Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage

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Mouse embryonic stem (ES) cells are pluripotent cells that differentiate into multiple cell lineages. The commitment of ES cells into the adipocyte lineage is dependent on an early 3-day treatment with all-*trans* retinoic acid (RA). To characterize the molecular mechanisms underlying this process, we examined the contribution of the extracellular-signal-regulated kinase (ERK) pathway. Treatment of ES cell-derived embryoid bodies with RA resulted in a prolonged activation of the ERK pathway, but not the c-Jun N-terminal kinase, p38 mitogen-activated protein kinase or phosphoinositide 3-kinase pathways. To investigate the role of ERK activation, co-treatment of RA with PD98059, a specific inhibitor of the ERK signalling pathway, prevented both adipocyte formation and expression of the adipogenic markers, adipocyte lipid-binding protein and peroxisome-proliferator-activated receptor γ. Furthermore, we show that ERK activation is required only during RA treatment. PD98059 does not interfere with the commitment of ES cells into other lineages, such as neurogenesis, myogenesis and cardiomyogenesis. As opposed to the controversial role of the ERK pathway in terminal differentiation, our results clearly demonstrate that this pathway is specifically required at an early stage of adipogenesis, corresponding to the RA-dependent commitment of ES cells.

Key words: adipocyte differentiation, mitogen-activated protein kinase, myogenesis, neurogenesis.

INTRODUCTION

The molecular mechanisms underlying the commitment of stem cells into a specific lineage remain largely unknown. Mouse embryonic stem (ES) cells are pluripotent cell lines and can be maintained in an undifferentiated state in the presence of leukaemia inhibitory factor (LIF) [1,2]. Removing LIF and adding appropriate differentiation agents results in the commitment of ES cells into a variety of cell types, including adipocytes [3], cardiac cells, skeletal-muscle cells [4] and neurons [5]. ES cells provide a pertinent model for analysing cell commitment to adipocytes and for studying the early stages of differentiation [3,6–9]. Adipocytes arise from mesodermal stem cells, a common precursor for myocytes, chondrocytes and osteocytes. Once committed to the adipocyte lineage, preadipocytes mature into adipocytes during the terminal stages of differentiation. Preadipocyte cell lines, such as 3T3L1 and 3T3-F442A, have been extensively used to investigate the molecular mechanisms during the late stages of adipogenesis, resulting in the isolation of many adipocyte-specific genes. One of these, the adipocyte-specific lipid-binding protein (aP2) gene, is regulated by a major adipogenic transcription factor involved in terminal differentiation, peroxisome-proliferator-activated receptor γ (PPARγ) [8,10–14].

The extracellular-signal-regulated kinases (ERKs) are involved in signalling cascades that regulate a number of major cellular functions, such as cell proliferation and differentiation [15]. The role of the ERKs during the late stages of adipocyte differentiation is controversial. On the one hand, results indicate that ERK phosphorylation of PPARγ inhibits adipogenesis [16,17] and PD98059 [a specific inhibitor of mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) 1, the enzyme responsible for ERK activation] does not affect adipogenesis in 3T3L1 preadipocytes [18]. On the other hand, the same compound inhibited terminal differentiation of Ob1771 cells [7]. Furthermore, prolonged inhibition of ERK expression using antisense oligonucleotides blocked adipogenesis in 3T3L1 [19]. An inhibitory role of ERK on adipocyte differentiation has also been described in adult human mesenchymal stem cells [20]. In this latter study, it has been shown that inducers of osteogenesis stimulate ERK activity from day 7–11 of treatment, and that addition of PD98059 blocks the osteogenic differentiation, resulting in adipogenic differentiation. The late activation of ERK observed in this particular cellular model suggests that this effect takes place during terminal differentiation. Furthermore, this effect is only obtained after treatment with osteogenic inducers and has been suggested to correspond to a transdifferentiation process from osteoblast to adipoblast. Therefore it may not be correlated to normal adipogenesis.

