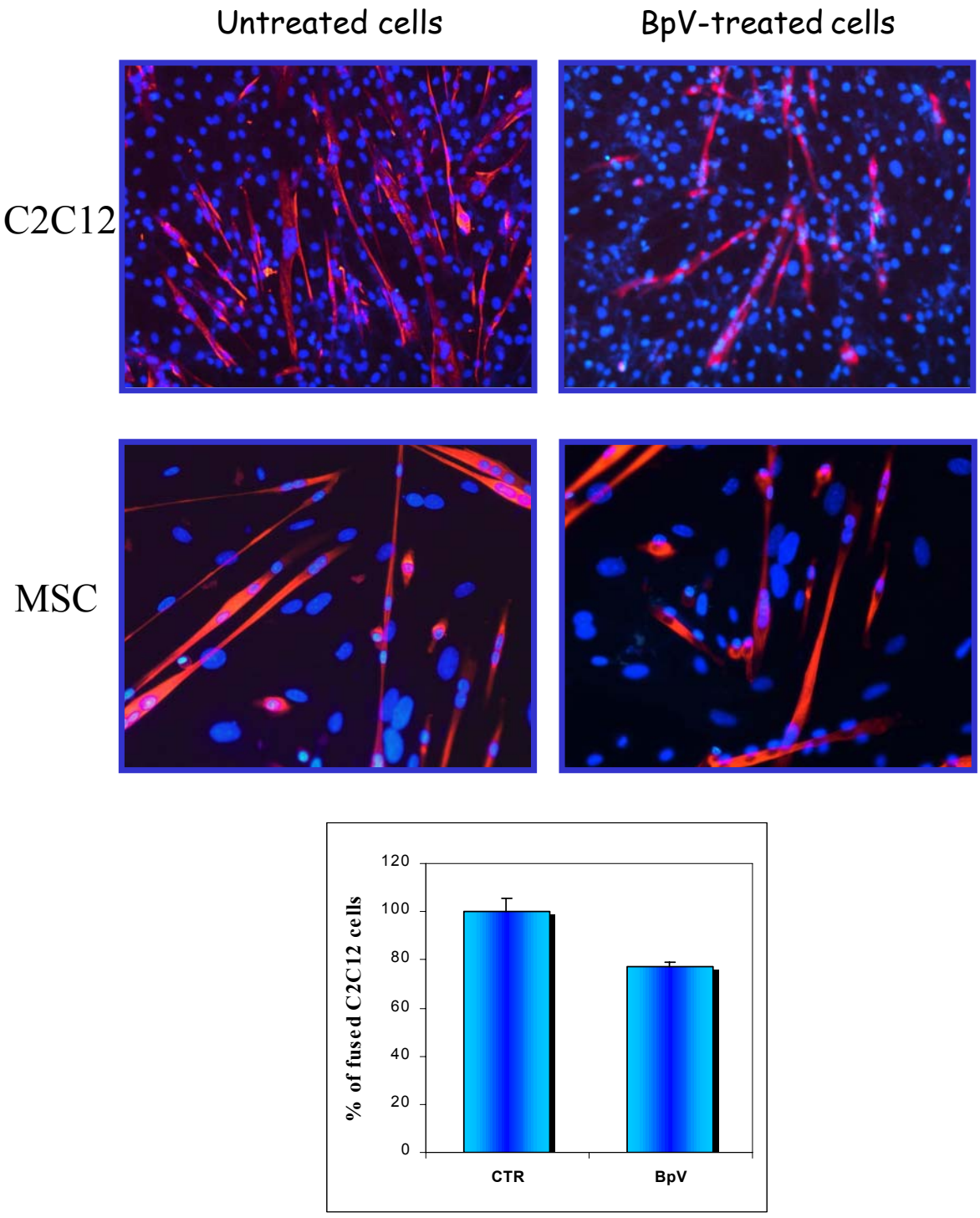


Fig.5 Differentiation of BpV-treated and untreated muscle cells (C2C12 and MSC cells). *The resulting untreated and BpV-treated cells (C2C12 and MSC cells) after a 24h BpV treatment were cultured in GM for 3 days, then shifted in DM for 3 additional days to allow differentiation to occur. The cells were then analysed by immunofluorescence for myosin expression. The appearance of multinucleated myosin positive cells is evident in the figure, when compared with untreated cells.*
The histograms indicate the fusion rate of BpV-treated C2C12 compared with untreated cells.

