# Article

# ERK1 and ERK2 are involved in recruitment and maturation of human mesenchymal stem cells induced to adipogenic differentiation

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**Adipocytes' biology and the mechanisms that control adipogenesis have gained importance because of the need to develop therapeutic strategies to control obesity and the related pathologies. Human mesenchymal stem cells (hMSCs), undifferentiated stem cells present in the bone marrow that are physiological precursors of adipocytes, were induced to adipogenic differentiation. The molecular mechanisms on the basis of the adipogenesis were evaluated, focusing on the MAPKinases ERK1 and ERK2, which are involved in many biological and cellular processes. ERK1 and ERK2 phosphorylation was reduced with different timing and intensity for the two isoforms in treated hMSCs in comparison with control cells until day 10 and then at 14–28 days, it reached the level of untreated cultures. The total amount of ERK1 was also decreased up to day 10 and then was induced to the level of untreated cultures, whereas the expression of ERK2 was not changed following adipogenic induction. Treatment with the specific ERK1/2 inhibitor U0126 during the whole differentiation period hampered hMSCs' adipogenic differentiation, as lipid droplets appeared in very few cells and were reduced in number and size. When U0126 was administered only during the initial phase of differentiation, the number of hMSCs recruited to adipogenesis was reduced while, when it was administered later, hMSCs did not acquire a mature adipocytic phenotype. ERK1 and ERK2 are important for hMSC adipogenic differentiation since any alteration to the correct timing of their phosphorylation affects either the recruitment into the differentiation program and the extent of their maturation.**

**Keywords:** mesenchymal stem cells, ERK, adipogenesis, MAPK, obesity, differentiation

## Introduction

Adipose tissue has long been considered merely as the depot for storing surplus energy, with useful properties, but without a key role for the organism besides being a mechanical support for more important structures. In mammals, two types of adipose tissue, with different morphological features and functions, have been described: brown adipose tissue, mainly involved in thermogenesis, and white adipose tissue (WAT). The main role of WAT is triacylglycerol storage and release, but it is considered to be also an endocrine organ because white adipocytes secrete adipokines (hormones and cytokines) influencing many biological processes such as adipogenesis, energy homeostasis, angiogenesis, and immune response (Vázquez-Vela et al., 2008). Nowadays, because of the increasing problem of obesity and related pathologies (like cardiovascular diseases, type 2 diabetes, some types of cancers and many others) (Gesta et al., 2007; Waki and Tontonoz, 2007; Gutierrez et al., 2009), the research landscape has changed and many studies focus on adipocytes' biology and on the mechanisms that control adipogenesis.

Adipogenesis is a biological process in which two stages occur: the first stage is named 'determination', while the second one is referred to as 'terminal differentiation'. The determination phase leads undifferentiated cells to enter the adipogenic differentiation program becoming pre-adipocytes. Subsequently, during the terminal differentiation, the committed cells—after a phase of clonal expansion—differentiate and acquire the phenotypical and molecular characteristics of mature adipocytes (Fevè, 2005).

Many of the findings on adipocytes and adipogenesis come from *in vitro* studies based on murine immortalized preadipose cell lines such as 3T3-L1, 3T3-F442A, and ob-17 cells, or on pre-adipocytes or adipocytes from human or rodent fat explants (Novakofski, 2004). Despite the meaningful results obtained in such studies, data emerging from the different models are often controversial. Moreover, the studies conducted on pre-adipocyte models shed light only on the second phase of differentiation, which leads the pre-adipocytes to acquire the characteristics of mature white adipocytes without accounting for the

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determination phase. The aberrant increase in fat mass that is observed in obesity is due to a dysregulation of both the phases of the differentiation process that results in an increase in the number of adipocytes (hyperplasia) and/or adipocyte size (hypertrophy). It follows that it would be important to understand the molecular basis of the entire adipogenic differentiation pathway, in order to find strategies to control it.

The mesenchymal stem cells (MSCs) are undifferentiated multipotent stem cells that reside in the bone marrow and in many other adult tissues (Minguell et al., 2001). These cells can be easily isolated and they possess a high proliferative and differentiative capacity being able to differentiate (both *in vitro* and *in vivo*) into mesoderm-type cell lineages and into non-mesodermal cells (Pittenger et al., 1999; Liu et al., 2009). In adult organisms, MSCs are the physiological progenitors of the adipocytes (Park et al., 2008) and, therefore, represent a feasible model for studying the adipogenic process from the earliest phases to a more mature phenotype, thus including both the determination and the terminal differentiation.