Adipocyte differentiation of ES cells can be achieved after a 3-week period [3], which includes the critical early treatment with retinoic acid (RA), followed by the application of classical adipogenic inducers. Under these conditions, large clusters of mature adipocytes are present in 70–80 % of the embryoid bodies (EBs). RA is involved *in vivo* in the regulation of many developmental processes and *in vitro* can modulate cellular differentiation in various experimental models. RA influences the pattern of differentiation of ES cells in a time- and concentration-dependent manner [21]. Regarding adipocyte differentiation, it is noteworthy that RA treatment induces opposite effects: stimu-

Abbreviations used: aP2, adipocyte-specific lipid-binding protein; ATF2, activating transcription factor 2; ERK, extracellular-signal-regulated kinase; EB, embryoid body; ES, embryonic stem; FBS, fetal-bovine serum; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; LIF, leukaemia inhibitory factor; MAP1B, microtubule-associated protein 1B; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK kinase; PI 3-kinase, phosphoinositide 3-kinase; PPARγ, peroxisome-proliferative-activated receptor γ; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor.

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latory during the early commitment of ES cells into the adipocyte lineage [3], and inhibitory at a later stage on terminal differentiation of 3T3L1 preadipocytes [22]. In the latter case, the inhibition seems to be due to inhibition of the expression of PPARγ and CAAT/enhancer-binding protein ('C/EBP') α genes.

In this present study, we attempted to determine the role of the ERK pathway during the commitment of ES cells into the adipocyte lineage. We show that RA activates the ERK pathway in EBs during the early events of adipocyte differentiation. Inhibition of ERK activation during this period using PD98059 or U0126 resulted in a strong inhibition of adipocyte formation and the expression of the adipocyte-specific genes aP2 and PPARγ. In contrast, neurogenesis and myogenesis were not affected by PD98059. Furthermore, addition of PD98059 after RA treatment did not alter adipogenesis. Taken together, these results show that ERK activation by RA is necessary for ES cell commitment into the adipocyte lineage.

EXPERIMENTAL

Cell culture and treatment

Mouse embryonic stem cells CGR8 [23] were grown on gelatin-coated plates and maintained in Glasgow minimal essential medium/BHK21 supplemented with 27 mM sodium bicarbonate, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 % (v/v) fetal-bovine serum (FBS) (Life Technologies, Rockville, MD, U.S.A.) and 100 μM 2-mercaptoethanol (Sigma, St Louis, MI, U.S.A). Cells were maintained at a pluripotent undifferentiated stage by the addition of 100 units/ml LIF.

For differentiation, cells were maintained in culture media without LIF as described previously [3]. Briefly, this protocol included two key steps; (1) from day 0–7, EBs were formed in suspension and treated daily from day 2–5 with 1 μM all-*trans* RA (Sigma); (2) from day 7–21, cells were seeded on to Petri dishes and treated with 85 nM insulin (Lilly, Paris, France), 2 nM 3,3′,5-tri-iodothyronine (Sigma) and 10 μM thiazolidinedione (compound #57135; Servier, Suresnes, France) to induce adipogenesis. Fresh medium containing the adipogenic inducers was applied every 2 days. Adipocyte colonies were analysed 21 days after the formation of EBs. For kinase inhibition, 40 μM PD98059 (Calbiochem Biosciences, La Jolla, CA, U.S.A.), 25 μM U0126 (Promega, Madison, WI, U.S.A.) or 5 μM SB2035580 (Calbiochem) were added to the culture media, which was renewed every day between day 2 and day 5 of the differentiation protocol. For protein kinase assays, EBs, 2 days after formation, were treated with either 1 μM RA, 50 nM PMA, 25 ng/ml anisomycin, 0.6 M sorbitol (Sigma) or 100 nM potassium bisperoxo(1,10-phenanthroline)oxovanadate [bpv(phen); Calbiochem].

Protein kinase assays

For the kinase assays, the formation of EBs was performed as follows: ES cells were seeded on to 10-cm bacteriological dishes at 2.6×10^6 cells/plate in culture media without LIF. First, 17 h prior to the addition, EBs were washed twice with pre-warmed PBS and transferred into culture media containing 0.5 % (v/v) FBS without LIF. For the inhibition of ERK activity, 40 μM PD98059 was added to the culture media 90 min prior to the addition of RA. After treatment with the different agents, EBs were washed twice with ice-cold PBS and suspended in lysis buffer [25 mM Hepes (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 100 μg/ml PMSF, 100 μg/ml tosylphenylalanylchloromethane ('TPCK'), 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 20 mM β-glycerophosphate and 0.1 mM Na_3VO_4]. The protein concentration of the cell extracts was determined by the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, U.S.A.).