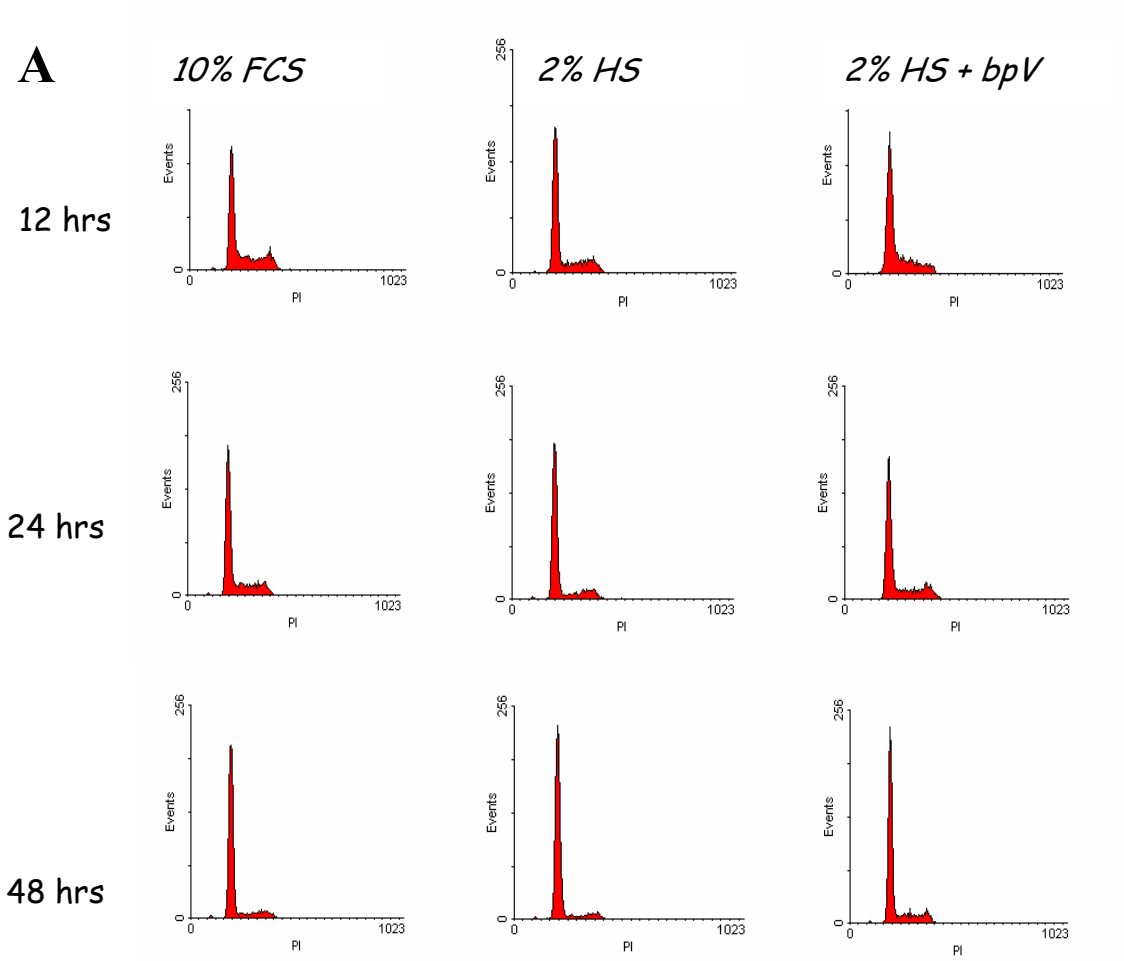


Table 1

		%G1		%S		%G2
10% FCS	*12h*	52,3 ± 0,9		35,7 ± 0,8 **		12,1 ± 1,8 **
2% HS		64,2 ± 6,2 ***		27,5 ± 3,3 ***		8,5 ± 2,9
2% HS + BpV		50,3 ± 11,1		43,1 ± 8,7		6,7 ± 2,4
10% FCS	*24h*	65,2 ± 1,5 **		27,9 ± 0,9		7,1 ± 0,5 ***
2% HS		75,8 ± 5,8 ***		18,1 ± 5,6 ***		6,2 ± 0,2 ***
2% HS + BpV		57,8 ± 10,1		30,1 ± 6,4		12,2 ± 3,7
10% FCS	*48h*	82,9 ± 0,9 ***		14,1 ± 0,2 ***		3,1 ± 0,7 *
2% HS		86,6 ± 1,9 ***		10,6 ± 3,4 ***		2,9 ± 1,5 *
2% HS + BpV		72,7 ± 3,5		22,9 ± 4,5		4,3 ± 0,9

*P<0.1
**P<0.02
***P<0.001

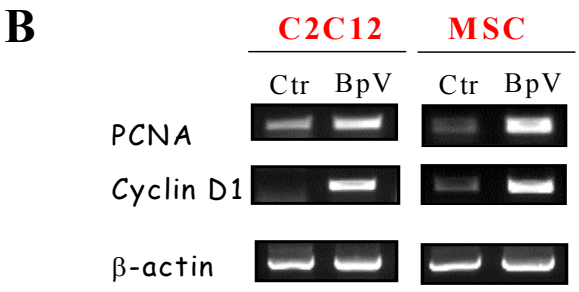


Fig.6 Cell cycle analysis in proliferating (10%FCS) untreated (2%HS) and BpV-treated (2%HS+ BpV) C2C12 cells*. A) FACS analysis was performed on proliferating, untreated and BpV-treated C2C12 cells at different time points (12-24-48 hours). B) RT-PCR shows the upregulation of PCNA and CyclinD1 expression in untreated and BpV-treated cells (C2C12 and MSC).*

Although cultured in differentiation medium, cell cycle progression of BpV-treated C2C12 cells, as well as primary satellite cells, was also confirmed by RT-PCR analysis of the expression of PCNA, a marker of the S phase, and Cyclin D1 which were both up-regulated upon BpV treatment (Fig. 6b).

To further demonstrate the proliferative activity of BpV treated C2C12 cells, a BrdU assay was performed on both untreated and BPV-treated C2C12 cells at different time points within 24 hours. While the percentage of BrdU positive cells decreases progressively until a significant decrement at 24 hours in untreated C2C12 cells, in BpV treated C2C12 the percentage of BrdU positive cells is not significantly altered (Fig.7a). Figure 7b shows the evident reduction, within 24 hours, of BrdU positive untreated cells; in contrast, upon BpV treatment many BrdU positive cells were still present. Figure 7c shows that even in the cell population that detached from the dish upon BpV treatment, many BrdU positive cells are present.

BpV modifies gene expression profile in C2C12 and primary satellite cells

We then analysed the expression of markers defining muscle lineage in BpV-treated cells (C2C12 and MSC cells) by RT-PCR. Figure 8 shows that the expression of the Muscle Regulatory Factors (MRFs: MyoD, Myf5 and Myogenin) was down-regulated after 24 hours of BpV treatment both in C2C12 cells and in MSC. By contrast the expression of markers defining muscle precursor cells such as Pax-3, Pax-7 and c-Met was up-regulated in both C2C12 and MSC cells, as well as Flt-1, whose expression is detectable in satellite cells and in regenerating fibres.

Taken together these results demonstrate that BpV treatment induces reprogramming of gene expression both in C2C12 and MSC cells.

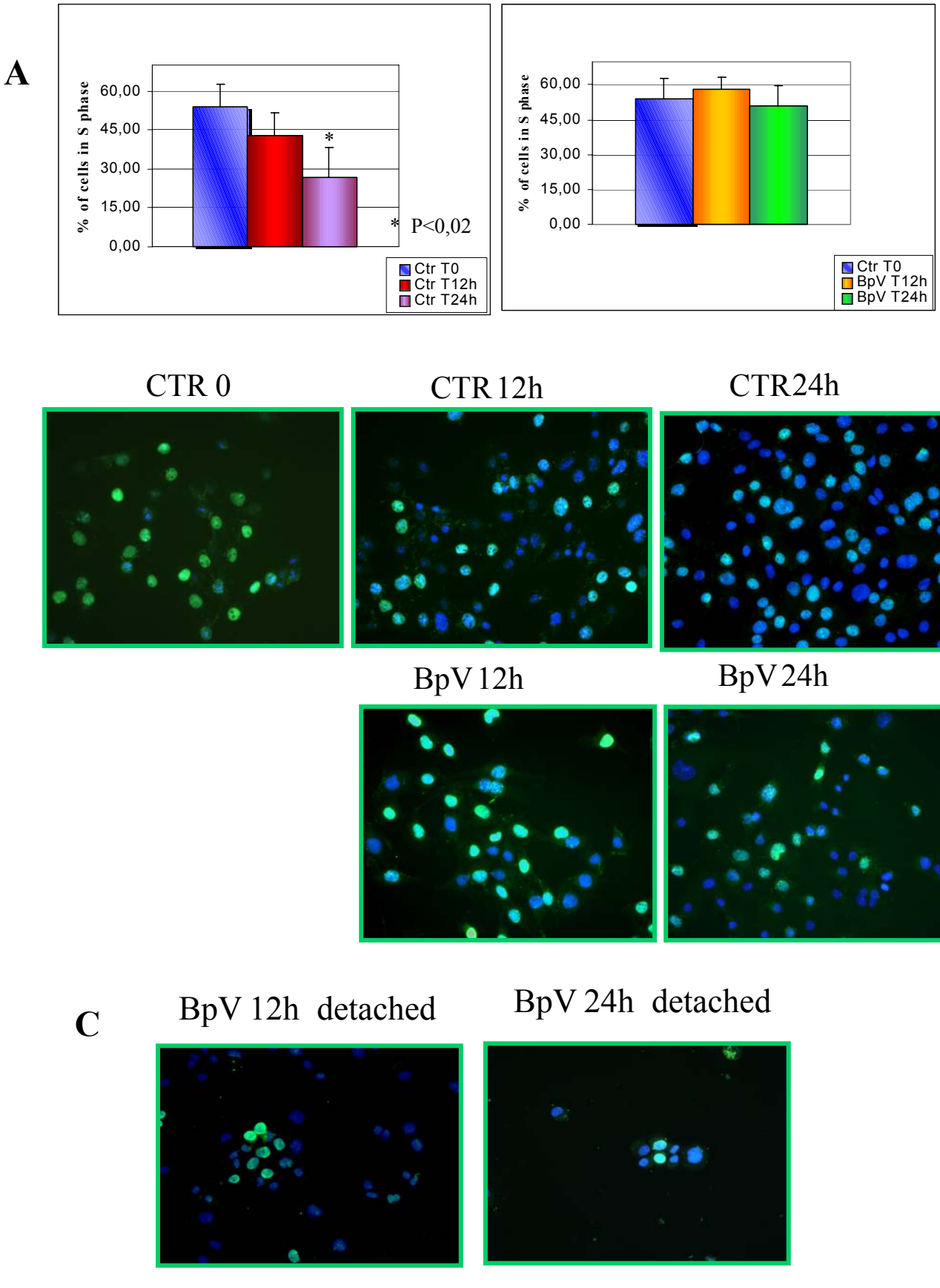


Fig.7 BrdU assay on BpV-treated and untreated C2C12 cells. *BpV-treated and untreated cells were incubated with BrdU labelling, for 1 hour, at different time points within 24 hours. A) The percentage of cells in S phase was calculated by determining the number of BrdU-positive cells with respect to the total number of cells in given fields. B) Fluorescence of BrdU labelling of BpV-treated and untreated C2C12 cells at different time points. C) Fluorescence of BrdU labelling of BpV-treated cells detached from the dish.*