The commitment of an undifferentiated cell to the adipogenic lineage requires the fine regulation of a complex network of transcription factors, cofactors, and signaling intermediates from numerous pathways (Rosen, 2005; Rosen and MacDougald, 2006). The mitogen-activated protein kinases (MAPKs) are a superfamily of serine/threonine kinases that regulate both cytoplasmic events and gene expression (Chang and Karin, 2001; Miloso et al., 2008) and are involved in many cellular processes such as proliferation, differentiation, and stress response (Zhang and Dong, 2007). A primary role of the MAPKs in the adipogenesis has been proposed (Aubert et al., 1999; Bost et al., 2005a), even if the significance of the involvement of these kinases is not clear. In fact, some authors suggest that the MAPKs—in particular, the extracellular regulated stress kinases 1 and 2 (ERK1/2)—could promote the adipogenic process (Benito et al., 1991; Sale et al., 1995; Aubert et al., 1999), while other groups propose an inhibitory role for ERK1/2 in the adipogenesis (Kim et al., 2002; Janderová et al., 2003). An understanding of the molecular regulation of adipogenesis is of key interest in developing therapeutic strategies to control obesity and the related pathologies. For this reason, in this study we evaluated the role of the MAPKs ERK1 and ERK2 in the adipogenesis in human mesenchymal stem cells (hMSCs) in the different phases of the differentiation process.

## Results

### *Adipogenic differentiation*

hMSCs were treated for adipogenic differentiation as described in 'Materials and methods' section. Differentiation was evaluated using morphological and molecular techniques. hMSCs treated with the adipogenic differentiation protocol (treated hMSCs) showed the presence of a few tiny intracellular lipid droplets starting from day 3 after adipogenic induction. The lipid droplets increased in number and size during the treatment with adipogenic induction medium until day 10, when the medium was switched to adipogenic maintenance medium. From this moment on, the lipid droplets begun to enlarge and, at the end of the treatment, many cells assumed a round shape and presented a single large droplet as it occurs in mature WAT adipocytes, with a size of 30–50 μm in diameter. The lipid nature of the droplets was confirmed by the Oil Red O staining. The hMSCs grown in culture medium alone (control hMSCs) did not show any lipid droplets at all the time points examined and maintained their typical fibroblast-like shape (Figure 1).

We evaluated by real-time RT–PCR the gene expression of specific adipogenic markers: (i) C/EBPβ and PPARγ2, two of the main transcription factors involved in the process of adipogenic differentiation, and (ii) adipsin and leptin, two of the adipokines that are produced by adipocytes. From the first day of induction to the end of the observation period (day 28), the gene expression of C/EBPβ, PPARγ2 and adipsin was higher in a statistically significant manner in treated hMSCs with respect to control hMSCs (Figure 2A–C). For the adipokine leptin, a similar increase was observed starting at day 7 and persisting until day 28 (Figure 2D).

The expression of PPARγ and C/EBPβ was evaluated also at the protein level by immunoblotting experiments. In treated hMSCs, C/EBPβ protein was already expressed at day 1 after induction and an increase in protein expression could be observed at day 3 and more consistently at later times. PPARγ2 protein was expressed starting at day 7 after induction and increased at

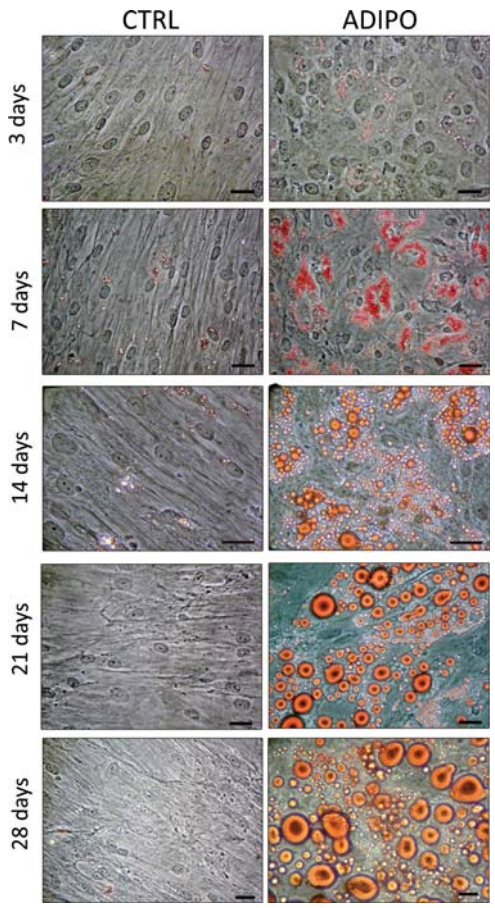


**Figure 1** Adipogenic differentiation of hMSCs. Control untreated hMSCs (CTRL) and adipogenic induced hMSCs (ADIPO) were stained with Oil Red O at 3, 7, 14, 21, and 28 days after induction and micrographs were taken. Bar = 30 μm. Micrographs are representative of different experiments performed with cells from three donors.