The ERK kinase assay was performed as described by Bost et al. [24]. Whole-cell protein extracts (50 μg) were immunoprecipitated with anti-ERK antibodies (SC-154; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 2 h at 4 °C. Immunoprecipitates were then washed three times and assayed for kinase activity using 5 μg of myelin basic protein (MBP; Sigma) as substrate in the presence of [γ-^{32}P]ATP. The phosphorylated MBP protein was resolved by SDS/PAGE on a 15 % (w/v) gel and quantified using a Molecular Dynamics PhosphoImager (Palo Alto, CA, U.S.A.).

The JNK kinase assay was performed as described by Hibi et al. [25]. Briefly, 50 μg of whole-cell extract was mixed with glutathione S-transferase (GST)–c-Jun(1–79) (a gift from Dr Michael Karin, University of California San Diego, La Jolla, CA, U.S.A.) for 3 h at 4 °C. After extensive washes, the beads were incubated with 20 μl of kinase reaction buffer {20 mM Hepes (pH 7.7), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 20 μM ATP and 5 μCi of [γ-^{32}P]ATP} for 20 min at 30 °C. The phosphorylated GST–c-Jun protein was eluted by boiling the sample for 5 min and resolved by SDS/PAGE on a 10 % (w/v) gel.

The p38MAPK assay was performed as follows: whole-cell extract (250 μg) was incubated with an anti-p38MAPK antibody (SC-535; Santa Cruz Biotechnology) for 2 h at 4 °C. Immunoprecipitates were then washed three times and assayed for kinase activity using a GST–activating transcription factor 2 (ATF2) fusion protein (a gift from Dr Benoît Dérijard, CNRS, Nice, France) as the substrate in the presence of [γ-^{32}P]ATP [26].

The phosphoinositide 3-kinase (PI 3-kinase) assay was performed as described by Ricort et al. [27]. EBs were lysed for 30 min at 4 °C in 150 μl of buffer A [20 mM Tris/HCl (pH 7.4), 137 mM NaCl, 100 mM NaF, 10 mM EDTA, 2 mM Na_3VO_4, 10 mM pyrophosphate, 1 mM PMSF and 100 units/ml aprotinin) containing 1 % (v/v) Nonidet P40. Lysates were centrifuged at 13000 ***g*** for 5 min at 4 °C. Supernatants were incubated for 2 h at 4 °C with phosphotyrosine antibodies (Upstate Biotechnologies, Lake Placid, NY, U.S.A.) and Protein A–Sepharose beads. Immune pellets were washed twice with each of the three following buffers: (1) PBS containing 1 % (v/v) Nonidet P40 and 200 μM Na_3VO_4; (2) 100 mM Tris/HCl (pH 7.4), 0.5 M LiCl and 200 μM Na_3VO_4; and (3) 10 mM Tris/HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA and 200 μM Na_3VO_4. Bead-associated PI 3-kinase activity was assayed by determining the phosphorylation of PtdIns in the presence of [γ-^{32}P]ATP as described previously [28]. The reaction products were separated by TLC.

Protein analysis

Proteins (50 μg) were resolved by SDS/PAGE on 10 % (w/v) polyacrylamide gels and ERK proteins were visualized by Western analysis with anti-ERK antibodies (SC-154; Santa Cruz Biotechnology).

Microscopic analysis of adipocytes, neurons and cardiomyocytes

Each EB was examined microscopically for the presence of adipocyte colonies (see Figure 3A), cardiomyocytes ('beating hearts') and neurons (cells with neurites, see [29]). Quantification was given as the percentage of EBs with adipocyte colonies, cardiomyocytes or neurons.

RNA analysis

Total RNA was prepared as described previously [30]. For Northern-blot analysis, 20 μg of total RNA per lane was blotted on to Biotrans nylon membranes (ICN, Costa Mesa, CA, U.S.A.), cross-linked with UV and hybridized with radiolabelled probes for aP2, PPARγ, myogenin and microtubule-associated protein 1B (MAP1B). The hybridization signal was quantified using a Molecular Dynamics radioimager. RNA was normalized to 18S levels determined by ethidium bromide staining.

