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Autologous Grafts of Mesenchymal Stem Cells – Between Dream and Reality

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1. Introduction

During the last decade, the characterization of Adult Stem Cells (ASC) incited extraordinary infatuation for the development of autologous cellular therapy. The number of directed cellular differentiation essays of hematopoietic and mesenchymal stem cells demonstrated, against the classical rules of embryology, unsuspected capacities to generate *ex vivo* practically all cellular types. Nevertheless, the difficulties of revealing these spectacular capacities during clinical applications of tissue reparation suggest a random evolution of cell cultures. The major influence on this reality may play fragmentary knowledge of transductional mechanisms controlling the cellular fate and above all, the quality of isolated cells. This last condition seems to represent the one of essential technical barriers. In fact, the process of cellular isolation principally residing in the employment of cellular adherence and/or magnetic field able to retain cells marked by tagged antibodies. Unfortunately, the proteins recognized by antibodies are not expressed by sole stem cells but also by committed progenitors. In fact, the bone marrow precursors represent very heterogenic population of mononuclear cells whose affiliation seemed to be recently questioned. For instance, the antibody against CD34 protein is employed for isolation of Hematopoietic Stem Cells (HSC) while the absence of CD45 (universal hematopoietic cell marker) is recognized as sufficient to qualify Mesenchymal Stem Cells (MSC). Consequently, if previous observations revealed promising potential of bone marrow stem cells to be used for development of cellular therapy, their utilization must be preceded by detailed studies of their biology with particular focus on specific markers and transductional pathways permitting a high purity of isolation and control of differentiation protocols. The proposed chapter is based on our recent work indicating the heterogeneity of mesenchymal stem cells isolated from rabbit bone marrow which, placed in the context of recent studies, allows to propose a novel hierarchic organization of bone marrow cells. As *ex vivo* differentiation of stem cells would be dependent on transductional mechanisms we also propose to discuss how pharmacological modulation of activity of molecular target implicated in calcium homeostasis may influence cellular differentiation.

2. Heterogeneity of MSC

2.1 Introduction

Besides tissues having properties of self-renewal such bone marrow, the liver represents in Man the sole internal organ endowed with a spectacular capacity of regeneration illustrated already by the ancient myth of Prometheus. Interestingly, this process intervenes only after physical damage of the hepatic parenchyma which, destabilizing the entirety of the extracellular matrix, highlights the crucial role of epigenetic modulation on the proliferation and cellular differentiation (Michalopoulos & DeFrances, 1997; for review). Even if this natural phenomenon does not seem to be reproduced in internal organs, the recent isolation of adult multipotent, dormant within the various organs and tissues, stem cells seemed open the way towards a Regenerating Cellular Therapy. Indeed, the bibliographical data indicate a great plasticity of stem cells and in particular those taking from bone marrow like hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). Certain reports, already conclusive in the rat and the mouse, indicate a possibility of directed in situ and in vitro differentiation of stem cells and open exciting therapeutic prospects for tissue and various organs repair, without exposing the host to the failure of an allogeneic transplant rejection. Thus, contrary to embryonic stem cells (ESC) whose clinical application is still not unanimously accepted, ASC initially appeared as an ideal solution to prepare various autologous graft. Nevertheless, this dream about the imminent clinical application of ASC to cure a number of diseases as diabetes, cystic fibrosis, myocardial infarction and many others physiopathological states appeared more difficult to accomplish than initially expected. With the perspective of recent dynamic works it is conceivable to think that this is just a problem of a better knowledge of their diversity as well on the fundamental level as from the point of view of their therapeutic use.

This observed *ex vivo* pluripotency of HSC and MSC was a cause of noted infatuation. Surprisingly, HSC known to date for their capacity of renewal of blood morphotical elements, were also able differentiated toward skeletal muscle (Ferrari, 1998), cardiac (Orlic et al., 2001), nervous (Mezey et al., 2000), liver (Lagasse et al., 2000) or epithelial cells (Krause et al., 2001). Despite the notable example of post infarct myocardium wall repair, these works did not open the way to the routine clinical application (Agbulut et al., 2004). Troublesome, these results appeared as not reproducible and the parabiosis experiments between exposed to radiation and green fluorescent protein (GFP)-transgenic mousses did not demonstrate this supposed regenerating power of HSC (Wagers et al., 2002).

The *ex vivo* experiments carried out with MSC reporting relatively similar observations. For a long time, MSC were considered as having potential of differentiation limited to mesenchymal family cells as osteoblasts, chondrocytes, adipocytes or muscle precursors (Ashton et al., 1980). More recently, MSC revealed *in vitro* abilities to generate cells distinguished also by ecto- and endodermal features (Reyes et al., 2002; Woodbury et al., 2000; Sato et al., 2005). However, this pluripotentiality was objected by certain unsettled findings. At first, Hardeman et al. (1986) showed formation of cellular hybrids like myofibroblast which was forming by a fusion of fibroblasts with myoblastes able to conserve muscular character (Hardeman et al., 1986). Recently, the fusion of neuronal stem cells with ESC yielded cells expressing both characters (Ying et al., 2002). Since labeling technique with DNA coding enzymes or fluorescent proteins, these observations question

the reality of observed differentiation *in situ*. On the other hand, the possibility of phenotype modification of gene expression according to culture conditions could contribute to observed *in vitro* differentiation (Discher et al., 2009). It is also plausible that these divergent observations reflect a greate heterogeneity of MSC characterized by a certain ability of multipotency revealed during *ex vivo* manipulation where large majority of them represent committed progenitor cells rather than really pluripotent stem cells. Then, the random results of directed differentiation may be explained by imperfect approach of cell isolation. In fact, majority of protocols used is based rather on adhesion capacity of MSC than on specificity of a membrane marker not yet identified.

At the first time, on the basis of morphological differences, the heterogeneity of MSC was brought up by Colter et al. (2000) which proposed three types of MSC. Two first, named RS-1 and RS-2, characterized by a little size and absence or presence of granulations, were considered as self-renewal cells. The third type, distinguished by apparently bigger size, seemed corresponded to already partially differentiated (CSMm) cells (Colter et al., 2000). Thus, the authors hypothesized that RS-1 and RS-2 cells were progenitors of CSMm but since more quiescent state, RS-1 population appeared as precursor of RS-2 that differentiated to CSMm cells. In the proposed schema, RS-2 population would have had the capacity to maintain equilibrium of CSMm production by the ability to reprogramming towards the ground RS-1 state (Colter et al., 2000). In reality, the ulterior antigenic study of these three cellular populations that matching these morphological differences revealed yet more important cellular heterogeneity than initially supposed (Colter et al., 2001).