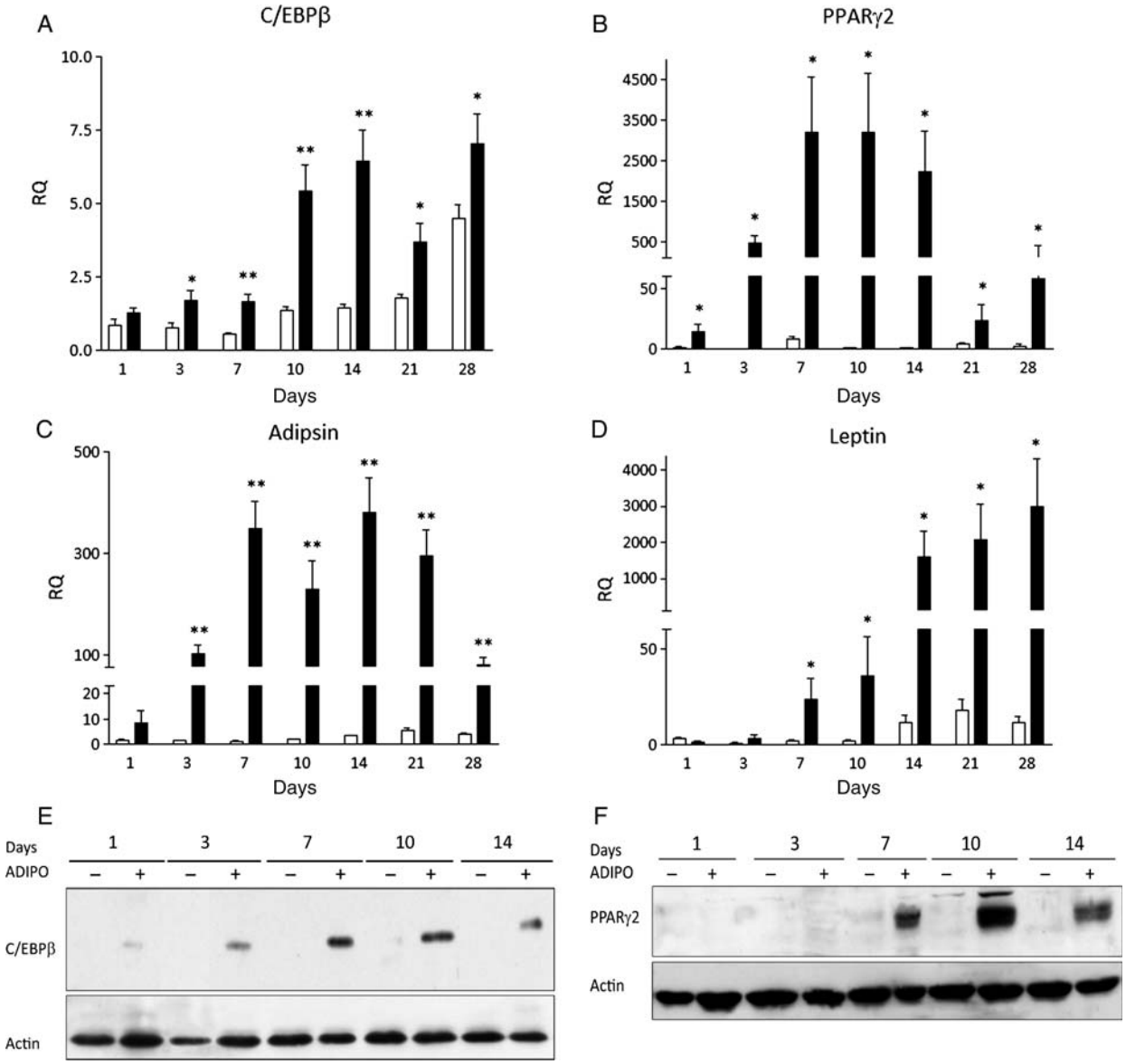


**Figure 2** Adipogenic markers' expression. Relative quantification of (**A**) C/EBPβ, (**B**) PPARγ, (**C**) adipsin, and (**D**) leptin expression was evaluated by real-time PCR in untreated hMSCs (white bars) and in adipogenic-induced hMSCs (black bars). Experiments were performed in triplicate with cells from three different donors and data are expressed as mean ± standard deviation. *$P < 0.05$ and **$P < 0.01$ with respect to CTRL cells. Protein expression of (**E**) C/EBPβ and of (**F**) PPARγ was evaluated in untreated hMSCs and adipogenic-induced hMSCs by immunoblotting experiments. Actin represents loading control.

day 10. In control hMSCs, C/EBPβ and PPARγ2 proteins were hardly detectable for the entire examination time (Figure 2E and F).

*ERK1 and ERK2 modulation*

Using immunoblotting experiments, we evaluated the modulation of ERK1 and ERK2 during the whole differentiation period, analyzing both the activation (i.e. phosphorylation) and the expression (total amount) of the two isoforms by using specific antibodies.

ERK1 and ERK2 were phosphorylated in control hMSCs from the beginning of the experiment to the end. In treated hMSCs, both ERK1 and ERK2 showed a reduction in the phosphorylation status in comparison with the control hMSCs, but the time and intensity of these reductions were different for the two isoforms. ERK1 phosphorylation was markedly reduced starting as early as 1 h after the induction and was hardly detectable until day 10. At day 14, an increase of ERK1 phosphorylation was observed that lasted until day 28. The reduction in ERK2 phosphorylation started later with respect to ERK1 since it became evident starting from 8 to 12 h. From day 10–14, ERK2 phosphorylation was similar to that observed in control hMSCs (Figure 3A and C).