RESULTS

RA activates the ERK pathway

RA treatment of EBs between day 2 and day 5 is required for the differentiation of ES cells into adipocytes [3,6–9]. Therefore we attempted to investigate the role of MAPK during this critical early period of adipogenesis. In order to determine if RA activated the ERK pathway, EBs (2 days after formation) maintained in 0.5 % FBS were treated with 1 μM RA or a known activator of the ERK pathway, PMA (50 nM). ERK activity was determined 1, 4, 8 and 24 h after RA treatment. RA applied for 30 min or 1 h failed to activate the ERK pathway (Figures 1A and 1B, and results not shown). However, RA induced a significant 3-fold activation of ERK appearing after 4 h and present up to 24 h after the addition of RA (Figures 1 A–C). As a control, PMA, applied 30 min before cell lysis, also resulted in a 2.5-fold activation of the ERK pathway. Western-blot analysis was performed and no change in ERK protein level was observed (Figure 1A), showing that ERK activation was not due to an increase in ERK expression. A time-course analysis was performed in order to determine ERK activation throughout the RA-treatment period. RA was added on day 2 and every day until day 5. The kinase assay revealed that ERK remained activated until day 3 (i.e. 24 h after the addition of RA). ERK was no longer activated by RA on days 4 and 5 (48 and 72 h after the first addition of RA respectively) and after RA removal on day 6 (Figure 1C). ERK activation was confirmed by two other approaches, using in gel-kinase assay and phospho-ERK antibodies (results not shown).

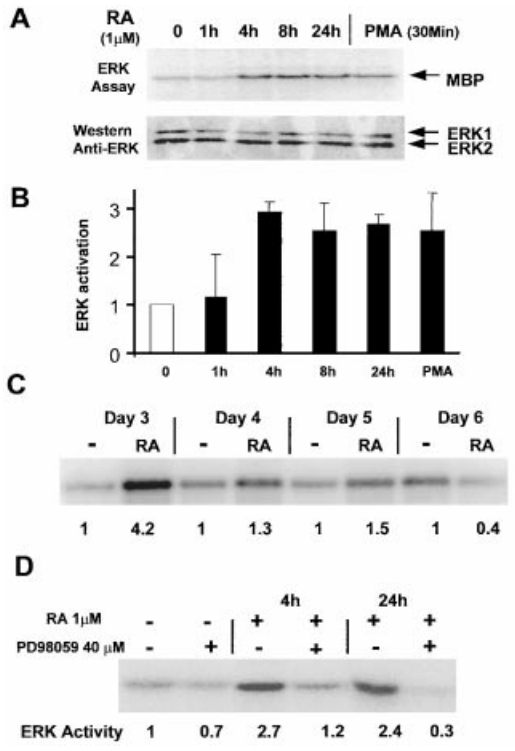

Figure 1 RA induces a prolonged activation of ERK in EBs

(**A**) EBs, two days after formation, were maintained in 0.5% FBS for 17 h prior to treatment. Cells were treated for the indicated times with 1 μM RA or with 50 nM PMA for 30 min. Cells were lysed and the whole-cell extract was used for the ERK kinase assay via the phosphorylation of MBP, as described in the Experimental section. The same cell extracts were used to perform a Western-blot analysis using anti-ERK antibodies. (**B**) Quantification of ERK activation from three independent experiments. Results are expressed as fold of basal activity. (**C**) Time course of ERK activation during RA treatment. RA (1 μM) was added to EBs every day (except on day 6) and ERK activity was determined at day 3 (24 h of RA treatment), day 4 (48 h), day 5 (72 h) and day 6 (96 h). (**D**) EBs were incubated for 90 min in the absence (−) or presence (+) of 40 μM PD98059 and then stimulated for 4 or 24 h with 1 μM RA. The cell extracts were assayed for ERK activation using MBP as a substrate. The activation is given in comparison with the ERK basal activity. Similar results were obtained in two separate experiments.

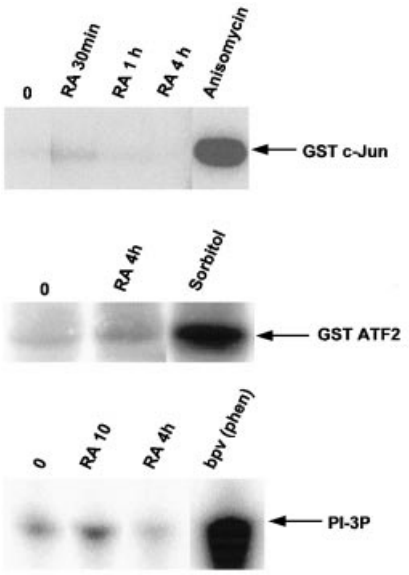

Figure 2 JNK, p38MAPK and PI 3-kinase pathways are not activated by RA

EBs, two days after formation, were treated with 1 μM RA, anisomycin (25 ng/ml for 20 min), sorbitol (0.6 M for 20 min) or bpv(phen) (100 nM for 10 min). JNK and p38MAPK activities were measured using GST–c-Jun and GST–ATF2 respectively as substrates. The PI 3-kinase assay was performed as described in the Experimental section and the phosphorylated PtdIns (PI-3P, PtdIns3*P*) is shown.