2.2 Evidence of rabbit MSC heterogeneity

The above data indicated the necessity to explore this proposed heterogeneity of MSC on the molecular level with particular insight into differences between clonal colonies which seems to be essential in elaboration of final approach of directed differentiation. Thus, we carried out the study having for objective the molecular characterization of colonies proliferating from individual CD45$^-$ mononuclear cells isolated from bone marrow of rabbit. This model was chosen for relative facility to obtain a biological material. This advantage being unfortunately associated with limited knowledge of rabbit genome, we have employed the Differential-Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) technique to analyze expressed respective mRNAs (Sturtevant, 2000). This approach resides in use of several non-specific primers able to hybridize with certain extracted mRNA during low temperature reaction (for more details see original paper of Sturtevant, 2000). In this way, the comparison of obtained patterns of mRNA in analyzed colonies showed differences in genes expression. The colonies were cultured separately after isolation of each clone proliferated from one cell on the surface delimited by cylinder (Figure 1A). After the harvest, the mRNA extract of each colony was analyzed with DDRT-PCR approach. In the figure 1B, we present the DDRT-PCR patterns of amplicons obtained after analyze of 14 colonies with couple 1 of DDRT-PCR primers purchased from Seegen (Seoul, Korea). Thus, these patterns, despite a certain similitude, vary by five differentially expressed mRNAs marked by the arrows. These genes correspond to proteins implicated in different cellular functions as follows: 1 – TBC1D7- cellular growth and proliferation; 2 – Filamine – cell migration; 3 – Cystatine 10 – chondrogenesis; 4- LUC7-like – inhibition of myogenic differentiation; 5 – MTHFR – inhibition of intracellular methylation. Their expression seem to be convergent with expression of OCT-4 gene (Figure 1C) considering as a marker of non differentiated cellular state (Tondreau et al., 2005).

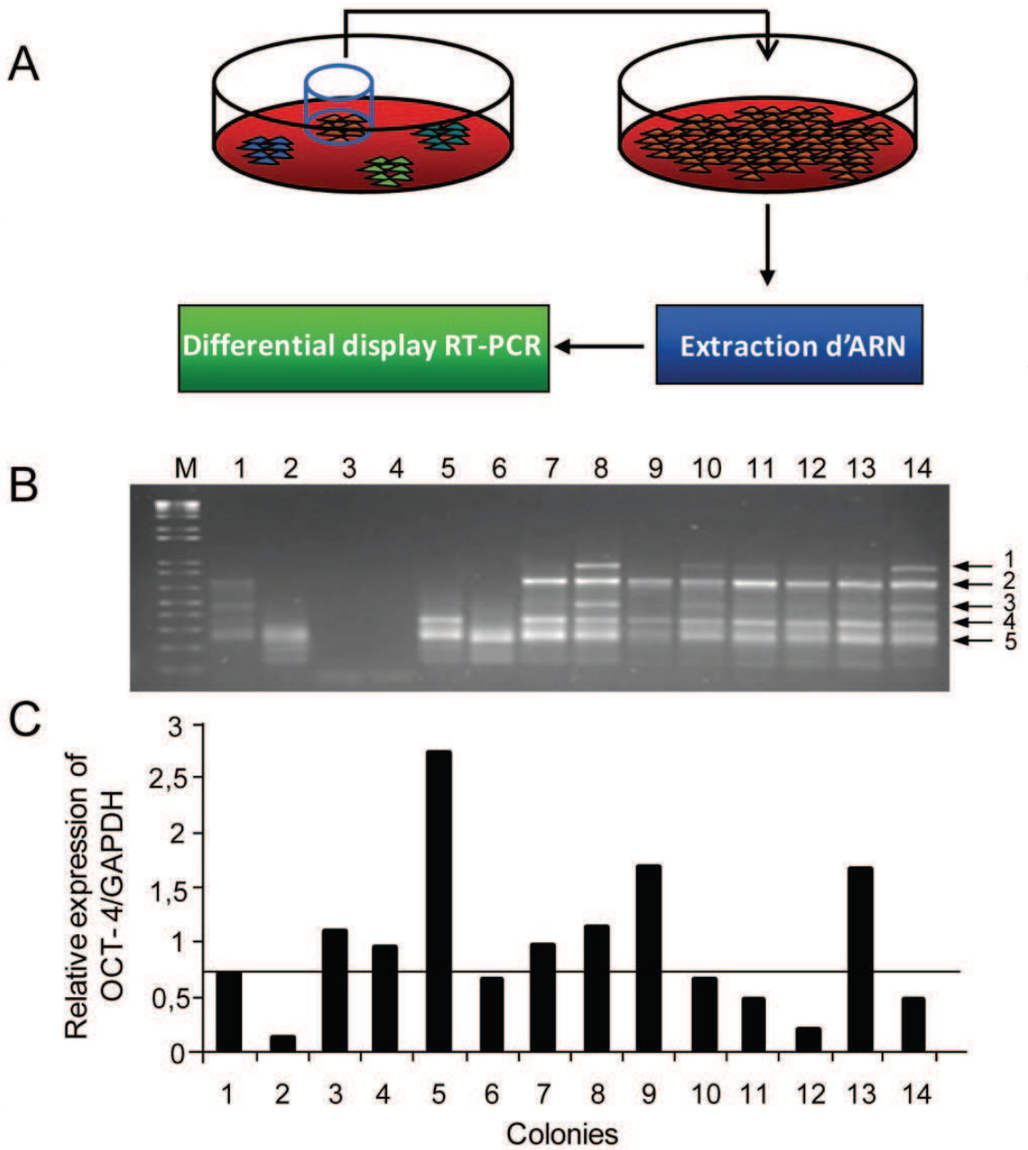

Fig. 1. **Contribution to the hypothesis of MSC cells heterogeneity.** A. Schematic representation of MSC clonal colonies development. After delimitation of one cell in the cylinder space and its proliferation, young colony is displaced in Petri dish where continues to proliferate. Just before confluence, cells are harvested for extract of RNAs. B. Differential Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) gel containing the amplicons obtained from 14 colonies extracts. The arrows indicate sequenced bands corresponding to TBC1D7, Filamine, Cystatine 10, LUC7-like, MTHFR gens. See the text for more details. C. Histograms representing the expression of OCT-4 gen. The line visualizes the mean level of relative OCT-4 expression.