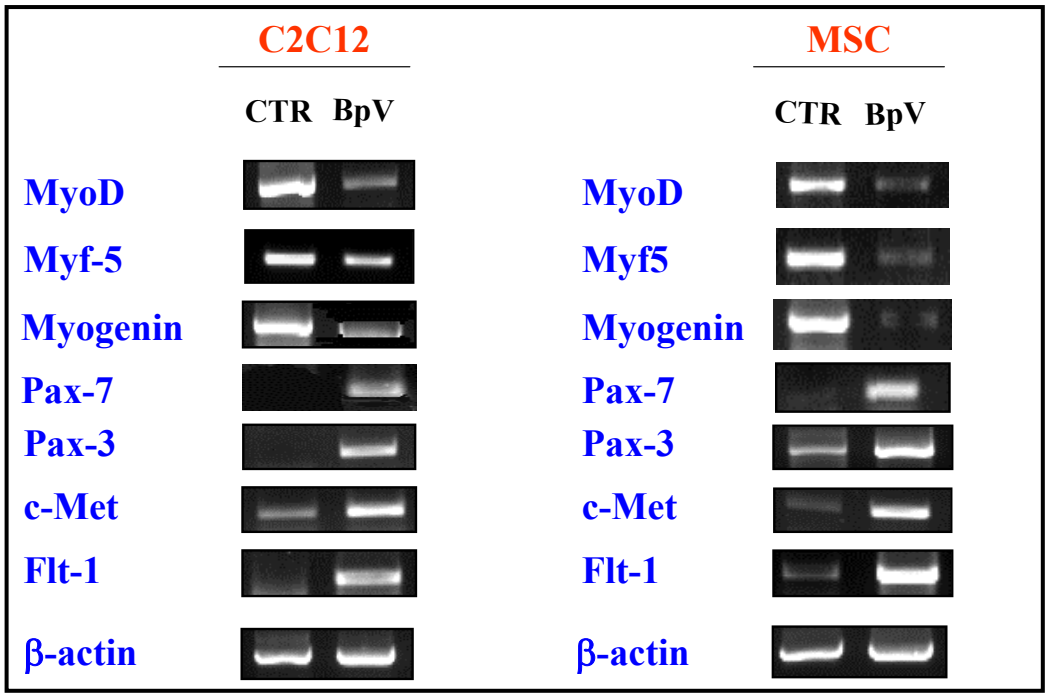


Fig.8 Gene expression profile in BpV-treated and untreated muscle cells (both C2C12 and MSC cells) by RT-PCR. *RT-PCR analysis shows in untreated and BpV-treated muscle cells the expression of MRFs, such as MyoD, Myf5 and Myogenin and of markers defining muscle precursor cells as Pax-3, Pax-7 and c-Met.*

BpV treatment induces SP phenotype in C2C12 cells

It is well known that the SP phenotype is a hallmark of stem cells in many tissues (Bunting, 2002). The SP phenotype is characterised by the ability of this population of stem cells to exert a "side" profile when analysed by FACS, and to exclude the Hoechst 33342 dye, due to the expression of ABC transporters. Recently, it was reported that also C2C12 cell population contains a subpopulation of cells exerting SP phenotype (Benchaouir et al., 2004b). We then verified whether BpV-treatment could increase the percentage of this SP population in C2C12 cells. FACS analysis of Hoechst 33342 stained C2C12 cells confirmed the presence of a very small sub-population (0.02%) characterised by low staining (SP). However, BpV treatment increases the percentage of this sub-population by 5 times (0.11%) when compared to untreated cells (Fig. 9).

SP phenotype, and even the Stem Cell phenotype, was also defined by the expression of two members of the ABC transporter superfamily: multidrug resistance-1 (MDR1) and breast cancer resistance protein (Bcrp1) (Bunting, 2002). We thus analysed the expression of both markers in both C2C12 and in MSC by RT-PCR analysis. Figure 9 shows that, in C2C12 cells, BpV treatment up-regulates both MDR1a and MDR1b isoforms but not Bcrp1, which is already expressed in untreated cells. Conversely Bcrp1 upregulation was evident in BpV-treated MSC, while the expression of MDR1a and MDR1b was fairly up-regulated slightly.

BpV induces stem and early hematopoietic markers in C2C12 and primary satellite cells

Untreated and BpV-treated C2C12 cells were then analysed for the expression of stem and early hematopoietic markers by FACS analysis, using conjugated specific antibodies. The results are summarised in Figure 10. Untreated cells already expressed low levels of the Stem Cell markers c-Kit, Sca-1 and CD34; however, BpV treatment induced a significant up-regulation of these markers. Among the early hematopoietic markers tested the expression of both CD45 and CD11b was very low in untreated cells; BpV treatment induced a low but detectable up-regulation of CD11b, while higher up-regulation of CD45 was evident; moreover, a macrophage lineage specific marker, Mac-3, was already expressed in untreated