In control hMSCs, ERK1 and ERK2 were expressed with minor variations throughout the entire experimental period. In treated hMSCs, the amount of ERK1 was reduced with respect to control hMSCs starting as early as 1 h after induction. From day 14, ERK1 expression increased and reached the level of control

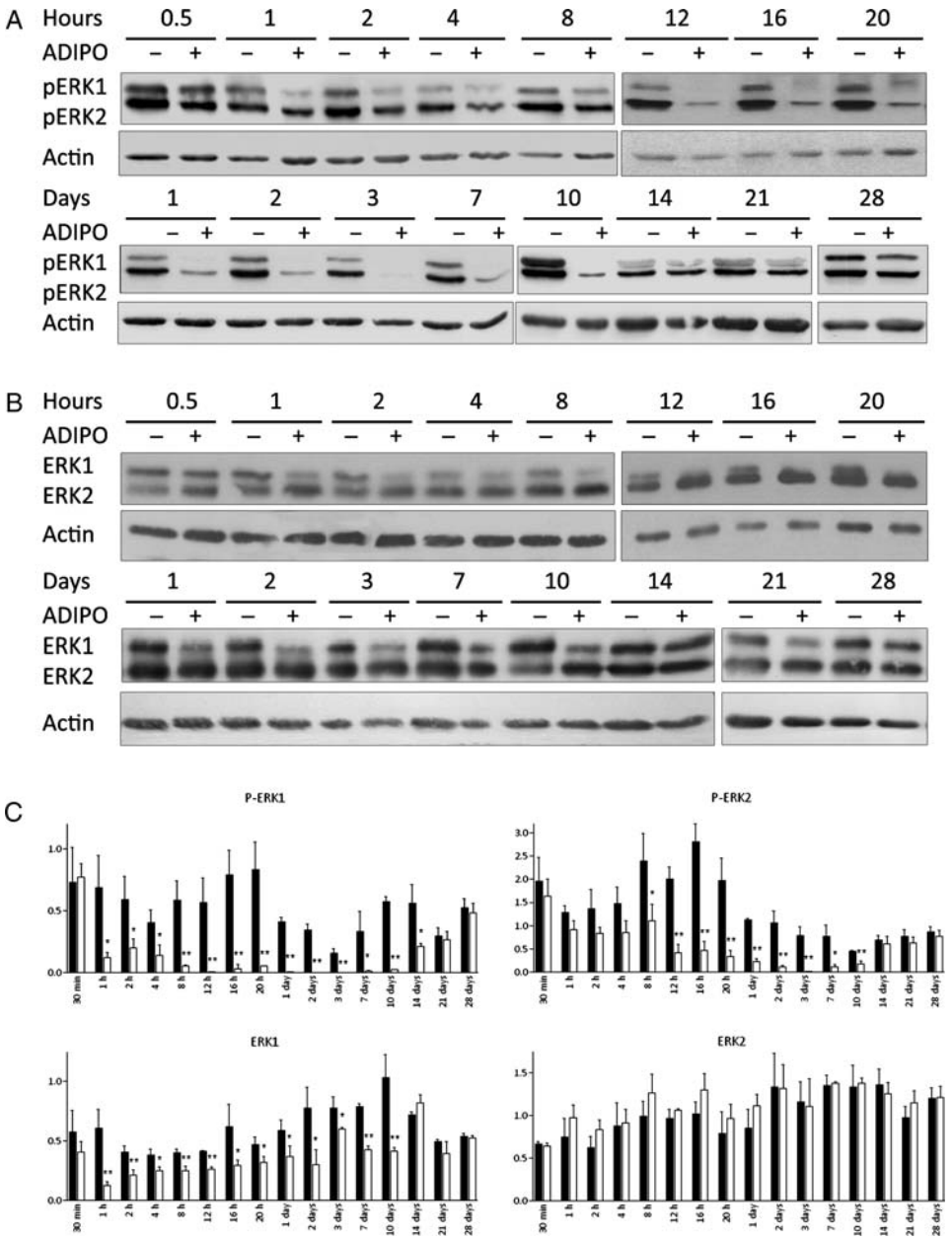


**Figure 3** ERK1 and ERK2 modulation during hMSCs' adipogenic differentiation. ERK1 and ERK2 (**A**) phosphorylation and (**B**) protein expression were evaluated in untreated hMSCs and adipogenic-induced hMSCs by immunoblotting experiments. Antibodies specific for ERK1 and ERK2 total and phosphorylated forms, respectively, were used. Actin represents loading control. The reported images are representative of the experiments repeated with cells from three donors. (**C**) Densitometric analysis of band intensities of phosphorylated ERK1/2 and of total ERK1/2 in untreated hMSCs (black bars) and in hMSCs treated for adipogenic differentiation (white bars) was performed. Values were normalized using actin protein as loading control. Experiments were performed on cells from three donors and data are expressed as mean ± standard deviation in arbitrary units. $*P < 0.05$ and $**P < 0.01$ with respect to CTRL cells.

hMSCs at day 28. The expression of ERK2 in treated hMSCs for all the time points considered was always similar to that of control cells without any change in expression (Figure 3B and C).

*ERK1 and ERK2 inhibition*

In order to examine the role played by ERK1 and ERK2 in hMSCs' adipogenic differentiation, we treated hMSCs with the ERK inhibitor U0126 (50 μM), which prevents phosphorylation of both ERK1 and ERK2. The 50 μM dose was chosen as the most effective one in inhibiting ERK1 and ERK2 phosphorylation without toxicity (see Supplemental Figure S1).