It was interesting to observe, that in the medium LIF-free (Leukemia Induced Factor is employed in view to preserve non differentiated state during cell proliferation) the colonies were able spontaneously differentiated to muscle precursor cells with unequal capacities (Figure 2).

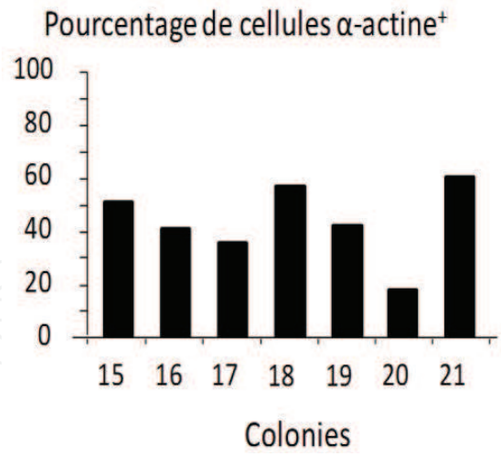

Fig. 2. **Relative spontaneous expression of α-actin gene in studied colonies.** The presence of actin was revealed by indirect immunocytochemistry where the presence of specific antibody was detected with horseradish peroxydase system.

All these results clearly indicate that mononuclear CD45- cells, still considered as MSC, form a heterogenic population characterized by different non differentiated and committed state. Our results did not determine the definitive number of cellular clones but suggest that currently practiced isolation of MSC may conducted toward random results of directed differentiation.

2.3 Toward a new hierarchy of bone marrow stem cells

Our conclusion seems to be strongly supported by the similar results obtained by microarray analysis of human MSC (Mareddy et al., 2009; Tormin et al., 2009). In addition, common distribution of certain membranes markers as CD44, CD73, CD90 or CD105 indicates that use of relative antibodies cannot be considered as discriminative tool for cell isolation. Thus, our results raised the question concerning hierarchy of organisation of bone marrow stem cells and place the observations previously published by groups of Verfaillie and Ratajczak at the special place. These key studies made mention of the very special cells named multipotent adult progenitor cells (MAPC) and very small embryonic-like cells (VSEL) respectively (Jiang et al., 2002; Kucia et al., 2006). Even if these results cannot be reproduced by other laboratories, MAPC possessing similar morphology to MSC are able generate mature cells characterizing by ecto-, meso- and endodermal features. This pluripotentiality, attesting their immature character, allows thinking that MAPC may be direct precursor of MSC as well as HSC (Jiang et al., 2002). Conceivably, this hypothesis may explain the random results of directed *ex vivo* MSC differentiation.

In contrast, VSEL cells are a very small, morphologically similar to embryo cells which being probably attracted by the chemical gradient of SDF-1, colonize bone marrow during embryogenesis (Kucia et al., 2006). Amazingly, the grafts of VSEL in irradiated mice indicated that their weak number seems to be responsible for acceleration of senescence process which suggesting their participation in internal organs and tissues regeneration (Kucia et al., 2008). Convergently, the increased number of VSEL, expressing myogenic Nkx2.5 protein, detected in general circulation in patients suffering from cardiac ischemia

suggests the possibility of their implication in cardiovascular repair (Wojakowski et al., 2009). In the case of definitive clinical confirmation, this observation may open extremely promising horizons of cellular therapy. Nevertheless, identification of these new cellular populations not responds to the question concerning the origin of MSC.

In this context, our results support hypothesis that MSC cannot be considered as pluripotent stem cells having the potential to generate all cells naturally deriving from tree embryonic layers. In the Figure 3, we propose to attribute this role to MAPC and VSEL cells. There are two possibilities, either we observe a coexistence of three cellular populations or existence an ontogenetic hierarchy. In the first situation, each cellular type possesses the variable potency of differentiation: i) VSEL that of committed precursor, ii) MAPC would be pluripotent and iii) MCS just mesodermal. In the second situation, VSEL would be direct precursor of MAPC generating MSC among other. In this way, the apparition of all cellular populations in bone marrow would reflect an ontogenic hierarchy formed during embryogenesis where initial number of VSEL cells determines a capacity of hypothetic organ repair during adult life. It appears that however the reality may be, the strategy of preparation of graft from MSC should be revised taking into account the recent clinical trials lacking therapeutic effects as published recently (Menasche, 2011). In fact, the clinical use of MSC is actually recognize for their immunomodulatory effects known in diminution of graft reject or graft versus host disease (Ringden et al., 2006; Ucceli et al., 2007; Le Blanc et al., 2008). In this way, two novel axes of fundamental research seem to be profiled: **i)** definite establishment of hierarchy of bone marrow cells in regard of MAPC and VSEL cells, **ii)** exploration of intracellular pathways in view to determine cellular fate during directed ex vivo differentiation.

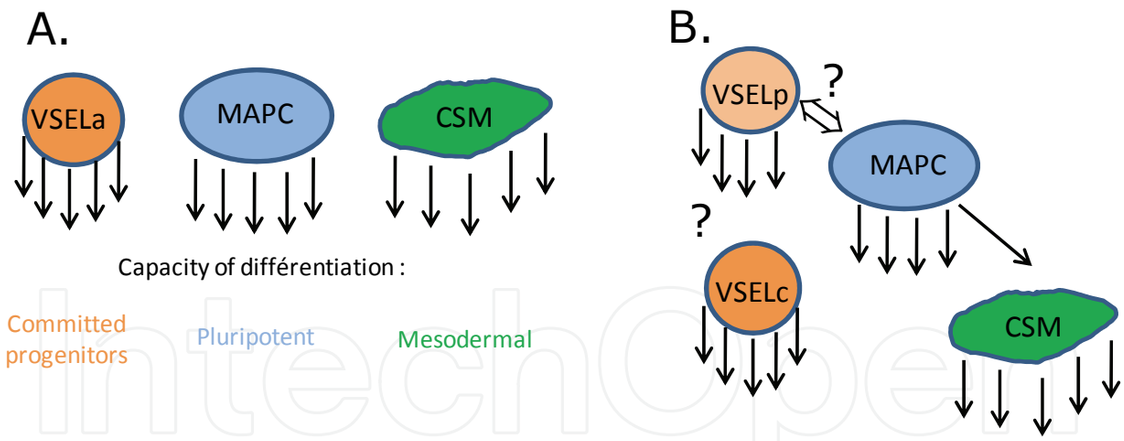

Fig. 3. **Schematic representation of bone marrow stem cells hierarchy.** A. Are we in the presence of three types of CSA which evolved separately according to the physiological regulation? B) Can one envisage a hierarchic ontogenic organization where VSEL cells would be pluripotent stem cells generating all bone marrow cells?