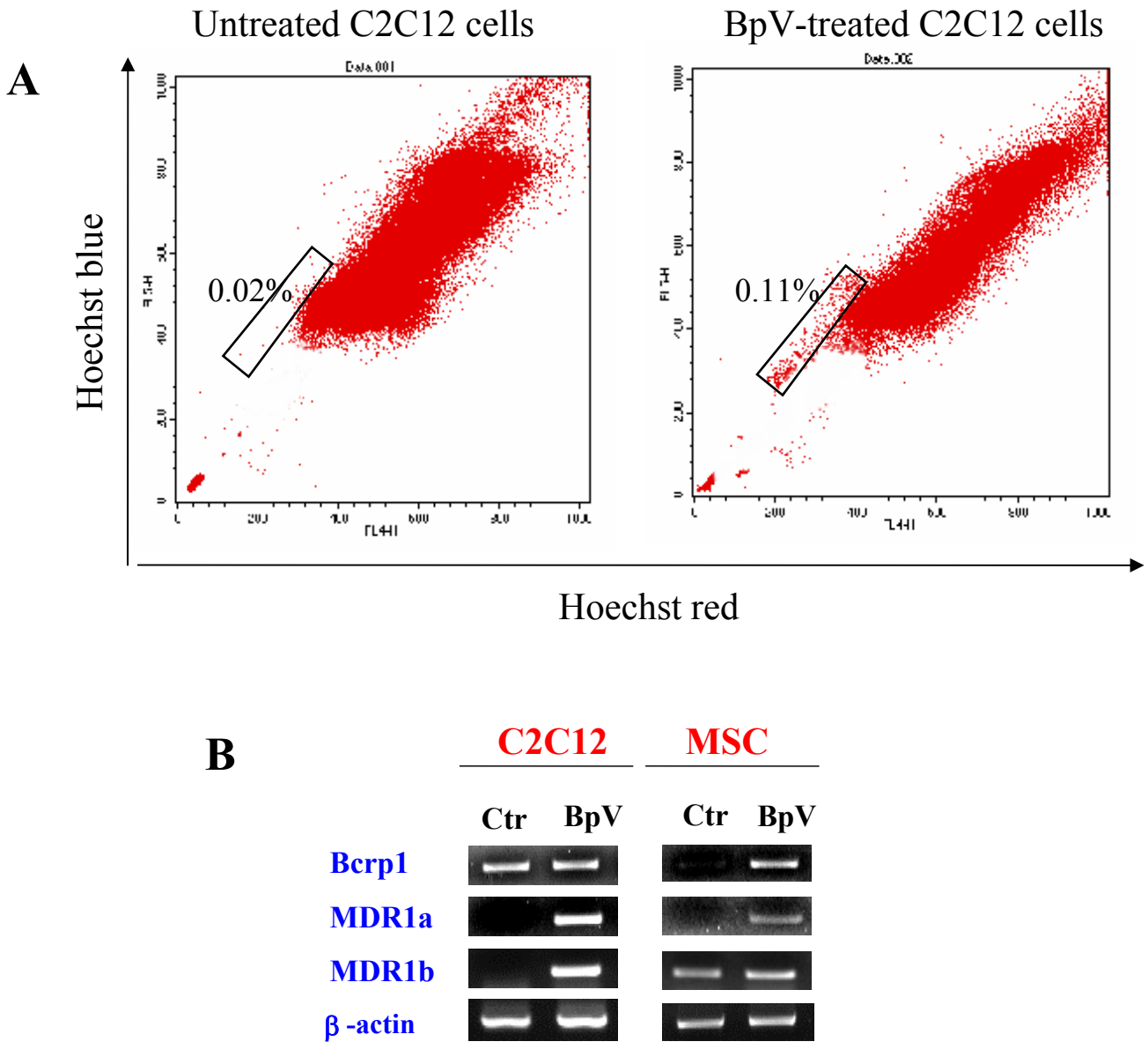


Fig.9 SP phenotype in untreated and BpV-treated muscle cells (both C2C12 and MSC cells). *A) FACS analysis of BpV-treated C2C12 cells using the fluorescence dye Hoechst 33342 staining. B) RT-PCR analisys of MDR1a, MDR1b and Bcrp1 expression in BpV-treated and untreated muscle cells (both in C2C12 and MSC cells).*

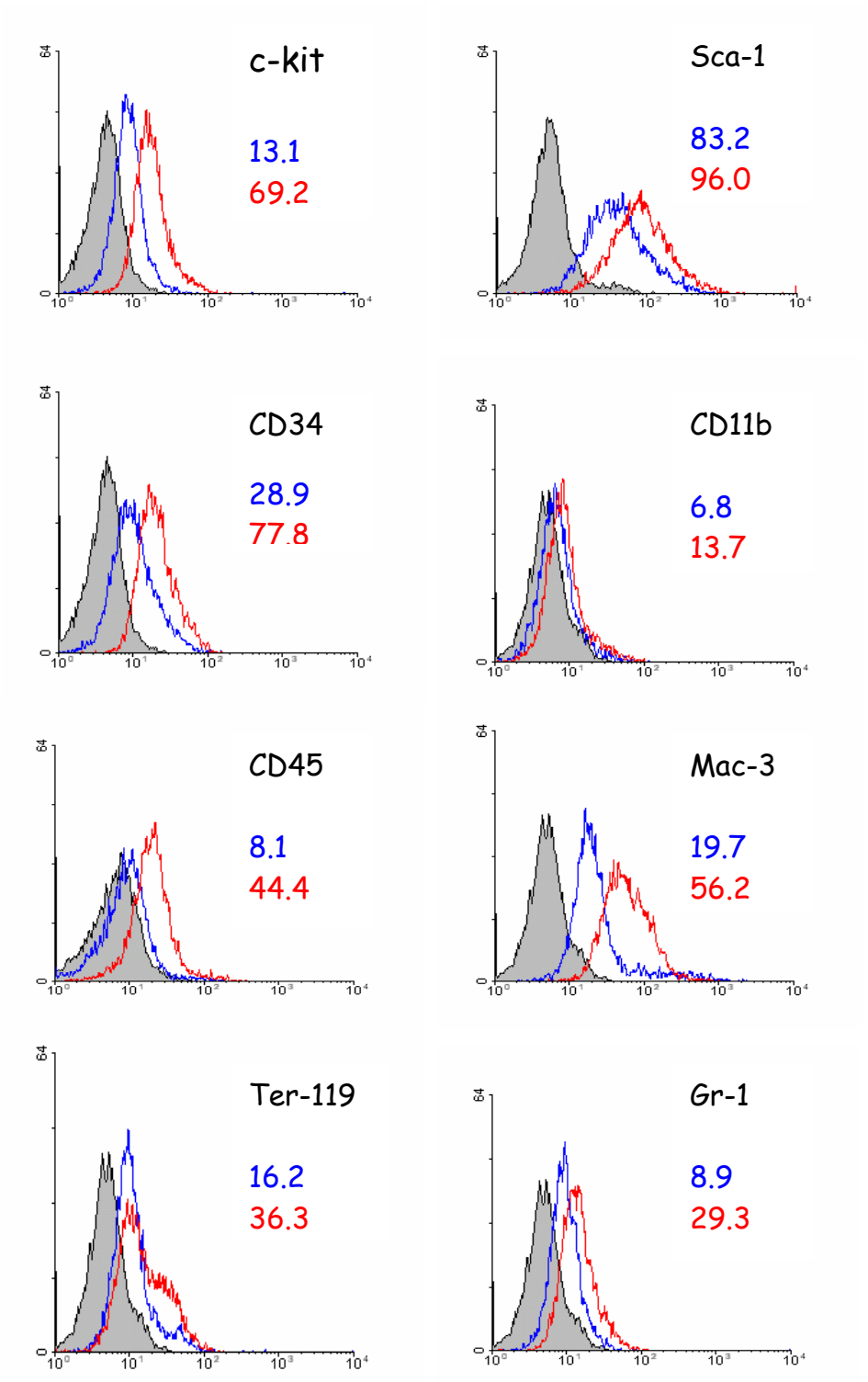


Fig.10 Expression of specific precursor markers for both muscle and hematopoietic lineages by FACS analysis of BpV-treated and untreated C2C12 cells*. C2C12 were treated with BpV for 24h and then analyzed by FACS for the expression of specific markers as indicated.*

cells, but its expression was highly up-regulated after 24 hours of BpV treatment. Finally the expression of Gr-1 and Ter119, granulocyte and erythrocyte lineage markers respectively, was also up-regulated upon BpV treatment.
Preliminary results for the expression of stem and early hematopoietic markers on MSC cells showed a similar profile.

Taken together these results suggest that BpV treatment promotes the exposed cells to an earlier phenotypic stage, inducing the expression of markers specific for precursor of both muscle and hematopoietic lineages.

BpV treatment induces multi-lineage potentiality in C2C12 cells

The gene expression profile acquired by the BpV-treated cells, prompted us to verify their possible multi-lineage potential.
As first, to verify the potential to give rise to cells of different lineages, for example of hematopoietic origin, C2C12 cells were cultured in the presence or in the absence of BpV for 24 hours; untreated and treated cells were then cultured for 5 additional days in a myeloid colony assay conditions, in the presence of low BpV concentration (2μM). After 5 days, BpV-treated cells formed hematopoietic-like colonies while untreated cells formed clones of adherent cells (Fig.11a). The hematopoietic-like colonies were then cytocentrifuged and the morphology of the obtained cells was analysed by Wright staining. Fig.11b shows that many of the cells acquired morphological phenotype characteristic of myeloid lineages (such as monocytes, macrophages or granulocytes). RT-PCR analysis performed on untreated and BpV-treated C2C12 cells cultured in myeloid conditions shows that the expression of the granulocytes macrophages colony stimulating factor receptor α (GM-CSFRα) and of the interleukin 3 receptor α (IL-3Rα) was induced in BpV-treated cells; no expression of these markers was detectable in untreated cells (Fig.11c). These two genes are expressed in both hematopoietic stem cells (HSCs) and in common myeloid progenitors (CMPs) (Miyamoto et al., 2002).

