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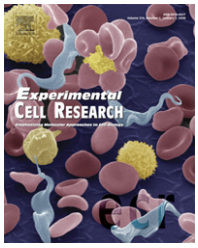

Research Article

ERK2 protein regulates the proliferation of human mesenchymal stem cells without affecting their mobilization and differentiation potential

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ABSTRACT

Human Mesenchymal Stem Cells (hMSC), derived mainly from adult bone marrow, are valuable models for the study of processes involved in stem cell self-renewal and differentiation. As the Extracellular signal-Regulated Kinase (ERK) signalling pathway is a major contributor to cellular growth, differentiation and survival, we have studied the functions of this kinase in hMSC activity. Ablation of ERK2 gene expression (but not ERK1) by RNA interference significantly reduced proliferation of hMSC. This reduction was due to a defect in Cyclin D1 expression and subsequent arrest in the G0/G1 phase of the cell cycle. hMSC growth is enhanced through culture medium supplementation with growth factors (GFs) such as Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF) or Epidermal Growth Factor (EGF). However, these supplements could not rescue the defect observed after ERK2 knockdown, suggesting a common signalling pathway used by these GFs for proliferation. In contrast, ERK1/2 may be dissociated from chemotactic signalling induced by the same GFs. Additionally, hMSCs were capable of differentiating into adipocytes even in the absence of either ERK1 or ERK2 proteins. Our data show that hMSCs do not require cell division to enter the adipogenic differentiation process, indicating that clonal amplification of these cells is not a critical step. However, cell–cell contact seems to be an essential requirement to be able to differentiate into mature adipocytes.

Introduction

Human mesenchymal stem cells (hMSC) are non-hematopoietic cells that reside mainly within the bone marrow stroma. These cells are multipotent and serve as precursors for various mesodermal tissues, allowing both the molecular basis of differentiation and the clinical potential for tissue regeneration and engineering to be studied [1,2]. Although a specific hMSC marker has yet to be identified, these cells form characteristic adherent, fibroblast-like colonies when grown

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in vitro and may be expanded further in the presence of serum. They are capable of differentiating *in vitro* and *in vivo* along multiple pathways that generate bone, cartilage, adipose and connective tissue. In order to acquire these new phenotypes, uncommitted hMSC must proliferate and differentiate, processes that require tight temporal control involving the activities of various transcription factors, cytokines, growth factors and extracellular matrix molecules.

One of the defining characteristics of MSCs, as for any stem cell, is a long-term capacity to self-renew. Conversely, the acquisition of lineage-specific differentiation characteristics is associated with an irreversible loss of multipotency, as well as a decrease in proliferation potential. From a clinical perspective, the identification of culture conditions that maximise hMSC growth but maintain differentiation capacity are desirable. In this regard, Fibroblast Growth Factor (FGF) [3], Platelet-Derived Growth Factor (PDGF) [4] and Epidermal Growth Factor (EGF) [5] have been shown to be potent mitogens for hMSCs, whereas a contrasting effect is seen after addition of interferon-alpha and interleukin-4 [6]. Under optimal conditions, hMSCs can be maintained in culture for many population doublings and still retain their differentiation capacity. However, extensive subcultivation of hMSCs impairs their functionality and the cells display evident signs of "*in vitro* aging", diminishing division capacity and an increase in senescence and/or transformation [7]. In this regard, it has also been shown that there is good correlation between the decline in replicative lifespan and donor age [8].

Although several biochemical processes and culture requirements for hMSC self-renewal and differentiation have been described, the exact mechanisms are poorly understood. One of the potential signal transduction pathways that might regulate these processes is the mitogen-activated protein kinase (MAPK) pathway. These kinases, including Extracellular signal-Regulated Kinase (ERK), c-Jun NH2-terminal Kinase (JNK), and p38, are the major signal transduction molecules regulated by growth factors, cytokines, and stress, which consequently trigger long-term cellular responses. Activation of MAPK family members is achieved through kinase cascades that serve to connect cell surface receptors to specific transcription factors and other regulatory proteins. Amongst them, the ERK1/2 pathway is probably the best characterized, belonging to an evolutionary conserved cascade defined by the RAS/RAF/ERK pathway [9]. Although ERK1 and ERK2 are coded by two different genes, they share an overall 83% identity at the aminoacid level [10]. ERK1 and ERK2 are involved in the control of many fundamental cellular processes that include cell proliferation, survival, differentiation, apoptosis, motility and metabolism. They are activated by dual phosphorylation on tyrosine and threonine residues which are regulated similarly. ERK1 and ERK2 contribute downstream to intracellular signalling by phosphorylating a largely common subset of substrates, both in the cytosol and in the nucleus.