3. Calcium signaling and fate of MSC

3.1 Introduction

The random results of *ex vivo* differentiation of MSC during preparation of autologous grafts indicate that promising potential may be revealed by the microenvironment of cellular

culture. This hypothesis seems to open a new area for proceedings of directed differentiation which may be based on modulation of activity of molecular targets of MSC. Given the dependence of phenotypical gene expression, self renewing, migration or proteolytical enzyme secretion on increase of cytosolic calcium concentration $[Ca^{2+}]_c$, pharmacological modulation of calcium signalling would represent a key to control cellular fate. The various membrane channels and ionic transporters having potential to modulate $[Ca^{2+}]_c$ appears then as appropriate molecular targets. Recent reports indicate that MSC express several ionic membrane channels generating sodium (Na), calcium (Ca) and potassium (K) inward and outward currents characterized by molecular biology and patch-clamp approaches (Li et al., 2005; Li et al., 2006; Deng et al., 2006; Kawano et al., 2003; Kawano et al., 2002; Heubach et al., 2004). Nevertheless, the capacity of these channels to modulate $[Ca^{2+}]_c$ in MSC was not yet evaluated. Some data indicate that in human MSC, the $[Ca^{2+}]_c$ may change upon cyclic oscillatory variations via a mechanism implicating inositol trisphosphate receptors (IP_3Rs), store operating channels (SOCs), L-type voltage dependent calcium channels (L-VDCaCs) as well as Na^+-Ca^{2+} exchangers (NCX) (Kawano et al., 2003; Kawano et al., 2002). We have recently shown that MSC express also several genes coding the proteins of transient receptor potential cation channel (TRPC1/2/4/6) family (Torossian et al., 2010) possessing a major role in cell proliferation as already documented in cancers (El Boustany et al., 2008). Over it, dependence of immature cell proliferation or myoblasts fusion (Lory et al., 2006) on activity of voltage dependent T-type calcium channel reinforces idea that pharmacological modulation of calcium signalling could reveal potential to improve efficiency of protocols employed in directed differentiation of adult stem cells.

In the present study, using functional and molecular biology approaches, we pursued two major objectives: *i)* evaluation of efficiency of membrane voltage dependent ionic channels (VDCaC, VDNaC, VDKC) and transporters (Na/K-dependent ATPase and NCX) to modulate calcium homeostasis on the basis of kinetics of $[Ca^{2+}]_c$ variations occasioned by selective activators and blockers, *ii)* demonstration that inactivation of chosen targets such T- or L- type VDCaC and TRPC1 reduced cellular proliferation and that high concentration of nifedipine activated neuroglial differentiation.

3.2 Efficiency of molecular targets to modulate calcium homeostasis

Figure N°4 illustrates that equilibrium state in single MSC is disturbed by modifications of the extracellular medium or by the presence of selective pharmacological agents which changing Ca^{2+}, Na^+ or K^+ gradients induce the $[Ca^{2+}]_c$ variations with different kinetics. The gathered histograms representing the areas under curves (AUC) were obtained from individual profiles whose averages are expressed in the Figures 4 and 5. The highest calcium mobilization was observed in the presence of depolarizing solution of KCl as well as 2-diazo-4,6-dinitrophenol (DDNP) or bepridil, well known respective blockers of BK_{Ca} channels and NCX. Even if each product activated this increase by different mechanism, the obtained AUCs were very similar and corresponded to 5.5, 5.25 and 5.1 µM/L (Fig. 4). The depolarizing solution of KCl imposing membrane potential to value inferior to -30 mV activates low threshold VDCaCs as "L"- and/or "N"-type channels. Similar effect obtained with DDNP (Fig. 5D) revealed a high capacity of the BK_{Ca} channel inactivation to membrane depolarization subsequent to cytosolic K^+ accumulation. The action of bepridil eliciting the reverse mode of NCX action is responsible for calcium influx which considered its proximity

with endoplasmic reticulum, induces intracellular calcium mobilization (Niggli et al., 1991). In the same way, figure 4 shows also that the action of Na/K-dependent ATPase having the capacity to modify sodium gradient induced cytosolic calcium increase by recruitment of NCX (Hilgemann et al., 1992). In contrast, the effect of other depolarizers as $CaCl_2$ solution, tetraethylammonium (TEA) or veratridine (Fig 5B, 4C and Fig 6) appeared as less efficient.

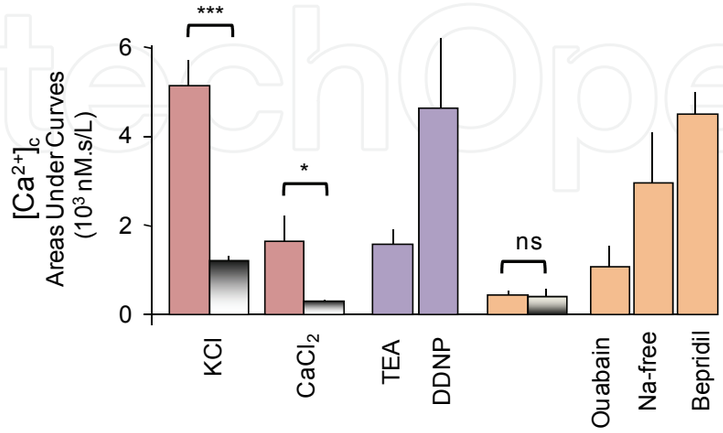

Fig. 4. **Mean values of area under curves (AUC) of cytosolic calcium mobilization in rabbit mesenchymal (MSC) stem cells.** KCl (25 mM) in the absence (n= 10) or in the presence of 10 µM nifedipine (▯ ; n= 15), $CaCl_2$ (10 mM) in the absence (n= 14) or in the presence of 10 µM nifedipine (▯ ; n= 17), TEA (3 mM; n= 14), DDNP (10 µM; n= 6), veratridine alone (100 µM; n= 29) or in the presence of 1 µM TTX (▯ ; n= 22), ouabain (10 µM; n= 6), Na-free (n= 11) and bepridil (100 µM; n= 17) were injected in the vicinity of MSC (***, $p<0.001$; **, $p<0.01$; *, $p<0.1$). The mean values (±SEM) of areas under curves were numerically integrated from individual microfluorimetric (Indo-1) recordings by trapeze method using Excel programme. Each recording represents the same number of points acquired every 250 ms by PC-assisted system developed by Notocord Systems (Paris, France).