Osteoclasts are of myeloid origin, thus the potentiality to give rise to this lineage was then analysed. Untreated and BpV-treated cells were cultured for 5 days in low serum

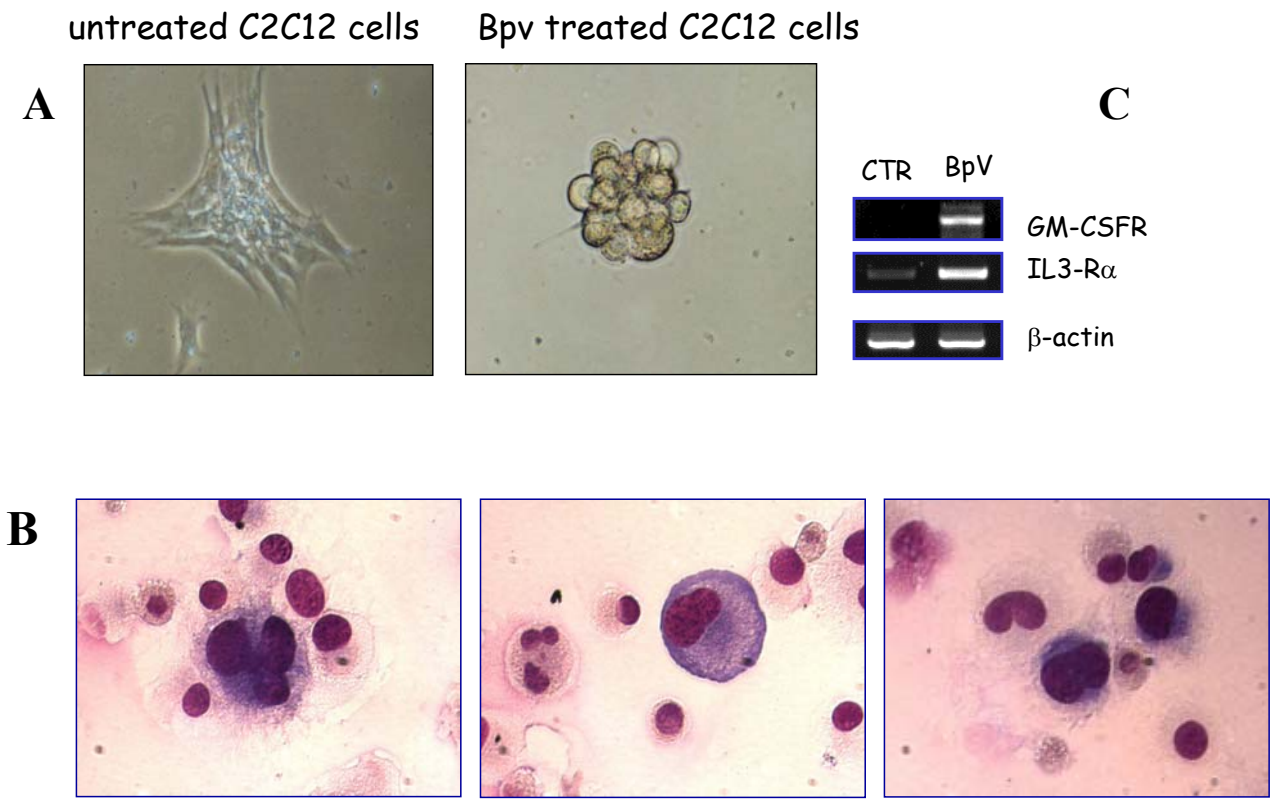


Fig.11 Methoculture of BpV-treated and untreated C2C12 cells. *C2C12 cells were treated with BpV (10μM) for 24h, and then cultured under myeloid conditions for 5 additional days; BpV was maintained at 2μM concentration through the whole period in culture. A) Hematopoietic-like clones are formed by BpV-treated cells, while untreated cells form clones of adherent cells. B) BpV-treated cells grown in methylcellulose culture were collected, cytospinned on to glass slides and stained with Giemsa for morphological analysis. The figure shows that the BpV-treated C2C12 cells show morphological characteristics of the myeloid lineages. C) RT-PCR analysis of the GM-CSFRα and IL-3Rα expression in untreated and BpV-treated C2C12 cells.*

containing medium supplemented with 100nM 1,25$(OH)_2$ vitaminD3 and 2μM BpV.

Conversion to osteoclast-like phenotype was then analysed by the histochemical assay of the acid phosphatase activity (TRAP). As shown in Figure 12, while no TRAP positive cells were detectable in untreated cells, many TRAP positive cells were present in BpV-treated cells. The acquisition of the osteoclast-like phenotype by the BpV-treated cells was also confirmed by RT-PCR analysis showing that BpV treatment up-regulates osteoclast specific markers such as Microphthalmia Transcriptor Factor (MITF), known to regulate transcription of TRAP (Luchin et al., 2001), and matrix metalloproteinase (MMP9), which has a critical role in osteoclast migration and bone reabsorption (Delaisse et al., 2003). Thus, BpV treatment can trigger C2C12 muscle cells towards an osteoclastic–like phenotype, which however is of hematopioetic origin.

To verify whether BpV-treated cells were capable of differentiating into phenotype of mesenchimal origin, rather than muscle, as osteoblast, untreated and BpV treated C2C12 cells were cultured for 5 days in low serum containing medium, supplemented with 50ng/ml Bone Morphogenetic Protein 2 BMP2 and 2μM BpV. Osteoblast conversion was then analysed by the histochemical assay of the alkaline phosphatase activity (ALP). It is already known that C2C12 cells, as well as primary satellite cells, can be converted to the osteoblast phenotype by treatment with a high concentration of BMP2 (100-300 ng/ml) (Katagiri et al., 1994a;Asakura et al., 2001b); however, at the lower concentration of 50ng/ml BMP2 used, very few untreated cells became positive for ALP activity, while a three times increase in ALP positive cells was observed in BpV-treated cells (Fig.13). Again, osteoblast conversion was confirmed by RT-PCR analysis for the expression of osteoblast specific markers: ALP, Osteocalcin, Collagen I and the transcriptor factor Cbfa1, which is considered the osteoblast master gene for its essential role in osteogenesis and osteoblast differentiation (Komori et al., 1997;Ducy et al., 1997). Thus BpV treatment enhances the BMP2 driven osteoblast conversion of C2C12 cells.