In order to determine if the RA-induced ERK activity was inhibited by the classical ERK pathway inhibitor PD98059 [31,32], we treated EBs (2 days after formation) with 40 μM PD98059 for 90 min prior to the addition of RA. As shown in Figure 1(D), this treatment completely inhibited ERK activity induced by RA between 4 and 24 h.

We then investigated the effect of RA on the JNK, p38MAPK and PI 3-kinase pathways by using specific radioactive kinase assays for each of these pathways. Figure 2 shows that RA had no effect on the activity of JNK, p38MAPK or PI 3-kinase. However, anisomycin, sorbitol and bpv(phen), activators of JNK, p38MAPK and PI 3-kinase pathways respectively, strongly induced the activation of these pathways (Figure 2). Therefore these pathways are unlikely to be involved during this specific stage of adipogenesis.

Our results clearly indicate that RA is a potent and specific

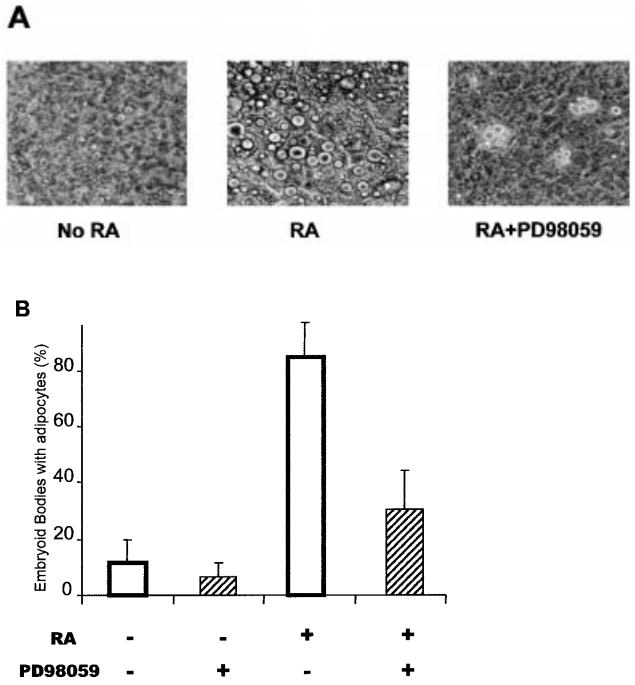

Figure 3 PD98059 prevents the formation of adipocytes

EBs were treated with or without 1 μM RA between day 2 and day 5 in presence or absence of 40 μM PD98059. (**A**) Photomicrographs of EB-derived adipocytes at 21 days after the indicated treatments. Magnification $\times 200$. (**B**) Percentage of EBs with adipocyte colonies at day 21. Results are means $\pm$ S.E.M. of three independent experiments.

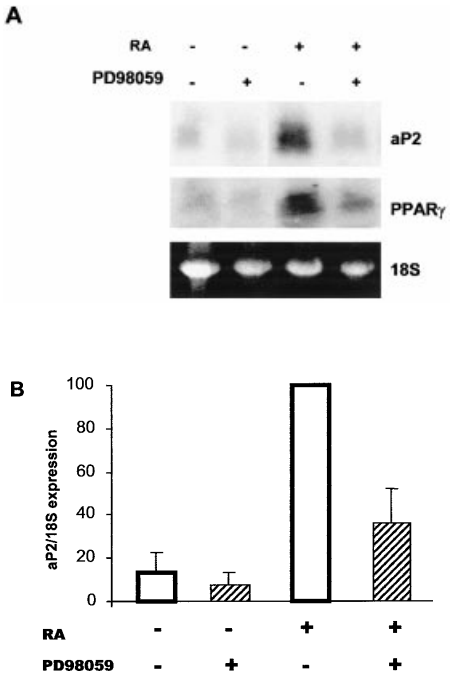

Figure 4 PD98059 strongly suppresses the expression of adipogenic markers

Adipogenesis was induced as described in Figure 3. (**A**) Expression of aP2 and PPARγ genes analysed by Northern blot. Equivalent amounts of RNA from EBs at day 21 were loaded in each lane, as indicated by ethidium bromide staining of 18 S on the agarose gel. (**B**) Quantification of the aP2 signal is represented as the mean of three independent experiments and normalized to the 18 S signal. Results are expressed as the percentage of maximal aP2 expression obtained with RA alone.