The type of VDCaCs was determined with nifedipine, a dihydropyridine derived L- type channel blocker and by RT-PCR experiments. The significantly reduced, but never totally abolished stimulatory effect of KCl and $CaCl_2$ solutions on calcium mobilization in the presence of the blocker indicating the involvement of both nifedipine-sensitive and insensitive VDCaCs. RT-PCR convergent experiments, carried out with Cav1.2, Cav2.2 and Cav3.3 specific primers, confirmed expression of L-, N- and T-type of VDCaCs in MSC (Fig. 4, boxes). L-type channels (Cav1.2 subunits) were already reported in human and rat MSC (Li et al., 2005; Li et al., 2006). In contrast, the existence of T channels in MSC are a matter of debate since contradictory reports concluding to the absence of Cav3.1 and Cav3.2 subunits (Li et al., 2005; Heubach et al., 2004) or to the presence of Cav3.2 whose functionality was however not determined (Kawano et al. 2002). The importance of expression of T channel in MSC is illustrated by observations in ESC where the sustained increase in $[Ca^{2+}]_c$ is responsible for cell proliferation (Lory et al., 2006) or fusion of differentiated myoblasts (Bijlenga et al., 2000). The expression of N-type VDCaCs in MSC is not surprising because its functionality in differentiating cells evolves through an expression pattern (Arnhold et al., 2000). Thus, during neuronal differentiation of ESC, transitory high expression of N-type

channel in initially apolar phenotype matched with cellular migration whereas its reappearance in differentiated neuron coincided, similarly to mature cells (Yokoyama et al., 2005), with synaptogenesis and modification of the exocytose level (Jones et al., 1997). As MSC are known for their secretory and migratory activities, similar functionality may be expected. Consequently, calcium fluxes in MSC can be modified by opening of three types of VDCaCs which filling up the different cytosolic microdomains with calcium can separately control gene expression, cellular proliferation and migration or exocytosis (Lory et al., 2006; Yokoyama et al., 2005; Yang et al., 2006; Yoo et al., 2007).

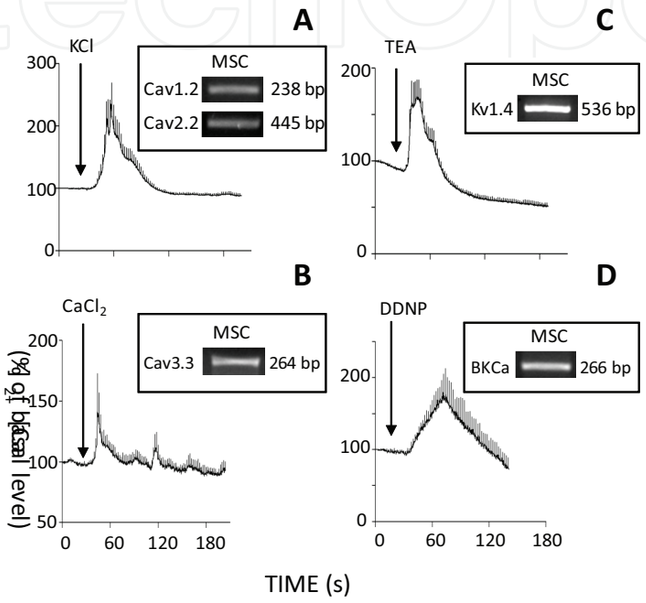

Fig. 5. **Effects of modulation of calcium and potassium channels on $[Ca^{2+}]_c$ in rabbit mesenchymal stem cells (MSC).** The arrows indicate the pressure-ejected administration of depolarizing solution KCl (25 mM) (A), $CaCl_2$ (10 mM) (B) and voltage or calcium dependent potassium channels blockers as TEA (3 mM) (C) and DDNP (10 µM) (D) in the vicinity of the cells. The right-placed boxes represent RT-PCR obtained amplicons of mRNA coding L, N, T-type voltage-dependent calcium channels (Cav1.2, Cav2.2 and Cav3.3 subunits), voltage dependent (Kv1.4 subunit) and calcium dependent (BK_{Ca}) potassium channels. The curves represent a mean from 10 (A), 14 (B), 14 (C) and 6 (D) individual cell recordings. The spontaneous level of $[Ca^{2+}]_c$ (100% basal level) was calculated for each experiment as the mean concentration during 30 s preceding the administration of ionic solutions or potassium channel blockers.

Interestingly, the activation of T-type VDCaC *in vivo* appears to be directly dependent on a potassium gradient demonstrating the crucial role of K^+ channels in the evolution of stem cell fate. This astute mechanism is based on cooperation between three types of ionic channels. Briefly, VDKC or/and CaDKC provoke transitory membrane hyperpolarization conducting to depolarizing potassium influx through delayed-rectifier potassium channel responsible for T-type VDCaC activation and [Ca2+]c increase. Such membrane hyperpolarization, detected in rat MSC (Deng et al., 2007) during progress of cell cycle from G(1) to S phase, seems to be dependet on the balance of expression between KCa3.1 and

delayed-rectifier (Kv1.2/Kv2.1) subunits which since their down-regulation with the specific RNAi appeared crucial for cell proliferation. Thus, the cooperation between IK_{Ca} and KDR channels that generate hyperpolarizing efflux and subsequently delaying influx of K^+ may vary membrane polarity near the threshold value of T-type VDCaC activation.

Amazingly, unlike the mechanism described above, we found pharmacological way to obtain *ex vivo* a similar effect on [Ca2+]c increase in MSC. Using functional and RT-PCR experiments, we observed that the blockage of Kv1.4 and BK_{Ca} channels (fig.4C, D) by TEA (51%; 32 of 63 cells) and DDNP (46%; 39 of 84 cells) induced $[Ca^{2+}]_c$-increase after VDCaC activation due to intracellular membrane depolarization triggered by cytosolic K^+ accumulation. Further studies are needed to show whether such blockage of BK_{Ca} channel would stabilize cell proliferation and immaturity.