We further analysed whether BpV treatment could convert C2C12 cells towards cardio-myocyte phenotype. To this purpose GFP-labelled C2C12 cells were cultured in the presence or not of BpV for 24 hours. Treated and untreated cells were then co-cultured for 4 additional days on a monolayer of cardiomyocytes, previously prepared from 1-2 day old

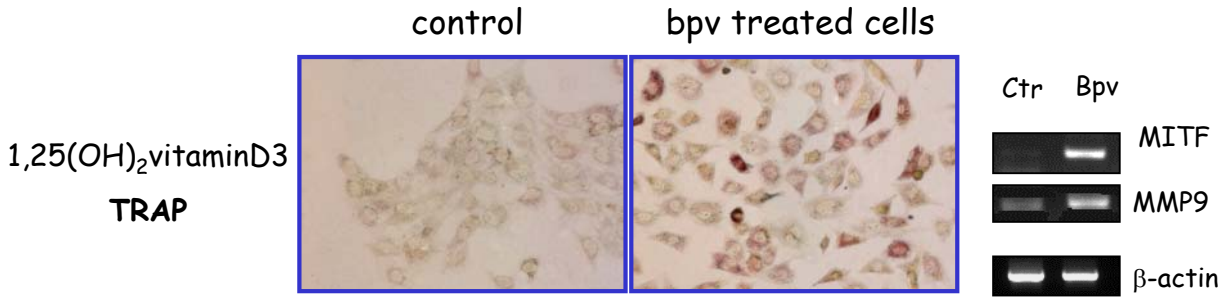


Fig.12 TRAP activity and RT-PCR analysis on untreated or BpV-treated C2C12 cells. *BpV-treated and untreated C2C12 cells were cultured in medium containing 1,25(OH)vitaminD3 and then analysed by the histochemical assay of the acid phosphatase activity (TRAP). The figure shows the evident induction of TRAP activity. RT-PCR shows the expression of osteoblastic specific markers such as MMP9 and MITF in both BpV-treated and untreated cells.*

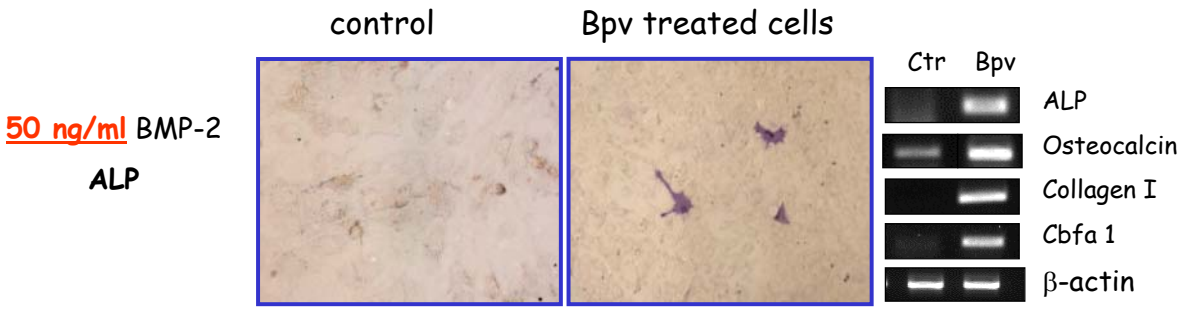


Fig.13 ALP activity and RT-PCR analysis on untreated or BpV-treated C2C12 cells. *BpV-treated and untreated C2C12 cells were cultured in medium containing BMP-2 and then analysed for ALP activity. The figure shows the evident induction of ALP activity. RT-PCR shows the expression of specific osteoblast markers such as Osteocalcin, Collagen I, Cbfa1, in BpV-treated and untreated C2C12 cells.*

mice. The conversion towards cardiac phenotype was then evaluated by the immunofluorescence analysis of cardiac troponin I (CTnI). The co-expression of cardiac troponin I (red) and GFP labelling demonstrate the conversion to the cardiac phenotype. The result shown in Figure 14 demonstrates that while no co-expression of GFP and CTnI was detectable in the absence of BpV, some cells co-expressing GFP and CTnI were evident upon BpV treatment. Thus, BpV treatment can trigger C2C12 muscle cells towards a cardiac phenotype.

Taken together, these results suggest that BpV treatment imparts to muscle cells the tendency towards different cell lineages (i.e. myeloid, osteoclasts, osteoblasts and cardiac cells), though maintaining their myogenic potential.

BpV treated muscle cells acquire the phenotype of a circulating progenitor

In order to verify whether the gene expression profile and the ability to trans-differentiate into different lineages may reflect the acquisition of the phenotype of circulating progenitors capable of systemic delivery and diapedesi to colonize different tissues, untreated and BpV-treated cells were transplanted into recipient mice via intra-arterial delivery. To this purpose, C2C12 cells were previously infected with a lentivirus carrying the GFP cDNA. The cells were subcultured for 2-3 times before use, to ensure the absence of free lentiviral DNA. To test this, medium was used to feed uninfected cells where no GFP-labeled cells were present. As first we investigated the possible BpV-induced homing into the skeletal muscle. $4x10^5$ GFP-labeled cells, untreated or treated with BpV for 24 hours, were injected into the femoralis artery *in vivo*. As the mouse model SCG$\alpha^{-/-}$ dystrophic mice were used. These mice represent a model for muscular dystrophy due to the absence of SCGα, thus degenerating muscle fibres need to be continuously replaced by muscle cell precursor. The capability of the transplanted cells to reach skeletal muscle via circulation was thus evaluated after transplantation, by the presence of GFP-cells in skeletal muscle. Five days after transplantation the mice were sacrificed and leg muscles (quadriceps, tibialis, soleus and gastrocnemius) were removed; GFP-labeled cells were visualised in cryosections under an epifluorescence microscope, while muscle fibres were visualised by immunofluorscence

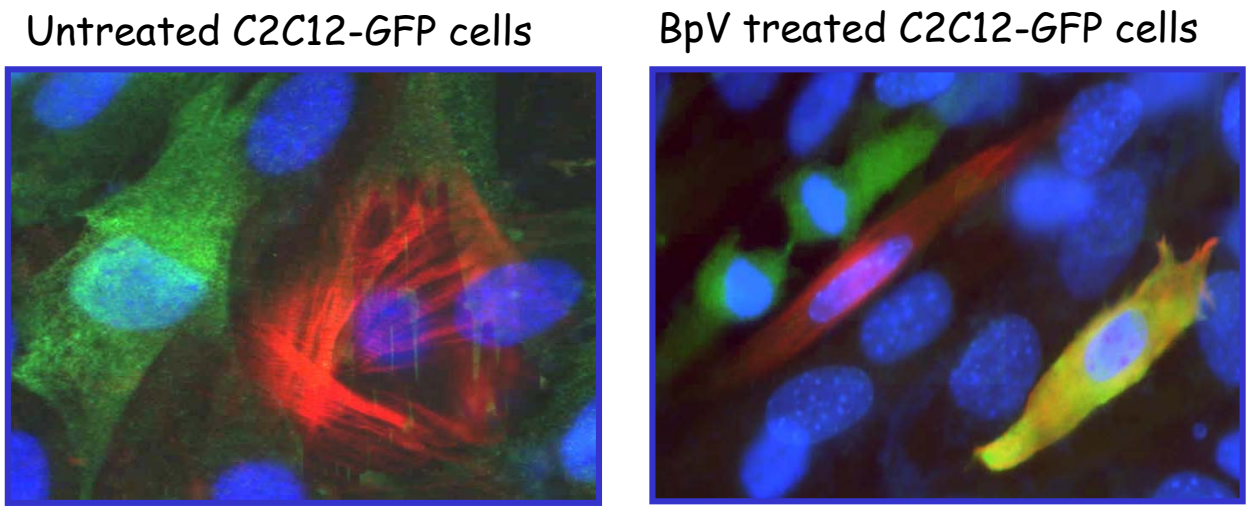


Fig. 14 Co-culture of GFP-C2C12 cells with neonatal cardiomyocytes. *GFP-C2C12 cells were treated with BpV for 24 hours and co-cultured with cardiomyocytes for 4 additional days. The cells were then analysed by immunofluorescence for the expression of Cardiac Troponin I (red). The figure shows that BpV treatment induces the expression of Cardiac Troponin I in GFP-C2C12 cells.*