The 50 μM U0126 was added to the adipogenic medium at different times, as summarized in Figure 4: (i) from the beginning of the experiment to the end (ADIPO + U0126 0–28); (ii) from day 0 to day 10 of induction (ADIPO + U0126 0–10); (iii) from day 10 until the end of the experiment (ADIPO + U0126 10–28). Adipogenic differentiation with or without the inhibitor was cytochemically evaluated by Oil Red O staining. Moreover, Oil Red O staining was quantified for a quantitative assessment of adipogenic differentiation.

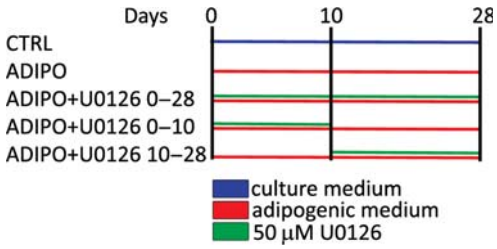


**Figure 4** Schematic representations of U0126 administration paradigms.

Only a few (<6%) of the hMSCs induced for adipogenic differentiation in the presence of U0126 for the whole period (ADIPO + U0126 0–28) showed the appearance of lipid droplets (Figure 5A). These lipid droplets were of small size (10–15 μm) and were 2–5 per cell. None of the hMSCs had the single large lipid droplet characteristic of the fully differentiated cell. When the inhibitor was removed after 10 days of induction (ADIPO + U0126 0–10), the few hMSCs that showed the presence of the lipid droplets were able to reach a more mature phenotype compared with ADIPO + U0126 0–28. At the end of the treatment,