In particular, several studies have looked at the effects of ERK1/2 on adipogenesis [11]. Prolonged inhibition of ERK expression using antisense oligonucleotides blocked adipogenesis in a pre-adipocyte cell line 3T3-L1 [12]. On the other hand, inhibition or ERK1/2 activation by a chemical inhibitor (PD98059) blocked the osteogenic conversion of hMSCs, resulting instead in the adipocyte formation in the presence of osteogenic inducers [13]. However, both studies were unable to discriminate between both proteins. Thus, the aim of the present study is to understand how ERK1 and ERK2 independent signalling affects the proliferation, migration and differentiation processes of hMSC.

Materials and methods

Culture and differentiation of hMSCs

Human bone marrow-derived MSCs (hMSCs) were obtained from Inbiobank Stem Cell Bank (www.inbiobank.org). Inbiobank is supported by the Spanish *Ministerio de Educación y Ciencia* and the Basque Country Government. Cadaveric bone marrow was harvested from brain-dead donors under the Spanish National Organization of Transplant (*Organización Nacional de Transplantes*, ONT) supervision. Informed consent was given by relatives. Each sample donor was tested and found negative for: HIV-1/2, Hepatitis B–C, Cytomegalovirus and Mycoplasma. All cells were processed at INBIOBANK following manufacturing procedures based on ISO9001:2000 under GMP conditions (Good Manufacturing Practices). Generated hMSCs display a typical CD29+, CD73+ (SH3 and SH4), CD105+ (SH2), CD166+, CD34–, CD45– and CD31– phenotype. In presence of specific differentiation factors, these cells were able to differentiate into osteocytes, condrocytes and fatty cells. hMSCs were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma, www.sigmaaldrich.com) supplemented with 10% fetal bovine serum (FBS). For proliferation assays, EGF, PDGF (Sigma) and bFGF (Biosource, Invitrogen, www.invitrogen.com) were used at 10 ng/ml. 1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxantine (IBMX) and 200 μM indomethacin (Sigma) were included in the adipogenic medium (AM).

Expression analysis

To evaluate the level of knockdown of used shRNAs, gene and protein expression was tested by relative quantitative Real Time-PCR (qPCR) and Western blot, respectively. For qPCR, total RNA was isolated from infected cells using Versagene™ kit (Gentra, Qiagen, www1.qiagen.com/) following the manufacturer's protocol. Same quantity of RNA was converted in total cDNA using a RT-Kit from Applied Biosystems (www.appliedbiosystems.com). ERK1 and ERK2 were amplified using predesigned TaqMan probes from Applied Biosystems (Assay IDs: Hs00946872_m1 and Hs00177066_m1, respectively). To compare the obtained values the DDCT method (User Bulletin #2, Applied Biosystems) was used. An endogenous control of β-actin gene was used (Assay ID: Hs99999903_m1) and the sample of empty pLVTHM as the calibrator. Reactions were carried out in an ABI 7300, following manufacturer's instructions. For Western blot, infected cells were counted and the same number of cells were directly lysated in sample buffer. Proteins were then separated in 10% (w/v) SDS-polyacrylamide gel (SDS-PAGE) and subsequently transferred onto polyvinylidene difluoride membrane (PVDF). ERK1/2, Phospho-specific (p) ERK1/2 (Cell Signalling Technologies, www.cellsignal.com), Cyclin-D1 and Sam-68 (Santa Cruz biotechnology, www.scbt.com) were used as primary antibodies. Secondary antibodies