The pharmacological activation of VDNaC represents another way to augment [Ca2+]c. Similarly to excitable cells like neurons or cardiocytes, the opening of VDCaCs in MSC results also from progressive membrane depolarization initiated by low threshold T-type VDCaC and/or VDNaC. In our experiments, veratridine (non-selective opener of VDNaCs) (Yang et al., 2006) started Na-induced depolarization which reaching activation threshold of VDCaCs was responsible for increase of $[Ca^{2+}]_c$ (84% given 54 of 64 cells). Not significant reduction of this effect by TTX, a VDNaCs blocker (t=0.38; 79% given 55 of 69 cells) (Fig. 5A) and identification of mRNA encoding Nav1.9 subunit (Fig 5A) indicated the expression of TTX-resistant VDNaCs in MSC. Noticeably, the type of VDNaC expression in MSC appears to be controversial. Using identical primer as Deng et al. (2006), we were unable to confirm their observation on expression of Nav1.1 subunit in rabbit MSC but we found relative transcript in extracts from rabbit nervous system which suggests non-expression of this subunit in our cultures. Divergent findings on the expression of VDNaC may also be noted in human MSC. While Heubach et al. (2004) failed to identify both TTX-resistant and TTX-sensitive channels, Li et al. (2005) detected a functional TTX-sensitive inward current. These discrepancies may result from the different experimental protocols used. In our study, mononuclear cells were separated with CD45 antibody instead of their capacity to adhesion already reported (Li et al., 2005). Moreover, our mRNA samples were obtained at the final stage of the first passage contrary to the 4^{th} or even the 8^{th} as previously described (Li et al., 2005; Deng et al., 2006). As expression of sodium channel unit *in vivo* changes throughout cellular maturation (Benn et al., 2001), these observed *in vitro* differences reveal modulation of gene expression by microenvironment. Nevertheless, the weak kinetics of calcium mobilization induced by veratridine seems indicate that VDNaCs did not appear as interesting target to modulate a fate of MSC.

On the contrary, NCX having capacity to exchange cytosolic/extracellular Ca^{2+} for Na^+ in normal or reverse mode (Niggli et al., 1991) within chemical gradient of both ionic populations, appears as powerful $[Ca^{2+}]_c$ enhancer in MSC. As shown in Fig.6, Na-free medium (38% given 17 of 45 cells), bepridil (44% given 31 of 70 cells) or ouabain (62% given 23 of 37 cells) led to transient increases in $[Ca^{2+}]_c$. The RT-PCR-detected expression of genes coding NCX and Na^+/K^+-ATPases (Figs. 6B, C) matched our functional observations. Similarly to other cellular models (Hilgemann et al., 1992), cytosolic overloading with sodium after ouabain-induced inactivation of Na^+/K^+-ATPase triggered a $[Ca^{2+}]_c$ increase resulting from exchange of sodium for calcium during reverse mode action of NCX (Niggli et al., 1991). In human MSC, the NCX seems to take part in the induction of calcium

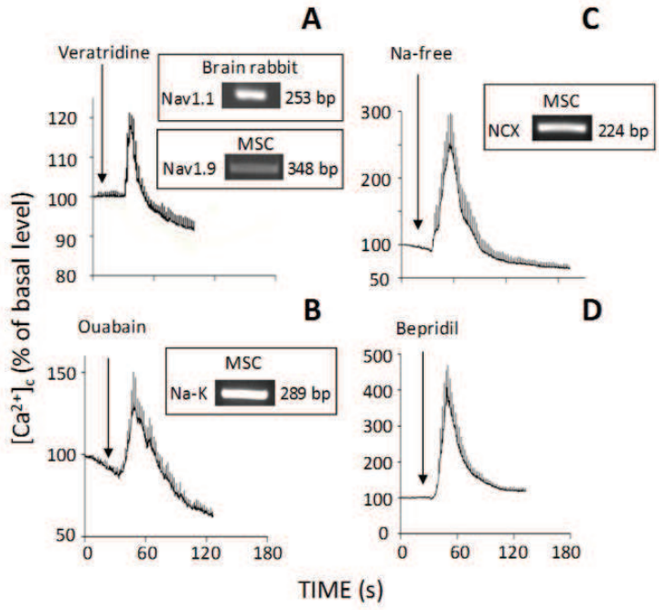

Fig. 6. **Effects of modulation of voltage dependent sodium channels, ATPase Na^+/K^+ dependent and sodium-calcium exchanger activities on $[Ca^{2+}]_c$ in rabbit mesenchymal stem cells (MSC).** The arrows indicate the pressure-ejected administration of Veratridine (100 µM) (A), Ouabain (10 µM) (B), Na^+-free medium (C) and Bepridil (100 µM) in the vicinity of MSC. The right-placed boxes represent the RT-PCR obtained amplicons of mRNA coding voltage-dependant sodium channel (Nav1.9 subunit) in MSC, Nav1.1 being detected only in brain rabbit extract, ATPase Na-K dependend (B) and Na^+-Ca^{2+} exchanger (NCX) (C). The curves represent a mean from 22 (A), 6 (B), 11 (C) and 17 (D) individual cell recordings. The spontaneous level of $[Ca^{2+}]_c$ (100% basal level) was calculated for each experiment as the mean concentration during 30 s preceding the administration of ionic solutions or potassium channel blockers.

oscillations (Kawano et al., 2003). In the present work, its activation induced a transient increase in $[Ca^{2+}]_c$ followed by a slow basal calcium level recovery. It is like during early stage of cardiomyocyte differentiation of mouse ESC, where without modifying transient calcium variations, NCX enhanced the basal level of $[Ca^{2+}]_c$ (Fu et al., 2006). This may indicate the crucial role of NCX in the stabilization of higher basal $[Ca^{2+}]_c$ in immature cells where its activity may be improved by direct intracellular phosphorylation or by increase of Na-gradient during opening of VDNaC or Na^+/K^+-ATPase inhibition.

3.3 Effects of VDCaCs inactivation on MSC cell culture

Taken account of highest capacity to modify calcium homeostasis, VDCaCs was chosen as more appropriate target to evaluate pharmacological modulation of MSC fate. Another choice is related to TRPC1 protein which being largely expressed by rabbit MSC (Torossian et al., 2010) is known as one of essential factors managing calcium distribution during cancer cell proliferation (El Boustany et al., 2008; El Hiani et al., 2009).

Then, the blockage of L- and T-type VDCaC pointed their implication in the control of cellular proliferation and differentiation. Mibefradil and nifedipine induced a dose-

dependent decrease in cell numbers corresponding to 25 and 15 % of cells respectively at 10 μM concentrations attaining very significant inhibition (65 and 50 %; $p<0.005$) when treated with 30 μM doses (Fig. 7). Similarly, inducing 45% inhibition of MSC proliferation, the specific siRNA demonstrated a major role of TRPC1 protein in this process.