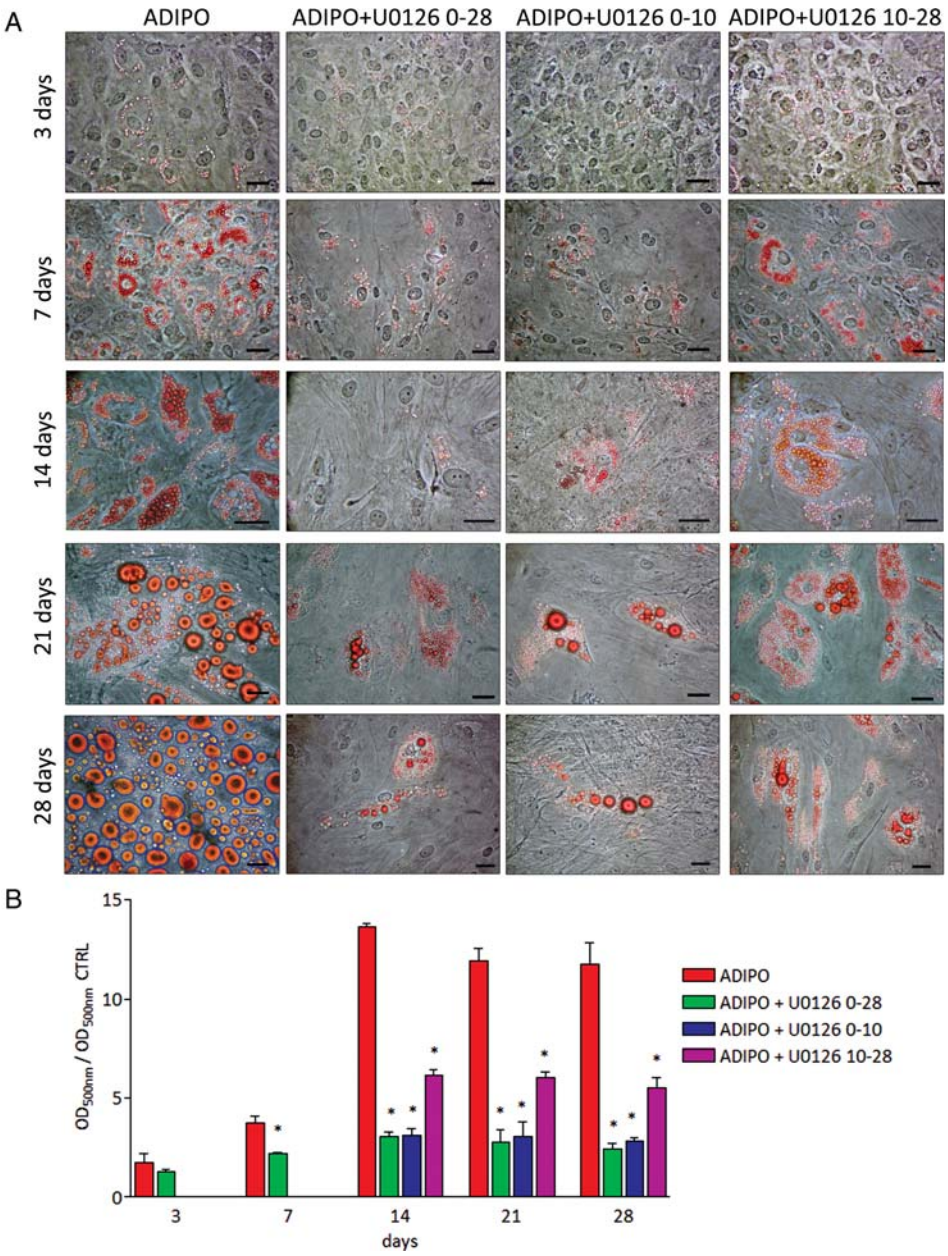


**Figure 5** Adipogenic differentiation of U0126 treated hMSCs. (**A**) Adipogenic-induced hMSCs (ADIPO) in the absence or presence of U0126 for the 0–28 day period (ADIPO + U0126 0–28) or for the 0–10 day period (ADIPO + U0126 0–10) or for the 10–28 day period (ADIPO + U0126 10–28) were stained with Oil Red O at days 3, 7, 14, 21, and 28 after induction and micrograph were taken (Bar = 30 μm). (**B**) Oil Red O was solubilized and optical density read at 500 nm to obtain a quantitative assessment of adipogenesis. The experiment was performed in triplicate with cells from three different donors and data are expressed as mean ± standard deviation. *$P < 0.01$ with respect to ADIPO cells.

cells with round shape and a single lipid droplet were 2–6/microscopic field (mean 4.2, C.I. 3.26–5.14). When the inhibitor was added from day 10 to day 28 (ADIPO + U0126 10–28), the cells, which at day 10 showed numerous tiny lipid droplets, were not able to mature their phenotype and, even at the latest times, the droplets were tiny and did not fuse to form the huge lipid droplets that are typical of adipogenic differentiation. Cells with round shape and a single large lipid droplet were 0–4/microscopic field (mean 1.8, C.I. 0.69–2.91). In CTRL cells, no sign of lipid droplet formation was observed for the whole experimentation time, while ADIPO cells underwent adipogenic differentiation as already described (see previous paragraph). At the end of the induction period, cells with round shape and a single lipid droplet were 100–130/microscopic field (mean 115.2, C.I. 104.3–126.1). After staining, the cultures underwent quantification of Oil Red O to assess lipid content (Figure 5B). ADIPO cells showed increasing levels of Oil Red O staining, which reached their maximum at day 14. In ADIPO + U0126 0–28 cells, Oil Red O deposition was reduced with respect to ADIPO cells. If the inhibitor was removed at day 10 (ADIPO + U0126 0–10), the Oil red O staining deposition was similar to that measured in ADIPO + U0126 0–28 cells. The cells induced for adipogenic differentiation and treated with U0126 from day 10 to day 28 (ADIPO + U0126 10–28) showed an intermediate level of Oil Red O deposition.

## Discussion

The increasing prevalence in the population of obesity and of its related pathologies makes the understanding of the critical mechanisms regulating the adipogenic process a matter of considerable importance. We focused our attention on the role of ERK1 and ERK2 in this process. The MAPKs have already been proposed as being key molecules for the adipogenic differentiation, but the significance of the involvement of such kinases in the differentiation process is far from being understood. Most of the studies on the adipogenesis process have been performed on pre-adipocytes, cells already committed to adipogenesis, which make it possible to study the later stages of differentiation. With respect to pre-adipocytes, MSCs (physiological precursors of adipocytes) represent a more suitable model for studying the early differentiation phase and, therefore, the whole differentiation process (Janderová et al., 2003).

Upon induction, hMSCs acquired morphological features of adipocytes with the formation of lipid droplets and expressed typical markers of adipogenic differentiation. The observed 6-day delay in PPARγ2 protein expression with respect to the increase in its transcript could at least be due in part to the higher sensibility of PCR than immunoblotting, but more likely it suggests the existence of regulator mechanisms that prevent PPARγ2's mRNA translation. MicroRNAs have already been demonstrated to be able to regulate adipogenesis (Wang et al., 2008; Xie et al., 2009), and recently a direct effect of a microRNA has been shown on PPARγ translation in human adipocytes (Karbiener et al., 2009). Despite the delay in the onset of PPARγ2 increase, triglyceride accumulation was evident starting from day 3. A basal low amount of active PPARγ, not detected by means of our immunoblotting procedures, could be present in hMSCs of their differentiative state, as reported by Janderová et al. (2003), and could be responsible for the onset of the differentiation.

In our study, during hMSCs' adipogenic differentiation, ERK1 and ERK2 maintained their phosphorylation status respectively for 1 and 8–12 h and, subsequently, their phosphorylation decreased, suggesting that such a reduction is required for adipogenic differentiation. Similar findings have been obtained also in different cellular models (Prusty et al., 2002; Tang et al., 2003; Chuang et al., 2007; Fuentes et al., 2010).

However, when we treated differentiating hMSCs with ERK1 and ERK2 inhibitor, thus further reduced ERK1 and ERK2 phosphorylation, surprisingly a marked reduction in adipogenic differentiation occurred. Inhibition of the ERK pathway with the ERK inhibitor U0126 in hMSCs hampered adipogenic differentiation, affecting not only the recruitment of hMSCs into the differentiation program, but also their ability to reach a differentiated phenotype, calling into question the real function of ERK1 and ERK2 in the differentiation process.