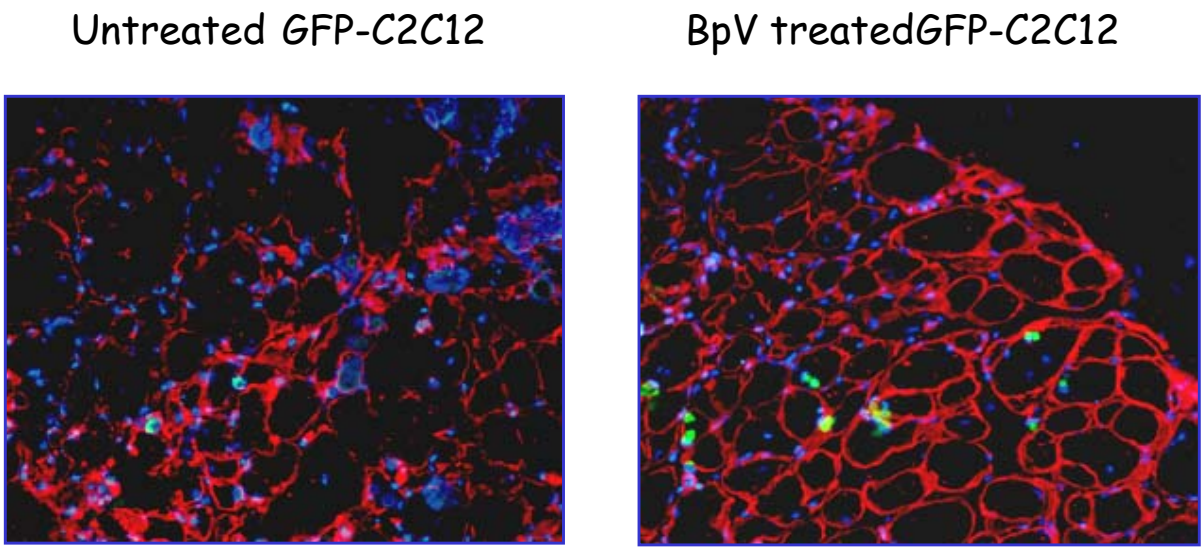


Fig.15 Intra-arterial injection of untreated and BpV-treated GFP-C2C12 cells in SCGα$^{-/-}$ dystrophic mice. *GFP untreated and BpV-treated C2C12 cells were transplanted via femoralis artery delivery into dystrophic mice. Mice were sacrificed 5 days after transplantation and muscle cryosections were analysed by immunofluorescence analysis. As showed in the figure, many more GFP-BpV treated cells migrate from femoral artery to skeletal muscle when compared with control cells.*

analysis using an antibody anti-laminin. As shown in Figure 15, very few GFP-labeled mono-nucleated cells are present in very damaged areas of skeletal muscle of mice injected with untreated cells; by contrast, many more GFP-labeled mono-nucleated cells were found even in undamaged skeletal muscle when BpV-treated cells were transplanted. This result demonstrates that BpV treatment induces C2C12 cells to acquire a phenotype of circulating progenitor capable of overcoming the endothelial barrier and reaching the muscle tissue.

DISCUSSION

In this study we demonstrated the capability of committed muscle satellite cells to acquire an earlier, pluripotent circulating "stem-like" behaviour, through a pharmacological treatment.

A number of recent results indicate that some plasticity can be induced in already committed cells (Hu et al., 1995a;Hakelien et al., 2002a;Odelberg et al., 2000;Kondo and Raff, 2000c). Thus, reprogramming of gene expression in committed cells, in order to obtain their reversion into earlier pluri/multipotent "stem-like" cells, might be a feasible option to develop innovative cell therapy strategies.

Searching for signalling pathways that could modulate self-maintenance, activation and expansion of muscle satellite cells we found that treatment of satellite muscle cells with BpV, a protein tyrosine phosphatase (PTP) inhibitor, primarily known to activate NFkB and AP-1 activities (Barat and Tremblay, 2003a), induces in both primary murine satellite cells and C2C12 satellite cells the acquisition of a "stem-like" phenotype. When cultured in appropriate conditions, BpV-treated satellite muscle cells are committed towards phenotypes belonging to distinct lineages. Moreover, BpV-treated cells acquire the ability to home, via circulation skeletal muscle.

At first we demonstrated that BpV induces both AP-1 and NFkB transcriptional activity. While BpV induces a 2-3 fold activation of luciferase reporter activity which is maintained through 24 hours of treatment, AP-1 is transiently activated within 8 hours. In contrast, TNFα, another well known activator of both pathways, induces a transient 2-3 fold induction which decreases below the basic activity within 12 hours. These results are in contrast with other reports (Guttridge et al., 1999b;Ladner et al., 2003) which show that TNFα induces, in the same cellular model, a biphasic activation of NF-kB which lasts for nearly 2 days following initial TNFα treatment.

Moreover, we showed that BpV treatment, in contrast to TNFα treatment, does not induce the well established NFkB activation pathway mediated by IkBα Ser32,36 phosphorylation (Boone et al., 2002a). It was previously shown that hypoxia, reoxygenation and PTPase inhibitor pervanadate activate NFkB via the "alternative" Tyr42 phosphorylation of IkBα, probably through the involvement of the catalytic subunits of PI3 kinase (Imbert et al., 1996;Beraud et al., 1999;Mukhopadhyay et al., 2000;Waris et al., 2003). Since BpV is a

pervanadate derivative we demonstrated, by Western Blot analysis, that it actually induces IkBα Tyr42 phosphorylation in satellite C2C12 cells which is not followed by its proteolitic degradation, and results in p65 NFkB subunit nuclear traslocation, as demonstrated by the immunoprecipitation analysis. These observations, including the different kinetics of NFkB activation, suggest that the two stimuli, BpV and TNFα, activate a qualitatively different NFkB response through different activation pathways.

Activation of these pathways is known to inhibit myogenesis; accordingly both BpV and TNFα prevent the formation of multinucleated myosin positive myotubes. Inhibition of differentiation is due to the inhibition of MRFs (MyoD, Myf5 and myogenin) expression, as demonstrated by RT-PCR analysis. However, BpV-treated cells, but not TNFα ones, shortly acquire a round shape morphology and continue to proliferate in low serum containing medium. It is well known that myoblasts, when cultured in differentiation medium, through MRFs activity, asynchronously and irreversibly exit the cell cycle and progress to the ultimate compartment of the myogenic lineage (Walsh and Perlman, 1997;Wei and Paterson, 2001). BpV treatment, on the other hand, maintains myoblasts proliferating, as shown by FACS analysis as well as by BrdU incorporation and by up-regulation of PCNA and Cyclin D1 expression. It is worth noting that, while PCNA is a marker of the S phase, Cyclin D1 is up-regulated during the G1/S transition, and that its expression is known to be induced by the NFkB pathway (Guttridge et al., 1999a). Importantly, BpV-induced inhibition of differentiation is reversible. In fact, when BpV-treated cells were cultured in absence of the drug they were still capable of forming multinucleated, myosin positive cells, with a fusion rate comparable to the untreated cells.

Thus, BpV appeared to exert the initial requisites required for the pharmacological treatment we were looking for: reversible inhibition of muscle differentiation associated with cell proliferation.

However, to determine whether BpV treatment may be a suitable approach to develop innovative cell therapy strategies, it is a must that BpV induces re-programming of gene expression. It is well known that quiescent and activated muscle satellite cells are characterised by the specific expression of the transcription factor Pax-7 and c-met, whose expression is down regulated upon myogenic differentiation (Seale et al., 2000a). Subsequently, activation of satellite cells is characterised by the rapid up-regulation of MRFs such as MyoD and Myf5, which then trigger myogenic differentiation (Cornelison and Wold,

1997a;Cornelison et al., 2000b;Cooper et al., 1999b). RT-PCR analysis, in both C2C12 and MSC cells, demonstrated that while BpV down-regulates the expression of the MRFs (MyoD, Myf5 and Myogenin), it up-regulates the expression of markers defining muscle precursor cells, such as Pax-7, Pax-3 and c-Met. Moreover, BpV treatment induces, in both C2C12 and MSC cells, up-regulation of the VEGF receptor Flt-1, whose expression has been shown to be detectable in activated satellite cells, but decreases during the differentiation program (Germani et al., 2003). Thus, the observed gene expression profile shows that BpV actually induces re-programming of gene expression and that the acquired gene expression profile resembles that of the muscle cell precursor.