Table 1 Effects of U0126 and SB203580 on adipogenesis

EBs were treated with or without 1 μM RA between day 2 and day 5 in presence (+) or absence (−) of 25 μM U0126 or 5 μM SB203580. Results are expressed as the percentage of EBs with adipocyte colonies at day 21. The number of EBs with adipocyte colonies compared with the total number of EBs is also shown.

	EB differentiation into adipocytes (% of EBs with adipocyte colonies)		[number of EBs with adipocyte colonies/total number of EB]	
	U0126		SB203580	
Treatment	−	+	−	+
Control	4.6 [5/108]	14.0 [14/100]	4.1 [2/48]	3.1 [1/32]
RA	73.4 [116/158]	38.1 [69/181]	79.0 [38/48]	62.0 [28/45]

inducer of the ERK pathway, but not the JNK, p38MAPK and PI 3-kinase pathways in EBs, and that this activation can be completely abolished by PD98059.

ERK activation is required for adipogenesis

To determine whether there is a causal relationship between RA activation of ERK and adipocyte formation in the ES cell model, we treated EBs with PD98059 and RA between day 2 and day 5, and adipocyte colony formation was observed at day 21 (Figure 3A). Quantitative analysis showed that 10 % of the non-treated EBs possessed adipocytes, which corresponded to spontaneous differentiation (Figure 3B). Blocking the ERK pathway with PD98059 between day 2 and day 5 resulted in a slight, but not significant, decrease in adipogenesis (Figure 3B). As described previously [3], treatment with RA promotes a high rate of adipogenesis resulting in the formation of adipocyte colonies in 80 % of EBs (Figure 3B). However, the co-treatment with PD98059 between day 2 and day 5 significantly prevented adipogenesis, with only 30 % of the EBs forming adipocytes. To confirm these results we used another specific inhibitor of the ERK pathway, U0126, which acts through a different mechanism to inhibit MEK1 than PD98059 [33]. Addition of U0126 during RA treatment induced a similar inhibition of adipogenesis as seen with PD98059 (Table 1). Conversely, addition of 5 μM SB203580, a p38MAPK inhibitor, concomitantly with RA treatment did not significantly affect adipogenesis (Table 1).

Expression of the specific adipocyte genes aP2 and PPARγ was investigated in parallel by Northern-blot analysis on EBs at day 21. mRNAs for both species were barely detectable in EBs treated in the presence or absence of PD98059 (Figure 4A). RA treatment induced a high level of expression of aP2 and PPARγ (Figure 4A), but addition of PD98059 during this treatment led to a 70 % reduction in aP2 expression (Figures 4A and 4B). Similarly, a large decrease in PPARγ expression was also observed (Figure 4A). These results clearly indicate that the inhibition of RA-induced ERK activity affects the expression of aP2 and PPARγ.

ERK pathway activation is required only during RA treatment

In order to determine whether ERK activation is required in other stages of differentiation of ES cells into adipocytes, we

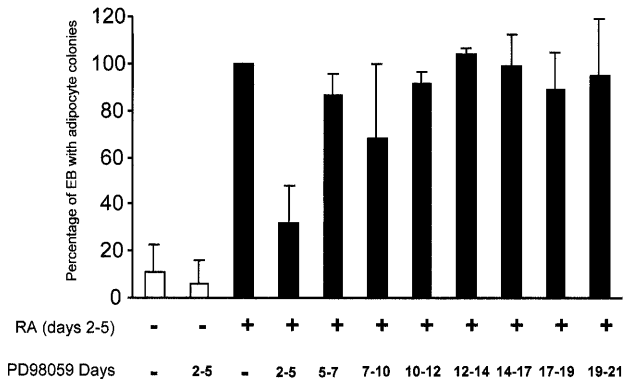

Figure 5 Addition of PD98059 after RA treatment does not affect adipogenesis

EBs were treated with or without 1 μM RA between day 2 and day 5. PD98059 (40 μM) was added to RA-treated EBs during the indicated periods and the percentage of EBs with adipocyte colonies at day 21 is shown. Results are expressed as the percentage of EB with adipocyte colonies (100% corresponds to the maximal differentiation obtained with RA alone). Results are means $\pm$ S.E.M. of three independent experiments.