The results of the three inhibition protocols clarify these unexpected findings. They emphasize the importance of the time course of the phosphorylation. In fact, after the removal of the inhibitor after the first phase of differentiation, no more cells were enrolled in the differentiation program, even if maturation of the few differentiating cells was not affected. On the contrary, when U0126 was administered only during the second phase, i.e. when ERK1 and ERK2 phosphorylation increased, the maturation towards a more differentiated phenotype was hindered.

These data suggest that activation of ERK in the very early phase of the adipogenic process is important for the engagement of hMSCs in the adipogenic program, probably acting on the transcriptional factors that trigger the differentiation process (Prusty et al., 2002; Farmer, 2005; Burns and Vanden Heuvel, 2007) and/or on the clonal mitotic expansion that is required for adipogenesis (Tang et al., 2003). The late ERK activation is also important for adipogenesis, influencing the maturation of differentiating cells, probably acting on lipid droplet maturation (Chung et al., 2005).

However, the reduction of ERK phosphorylation that occurs between early and late phosphorylation peaks is important since during osteogenic differentiation the reduction of ERK phosphorylation switches the process to adipogenesis (Jaiswal et al., 2000; Fu et al., 2008). Moreover, different compounds exert an antiadipogenic effect by increasing ERK phosphorylation (Chen et al., 2007; Fuentes et al., 2010; Wang et al., 2010). Therefore, we can affirm that in human adipogenesis, a fine modulation of ERK activity is essential for the correct outcome of the adipogenic process.

The different time course of the expression and phosphorylation of ERK1 with respect to ERK2 suggests that the two isoforms play different roles during the adipogenic process as it has already been demonstrated in mouse adipogenesis (Bost et al., 2005b). Further studies are necessary to elucidate this point.

In conclusion, in this study, we have demonstrated that ERK1 and ERK2 are important for hMSC adipogenic differentiation since any alteration to the correct timing of their phosphorylation affects adipogenesis. ERK1 and ERK2 play a key role in determining how many cells enter the adipogenic differentiation program and their phenotypical maturation towards adipocytes,

suggesting the possibility of controlling adipogenesis through the use of drugs that act selectively on ERKs.

## Materials and methods

### *Isolation and culture of hMSCs*

hMSCs were obtained from the 'Stefano Verri' Cell Therapy Laboratory, Monza, Italy. hMSCs were isolated from bone marrow harvested from the iliac crest of healthy donors as described previously (Salvadè et al., 2007). In brief, after a bone marrow transplant, collection bags were washed with PBS (Listar Fish, Milan, Italy) to obtain residual bone marrow. Mononuclear cells were isolated through from day 10 until the end density-gradient separation (Ficoll-Hypaque; GE Healthcare, Milan, Italy) and were resuspended in a culture medium composed of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG, Lonza, Verviers, Belgium) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml fungizone (Lonza), and 10% defined fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Cells were plated in culture flasks at a high density and were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. After 24 h, non-adherent cells were removed by medium change; attached cells were cultured until they reached 80%/90% confluence and subsequently were detached with trypsin (Lonza) and subcultured at $4 \times 10^3$ cells/cm$^2$. The medium was changed twice a week.

According to the criteria proposed in the definition of MSCs (Dominici et al., 2006), and as demonstrated elsewhere (Salvadè et al., 2007), the hMSCs used in our experiments were: (i) plastic-adherent and capable of extensive proliferation when maintained in standard culture conditions; (ii) positive for specific surface antigens; (iii) able to differentiate into osteogenic, adipogenic, and chondrogenic lineages under specific *in vitro* differentiating conditions. The genomic stability of hMSCs during the culture period was assessed by monitoring the chromosomal status at several passages *in vitro* and no abnormalities were observed (data not shown).

### *Adipogenic differentiation*

hMSCs at culture passage p5–p7 were seeded at $2 \times 10^4$ cells/cm$^2$ on culture dishes in DMEM-LG. The following day, the medium was switched to adipogenic induction medium (AIM) composed of complete DMEM-high glucose (DMEM-HG, Lonza), i.e. plus 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml fungizone, and 10% FBS, supplemented with 1 μM dexamethasone (Applichem GmbH, Darmstadt, Germany), 10 μg/ml insulin, 100 μM indomethacin and 500 μM isobutylmethylxantine (Sigma-Aldrich, St. Louis, MO, USA). On day 10, AIM was replaced with adipogenic maintenance medium (AMM), which consisted of complete DMEM-HG supplemented with 10 μg/ml insulin without other adipogenic factors. Treatment with AMM was extended until day 28. hMSCs treated with complete DMEM-HG without adipogenic supplements represented control cultures. Media were changed twice a week.

### *Treatment with ERK inhibitor*

ERK inhibitor U0126 (Promega Italia, Milan, Italy) was dissolved in dimethyl-sulfoxide and diluted in adipogenic medium (AIM or AMM) at a final concentration of 25–50 μM. Preliminary studies were performed to evaluate the toxicity and efficacy of U0126 (see Supplemental Figure S1). The cell viability of hMSCs treated with different doses of U0126 was evaluated by MTT assay, as described elsewhere (Donzelli et al., 2004) and the effect of U0126 on ERK1/2 phosphorylation was assessed by immunoblotting.