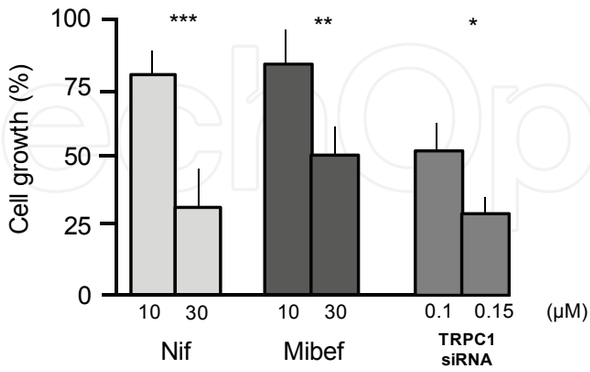

Fig. 7. **Relative mesenchymal stem cells (MSC) proliferation in the presence of calcium channel blockers.** MSC were cultured during 48 hours in the medium containing nifedipine, mibefradil (10 and 30 μM, both) or siRNA of TRPC1 (0.1 or 0.15 μM). The results represent the means (±SEM) from four independent experiments expressed as a percentage of proliferating cells. Each culture contained initially 20000 MSC and after 24h incubation period in expansion medium, the blockers at respective concentrations were administered in the plates. After 48h period of incubation the cells were fixed in acetic alcohol, stained with crystal Violet and extracted with acetic acid after drying. The optical density of extractions was evaluated using spectrophotometric measurement at 570 nm and compared to the standard range to obtain the number of cells. The relative effect of the drugs on cell proliferation was evaluated in comparison to non-treated cells (***, $p<0.001$; **, $p<0.01$; *, $p<0.1$).

Noticeably, the presence of a higher concentration of nifedipine (100 μM) induced apparition of two types attached irregularly shaped cells. The first type, representing about 85%, was characterized by expression of Glial Fibrillary Acidic Protein (GFAP) (Fig. 8B, D, F) whereas the second remained GFAP negative. This result may be particularly relevant in comparison to the control LIF-free culture where cells showed varied morphology and ability to spontaneously differentiate into myogenic precursor cells since relative to α-smooth muscle actin staining (Fig. 8A)

For the first time, we show that blockade of L-type channels in MSC may generate neural precursor cells already shown for their GFAP staining (Imura et al., 2003). We observed two kinds of $GFAP^+$ cells corresponding to a low number of neural-like cells accompanied the large majority of staining cells displaying astrocyte-like morphology. The absence of GFAP staining in the LIF-free expansion medium and the disappearance of myogenic character after treatment with nifedipine, fully support the idea that pharmacological modulation of calcium homeostasis would reinforce strategy for directed differentiation of stem cells. These observations suggest that the reduction of higher and persistent $[Ca^{2+}]_c$ appears like a turning point between proliferation and differentiation where favouring proliferation, the persistent calcium level avoids differentiation.

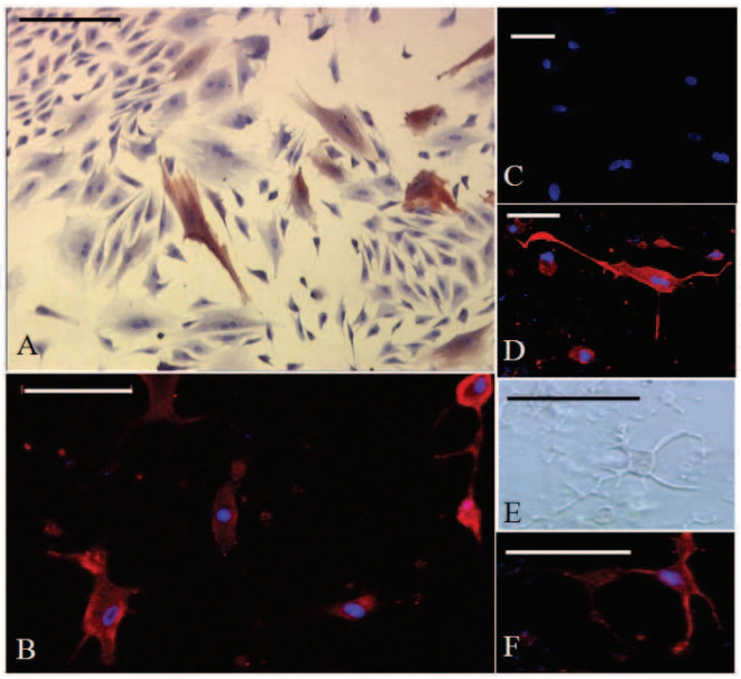

Fig. 8. **Spontaneous myogenic and nifedipine induced GFAP⁺ cells derived from mesenchymal stem cells (MSC).** (A) MSC stained with α-smooth muscle actin cultured in the LIF-free expansion medium. (B) Expression of GFAP in MSC cultured in the LIF-free expansion medium supplemented with 100 µM nifedipine. (C) Absence of GFAP expression in MSC cultured in the LIF-free expansion medium in the absence of nifedipine and counter staining with nuclear dye Hoechst. (D) Neural-like GFAP⁺ **cells**. (E,F) Morphological aspects of astrocyte-like cultured MSC in the LIF-free expansion medium supplemented with 100 µM nifedipine (E, viable cell; F, GFAP⁺ fixed cell). Scale bars, 100 µm.

The results obtained during prolonged exposition of MSC on both anticalcics seem to corroborate this hypothesis. As shown in the Fig. 9, the 10 µM doses were able introduce morphological modifications indicating the initiation of differentiation process. Cells growing in the presence of mibefradil (Fig. 9B) seem to display a more elongated and spindle shape while nifedipine favoured formation of cell extensions (Fig. 8C). Theirs action coincided with apparition of numerous vacuoles apparently more large and swollen in the presence of nifedipine (Fig. 8B, C). Since negative staining with oil red O, hematoxylin-eosine, toluidine blue or periodic acid-Schiff (data not shown) these vacuoles did not contain lipids, glycoproteins nor mucopolysaccharides. Such formation, attributable to an intensification of autophagy process (Mizushima & Levy, 2010), was transiently observed during erythrocyte or lymphocyte differentiation (Kundu et al., 2008; Mortensen et al., 2010) and appeared crucial to adipogenesis (Baerga et al., 2009). According to information recently reported in human U-251 glioblastoma cells (Johnson et al., 2006) or maturating foetal hepatocytes (Matsunga et al., 2008), the mechanism of this process may be explain by not well understood dependency of initial stage of differentiation upon Ca-dependent PI3-kinase activity. Interestingly, the siRNA-inactivation of TRPC1 expression did not modify cell morphology suggesting that unlike T and L type channels this protein is not implicated in MSC differentiation.