Which is the real border line between a "cell precursor" and a "stem cell" and how these stages are finely regulated, is not clear yet. It is well known that SP phenotype is a hallmark of stem cells in many tissues; it has been recently reported that C2C12 cells comprises a cell subpopulation actively excluding Hoechst 33342, through the activity supported by ABC transporters (Benchaouir et al., 2004a). By FACS analysis we confirmed this result, but a 5 times increase in the percentage of this population was also observed upon BpV treatment. This result was also supported by the observation that BpV up-regulates MDR1 and Bcrp1, markers characteristic of the SP population, as shown by RT-PCR analysis.

The acquisition of a "stem cell-like" phenotype in BpV-treated satellite cells, was also confirmed by the BpV-induced up-regulation of additional markers characteristic of adult muscle stem cells, such as CD34, Sca-1 and c-kit. As shown by FACS analysis, some of these markers are already expressed in satellite cells, however, BpV treatment significantly enhances their expression. This result confirms that BpV treatment induces, on the exposed cells, a reprogramming of gene expression, inducing the up-regulation of markers specific of muscle cell precursors and triggers the cells towards a "stem-like" phenotype.

Moreover, FACS analysis shows that BpV treatment induces the expression of early markers of the hematopoietic lineage, such as CD45, the leucocyte common antigen, CD11b, an integrin also known to define early hematopoietic cells, Mac-3 and Gr-1, early markers of the myeloid lineage and Ter-119, which defines the erythroid lineage.

The gene profile expression acquired prompted us to verify the full differentiation potential of the BpV-treated cells. Previous works have demonstrated that myogenic cells, such as C2C12 myoblasts (Teboul et al., 1995;Katagiri et al., 1994b) as well as muscle

satellite cells (Asakura et al., 2001a), display osteogenic or adipogenic differentiation capability upon treatment with BMPs or adipogenic inducers respectively; recently, it was also demonstrated that C2C12 cells may be converted to a neuronal phenotype (Watanabe et al., 2004a); however, there is no evidence of their hematopoietic or myo-cardiac differentiation capability. We presented direct evidence that BpV-treated C2C12 cells, when cultured in the appropriate conditions, were able to differentiate into phenotypes of hematopoietic and mesenchymal origin, other then muscle cells, while retaining their muscle differentiation potentiality. In particular, we demonstrated that BpV-treated C2C12 cells, when cultured in myeloid colony assay conditions, can form hematopoietic-like colonies, whose cells display morphogical characteristics of the myeloid lineages; this result was also confirmed by the observed upregulation of markers of myeloid cells such as IL3-Rα and GM-CSFRα, as shown by the RTPCR analysis. The ability of BpV-treated cells to give rise to myeloid-derived cells was further demonstrated by the BpV-induced up-regulation of the activity and/or the expression of markers characteristic of the osteoclast phenotype, such as TRAP, MMP9 and MITF, when the cells were cultured in medium containing 1,25$(OH)_2$ vitamin D3.

Also the ability to differentiate into mesenchymal lineages different from muscle, such as osteoblasts, is significantly enhanced by BpV treatment. Both C2C12 and MSC cells are known to differentiate into osteoblasts when cultured in the presence of a high concentration of BMP2 (100-300 ng/ml) (Katagiri et al., 1994c). At a lower BMP2 concentration (50ng/ml) very few, if any, ALP positive cells were detected; however, if the cells were pre-treated with BpV, a 5-10 times increase in ALP positive cells was observed, as the up-regulation of both "early" (Cbfa1, considered as a "master gene" for the osteoblast lineage) and "late" (collagen 1 and osteocalcin) osteoblast-specific markers.

Importantly, when BpV-treated C2C12 cells were co-cultured over a monolayer of cardiomyocytes, they express the cardiac specific isoform Troponin I. Although no beating was observed in CTnI-positive C2C12 cells, this result may open new perspectives for therapeutical approach in heart diseases; in fact, transplantation of skeletal muscle cells into infarcted hearts is already used in experimental clinical approaches; however, early clinical data suggest that myoblast transplantation may be associated with a transient period of electrical instability (Taylor, 2004d). The ability of BpV-treated muscle cells to acquire a cardiomyocyte-like phenotype may overcome the observed disadvantages.

It was recently shown that C2C12, HUVEC, fibroblasts and bone marrow cells express cardiac markers when co-cultured with cardiomyocytes, but this expression is the result of cell fusion (Reinecke et al., 2004;Matsuura et al., 2004). In our conditions no CTnI expressing C2C12 cells were detected when untreated cells were co-cultured with cardiomyocytes. However when BpV-treated cells were co-cultured with cardiomyocytes many of these expressing cells were detected. Whether this is a result of a real trans-differentiation or a BpV induced enhancement of fusion is still not known.

These results demonstrate that BpV treatment confers on C2C12 cells a multi-potential plasticity, as a result of the acquisition of a "stem cell-like" phenotype and behaviour. An additional requirement to determine whether BpV treatment may be a suitable approach to develop innovative cell therapy strategies, is that the cells should acquire the behaviour of a circulating progenitor, thus allowing transplantation through systemic delivery and home and colonise different tissues. The BpV-induced re-programming of gene expression and plasticity is already a strong starting point. However, the observation that, upon transplantation via femoralis artery, BpV-treated C2C12 cells, but not untreated cells, are able to migrate outside the vessels in the presence of inflammation, as in the case of dystrophic muscle, makes BpV a very promising pharmacological tool for the establishment of cell therapy protocols. The precise pathway used by transplanted cells to reach host muscle tissues is not known, however the possible BpV-induced expression of specific inflammatory cytokines receptors should be further investigated. Thus, BpV-treated C2C12 cells are able to circulate, migrate outside the vessels and reach the muscle tissue, although more quantitative and statistical analyses are required. Whether the cells are also capable of contributing to muscle regeneration is still under investigation.

In conclusion, BpV treatment appears to respond to most of the requirements needed for a pharmacologically-based cell mediated therapy, using already committed cells: reversible inhibition of differentiation, maintenance of cell proliferation, re-programming of gene expression towards an "earlier" "stem cell–like" phenotype, cell plasticity and systemic delivery. Future work is obviously needed to determine the actual feasibility of this approach for tissue repair in vivo, as well as to fine tune the treatment conditions (qualitative and/or quantitative enhancement of the response by modifying culture conditions or by synergy with other factors, or the efficacy of the pharmacological treatment in vivo).

Moreover, BpV treatment represents a good tool for studying the molecular mechanism and chromatin remodelling responsible for genome reprogramming and for the establishment of a “stem cell” phenotype.

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Manuscript in preparation

PHARMACOLOGICAL ACTIVATION AND REPROGRAMMING OF MUSCLE SATELLITE CELLS TO ACQUIRE AN EARLIER, PLURIPOTENT, CIRCULATING "STEM-LIKE" BEHAVIOUR, SUITABLE FOR THERAPEUTICAL APPLICATIONS

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During my PhD I have been also collaborating to a different project run in the laboratory aimed to study the possible role of the PKC-theta in regulating skeletal muscle homeostasis.

The results of this study are included in a manuscript to be submitted.

Manuscript to be submitted

PKC-THETA SYNERGIZE WITH CALCINEURIN FOR THE ACTIVATION OF MUSCLE-SPECIFIC PROMOTERS

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