U0126 at 50 μM was administered according to three different paradigms: (i) during the whole differentiation time (ADIPO + U0126 0–28); (ii) during the first 10 days of differentiation (ADIPO + U0126 0–10); (iii) from day 10 to the end of the experiment (ADIPO + U0126 10–28). hMSCs cultured with DMEM-HG but without either adipogenic factors or U0126, and hMSCs induced for adipogenic differentiation (AIM and AMM) without the inhibitor represented the control cultures. Media were changed twice a week and U0126 was added at any medium change.

### *Oil Red O staining and quantification*

Adipogenic differentiation was evaluated using Oil Red O staining (Sigma), which shows the presence of triglyceride deposits. In brief, at 3, 7, 10, 14, 21, 28 days from the adipogenic induction, hMSC cultures were washed with PBS (Sigma), fixed in paraformaldehyde 4% in phosphate buffer 0.12 M for 10 min and stained with three volumes of Oil Red O (0.3% in isopropanol) and two volumes of $H_2O$ for 15 min at room temperature. Adipogenic-differentiated cells were recognized by their characteristics being round shaped and containing a single large lipid droplet. For countings randomly selected, microscopic fields were photographed at a magnification of 10×. The total number of cells (nuclei/microscopic field) were: (i) ADIPO = mean 140.6, C.I. 130.0–151.2; (ii) ADIPO + U0126 0–28 = mean 116.8, C.I. 101.1–132.5; (iii) ADIPO + U0126 0–10= mean 128.6, C.I. 112.8–144.4; (iv) ADIPO + U0126 10–28 = mean 123.2, C.I. 107.7–138.7. To perform the quantification of triglyceride accumulation, Oil Red O was solubilized with 100% isopropanol and the optical density was measured with a spectrophotometer at 500 nm. Experiments for the quantitative assessment of adipogenic differentiation were performed in triplicate in cells from different donors and results were normalized to the absorbance of untreated control cells. Statistical analysis was performed using the one-way ANOVA test and Tukey's multiple-comparison test as a post-test with the Graph Pad Prism statistical package (Version 3.03 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com).

### *Real-time RT–PCR*

To analyze the gene expression of hMSCs, total RNA was isolated using the GeneElute Mammalian Total RNA Kit (Sigma) according to the manufacturer's instructions. RNA was quantified and 0.6 μg of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy) to obtain 20 μl of cDNA. The analysis of gene expression was carried out in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) in 25-μl reaction volume using Power SYBR Green PCR Master Mix (Applied Biosystems) and specific primers. All primers were designed

**Table 1** Primers used for real-time PCR.

| Gene | Primer sequences | Accession no. |
|---|---|---|
| 18S | F: 5′-TAGCCTTTGCCATCACTGCC-3′ | NM_022551 |
| | R: 5′-CATGAGCATATCTTCGGCCC-3′ | |
| Adipsin | F: 5′-CACCATCGACCACGACCTC-3′ | NM_000230 |
| | R: 5′-AGTGTGGCCTTCTCCGACAG-3′ | |
| C/EBPβ | F: 5′-GGGCCCTGAGTAATCGCTTAA-3′ | NM_005194 |
| | R: 5′-ATCAACAGCAACAAGCCCG-3′ | |
| PPARγ2 | F: 5′-TGGGTGAAACTCTGGGAGATTCT-3′ | NM_015869 |
| | R: 5′-TGAAGGAATCGCTTTCTGGGT-3′ | |
| Leptin | F: 5′-CTGATGCTTTGCTTCAAATCCA-3′ | NM_000230 |
| | R: 5′-GCTTTCAGCCCTTTGCGTT-3′ | |

using primer-Express 2.0 software (Applied Biosystems). 18S ribosomal RNA was used to normalize gene expression and relative quantification was performed with the ΔΔCt method, using as calibrator hMSCs cultured in DMEM-LG. The primers used for the real-time PCR are listed in Table 1.

*Immunoblotting analysis*

After adipogenic induction, hMSCs were washed twice with ice-cold PBS and solubilized in lysis buffer containing freshly added protease and phosphatase inhibitors. The lysates were clarified by centrifugation at 4°C at 13000 rpm for 15 min. Total proteins were measured with a Coomassie Protein Assay Reagent Kit (Thermo Scientific, Rockford, USA). Protein aliquots were solubilized in Laemmli buffer 5x, boiled for 5 min, and run onto 13% SDS–PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters.

For each primary antibody used, immunoblotting analysis was done according to the manufacturer's instructions. Anti-ERK1/2 antibody (1:5000) was purchased from BD Biosciences (San Jose, CA, USA); anti-phospho-ERK1/2 (1:1000) was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-C/EBPβ antibody (1:250) and anti-actin antibody (1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-PPARγ (1:500) was purchased from Abcam (Cambridge, UK). The immunoreactive proteins were visualized using a chemioluminescence system (GeneSpin, Milan, Italy).

Densitometric analysis was performed using the dedicated software Kodak ID 3.6 (Kodak, Rochester, New York, USA). Values were normalized using actin protein as loading control and data were calculated as mean ± standard deviation and reported in arbitrary units. Statistical analysis was performed using the Student's *t*-test with the Graph Pad Prism statistical package, and $P < 0.05$ was considered significant.

## Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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**Conflict of interest**: none declared.

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