added PD98059 at different time periods. EBs grown in presence of RA between day 2 and day 5 were treated with PD98059 for different periods from day 2–21 (Figure 5). Adipocyte colonies were analysed on day 21. As described above, PD98059 affected adipocyte differentiation during RA treatment between day 2 and day 5. Interestingly, addition of PD98059 during a window of 2 or 3 days at other periods did not significantly affect adipogenesis (Figure 5). A potential toxic effect of PD98059 on EB between day 2 and day 5 is thus unlikely, since addition of the inhibitor later on had no effect on differentiation, regardless of the number (Figure 5) or size (results not shown) of EBs with adipocyte colonies considered. More importantly, our results show that ERK activation is strictly required during the early stages of ES cell differentiation into adipocytes and not during the terminal stages.

Early activation of ERK is not required for neurogenesis, myogenesis or cardiomyocyte formation

We investigated therefore if PD98059 has a general inhibitory effect on ES cell commitment and differentiation and if the ERK pathway may be a regulator of other lineages during the early RA-treatment period. In order to address these questions, we analysed the cellular commitment of ES cells to neuroblasts, myoblasts and cardiomyocytes in the absence or presence of PD98059 and RA between day 2 and day 5. Neurogenesis was examined by measuring the expression of a specific and early neuroblast marker, MAP1B [34]. In the presence of RA, the addition of PD98059 not only did not inhibit the expression of the neuroblast marker, but an increased amount of MAP1B mRNA was detected in the presence of the inhibitor (Figure 6A). In the absence of RA, a similar expression of MAP1B was observed and this expression was not affected by PD98059 (Figure 6A). Complete neuronal differentiation of ES cells is achieved by an early treatment with RA [5], and results in the formation of visible axons. In the absence of RA, neurogenesis was partial, since no axons were visible despite a detectable expression of MAP1B (Figures 6A and 6B). After RA treatment, neurons with axons were present in 80% of the EBs (Figure 6B). Simultaneous treatment with RA and PD98059 resulted in the formation of axons in 85% of EBs (Figure 6B), showing clearly

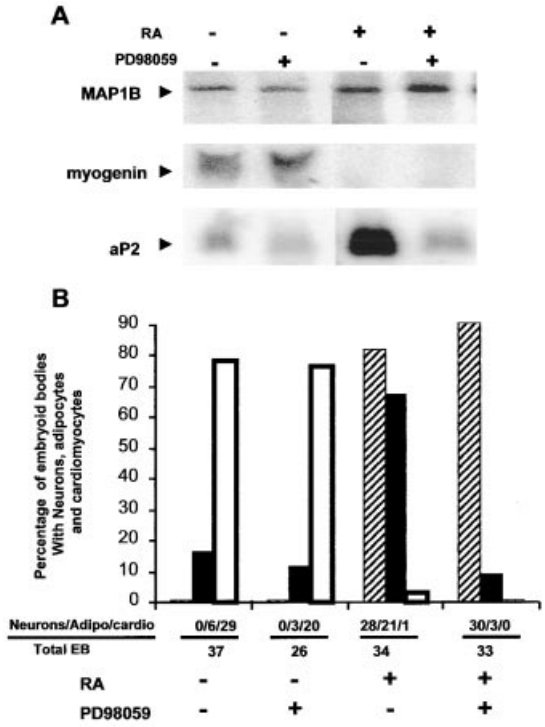

Figure 6 PD98059 does not affect neurogenesis, myogenesis or the formation of cardiomyocytes

(**A**) MAP1B, myogenin and aP2 expression was examined by Northern-blot analysis in EBs, 21 days after formation, previously treated with the indicated agents between day 2 and day 5, as described in Figures 3 and 4. The membrane was hybridized with the three radiolabelled probes. (**B**) The percentage of EBs with neurons (hatched bars), cardiomyocytes (cardio, white bars) and adipocytes (adipo, black bars) was determined at day 21 in the same experiment. The absolute numbers of EB containing neurons, adipocytes colonies and cardiomyocytes and total EBs are indicated. This experiment is representative of two independent experiments.

that inhibition of RA-induced ERK activation did not alter neuronal differentiation, whereas it inhibited adipogenesis within the same EBs.