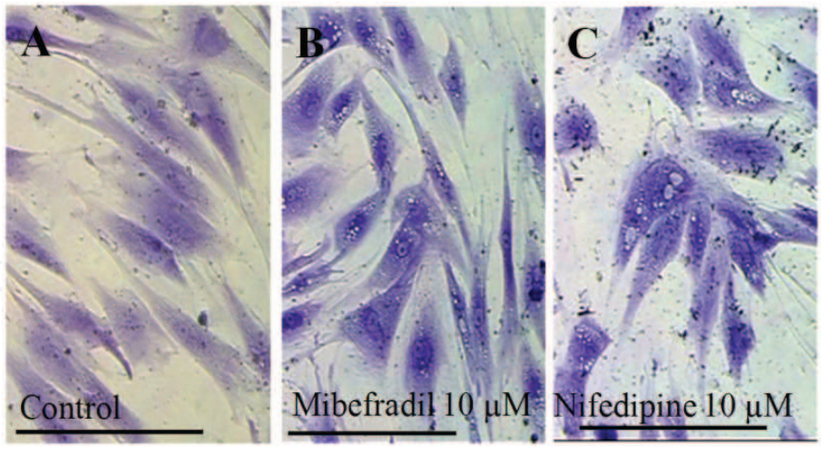

Fig. 9. **Morphological modifications of mesenchymal stem cells (MSC) induced by calcium channel blockers.** Culture of MSC in the LIF-free expansion medium in the absence (A) and in the presence of mibefradil, "T-type" (B) or nifedipine, "L-type" calcium channel blockers (C). Both blockers were employed at 10 µM concentration. Scale bar, 100 µm.

3.4 Conclusion

Taken together, our data demonstrated that pharmacological modulation of ionic carriers' activity, as particularly T- and L-type VDCaC or TRPC1 protein, may reinforce the strategies employed *ex vivo* for directed differentiation of stem cells. Further studies should demonstrate whether pharmacologically induced modulation of $[Ca^{2+}]_c$ in stem cells would maintain their immaturity or begin their differentiation.

4. General discussion and perspectives

The heterogeneity of MSC and their various commitments, as discussed above, perfectly explain why clinical application appears restraint their supposed pluripotentiality on immunological and mesenchymal capacity. In fact, not expressing II class MHC molecule, MSC are therefore not antigen-presenting cells and would be ignored by the host's immune system (Tse et al., 2003; Krampera et al., 2003). By their constitutive secretory activity (Caplan, 2009), MSC have capacity to create microenvironment favourable to combat graft-versus-host-disease (Koc et al., 2000) as well as attenuate inflammatory bowel symptoms in Crohn disease grafted patients (Caplan, 2009). Their aptitude for differentiate into osteoblasts was exploited in clinical trial for the treatment of *osteogenesis imprfecta* patients (Horowitz et al., 1999; 2002). One of very interesting work representing the regeneration of surgically amputated meniscus in goat by knee injection of MSC with hyaluronan delivery vehicle, provides perspective in the treatment of arthritis (Murphy et al., 2003). In contrast, use of MSC in view of cardiac post infracted reparation which seems to provide therapeutic improvement appeared to be not exerted by cardiomyocyte differentiation (Caplan, 2009). Convergently, the results of clinical trials realized with autologous MSC, HSC and mononuclear bone marrow cells (MNC) in about 1600 (Menasche, 2011) patients suffering from acute myocardial infarction, refractory angina or chronic heart failure did not give expected benefits indicating that heterogenic MNC of bone marrow, while remaining immunologically neutral, appear to be not therapeutically reliable to repair other than hard

tissues like bone or cartilage. Nevertheless, the existence of great variability in the functionality of MSC retrieved from patients indicating that pluripotent differentiation would be ascribed to more immature cells which are able generating MSC. Our study shows clearly that MSC should be considered as heterogeneous and composed by lineage-committed cells that may be multipotent but certainly non pluripotent cells. In addition, parallelism between decrease in MSC number with age (Lennon et al., 1996) and acceleration of the senescence process in mouse grafted with a low number of VSEL cells (Kucia et al., 2008) strongly suggests that this role may be ascribed to VSEL cells which would represented this pluripotent cellular population. In this way, the unequal number of VSEL cells in isolated samples may explain the random results of *ex vivo* differentiation. It is therefore conceivable that specific isolation of this cell population represents the first problem to resolve. In fact, the number of data suggest that bone marrow may be considered as reserve of pluripotent cells but this property cannot be attributed to MSC.

Our study of calcium signaling raises a second problem of directed differentiation representing by epigenetic reprogramming of gene expression which in an unpredictable manner would change the cellular fate. This conclusion is supported by divergence concerning the expression of VDCaCs and VDNaC in MSC. This inconvenience could be avoided in the cultures composed of a homogenous population of stem cells able reproducing stable microenvironment. Microenvironmental stability appears then as one of the more important conditions allowing prediction of cellular evolution and an objective comparison of the effects occasioned by experimentally introduced modifications. Our results indicated that pharmacological modulation of calcium homeostasis may influence cellular behavior seem open the perspectives for research of experimental protocols having potential to control the cell proliferation and differentiation.

Taken together, it can be concluded that in view to realize a dream about autologous regenerative grafts it would be necessary to direct the basic research toward two major objectives: i) to find the strategy to facilely isolate pluripotent stem cells from the bone marrow and ii) to perfect protocols allowing control the evolution of cellular cultures.

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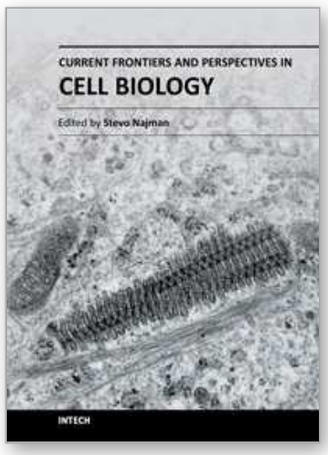

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