Adipoblasts and myoblasts derive from a common mesenchymal precursor. We analysed the cellular commitment of ES cells to myoblasts by measuring the expression of myogenin, a myoblast marker. Myogenin was not expressed when EBs were treated with RA (Figure 6A), since RA applied between day 2 and day 5 inhibits skeletal and cardiomyogenesis [3]. However, as expected, it was expressed when EBs were not treated with RA. The addition of 40 μM PD98059 between day 2 and day 5 did not affect myogenin expression, showing that it does not interfere with myogenesis. Finally, ES cells also differentiate into cardiomyocytes and 'beating hearts' were observed 10 days after the formation of EBs. We analysed the effect of PD98059 on cardiomyocyte formation. Addition of RA inhibited cardiomyocyte formation as previously described [3]. In the absence of RA, 78% of the EBs formed cardiomyocytes (Figure 6B), and interestingly, the addition of PD98059 did not affect cardiomyogenesis, since 77% of EBs still possessed cardiomyocytes.

DISCUSSION

In the present study, we have analysed the early molecular mechanisms involved in the commitment of pluripotent ES cells into the adipocyte lineage. We found that the ERK pathway is

activated by RA treatment between day 2 and day 5, a period critical for RA-induced adipocyte differentiation, and that this activation is specifically required for adipogenesis.

Despite several studies showing that RA activates MAPK pathways in different cellular models [35–38], the precise mechanism by which RA regulates these pathways is unknown. The RA signal is transduced through two families of RA receptors (RAR), which act as transcription factors. RARα, β and γ and their isoforms are activated by both all-*trans* RA and 9-*cis* RA, whereas the retinoid X receptor (RXR) family (RXRα, β and γ) is activated only by 9-*cis* RA [39]. To explain how RA regulates MAPKs, a direct interaction between RA and its receptors with the signalling elements of the pathways could be suggested. However, such a mechanism would imply a rapid activation of the pathway. Indeed, most extracellular signals typically activate signal transduction pathways within 30 min, as shown for PMA in Figure 1(A). Since the activation of ERK occurs 4 h after the addition of RA, this hypothesis is very unlikely. Instead, this delay may correspond to the regulation of expression of an intermediate protein involved in the ERK pathway. Indeed, RA, acting through RAR and RXR, controls the expression of numerous genes [40–42]. For example, Lee et al. [43] have shown that RA induces the expression of MAPK phosphatase 1 ('MKP-1'), which is involved in regulating MAPKs.

The cellular response to the activation of signal transduction pathways is determined by the duration of this activation. There is considerable evidence showing that differentiation of cells requires sustained activation of ERK, whereas transient activation of ERK1 leads to proliferation (for review see [35]). In the present study, we observed a sustained activation of ERK by RA over a period of 20 h, which is in agreement with this conclusion. We also noticed that RA does not activate the ERK pathway on day 4 or day 5. Considering that RA is required between day 2 and day 5 [3] and that RA-induced ERK activity is necessary for adipocyte differentiation, we suggest that RA exerts other effects in addition to ERK activation.

Using two different inhibitors of the ERK pathway, we found that the early RA-induced ERK activation is required for the commitment of ES cells into mature adipocytes (Figures 3, 4 and Table 1). This result is related to the established role of the different MAPKs in RA-induced differentiation in other cell lines. For example, Yen and co-workers [36,37] described that RA specifically activates ERK, but not JNK or p38MAPK, during HL60 differentiation. In P19 murine EC cells, RA causes sustained activation of JNK, but not ERK or p38MAPK, leading to endodermal differentiation [38], and this effect is mediated by the activation of MEK kinase 4 (MEKK4), an upstream regulator of JNK [44]. Therefore the potent regulation of cellular differentiation by RA is a complex process that can be mediated in various cellular models by regulation of different MAPKs, with activation of one particular pathway being associated with the induction of a specific cellular differentiation process.

In the present study, we show that activation of the ERK pathway is required specifically during the early stages of ES cell differentiation into adipocytes. In fact, we demonstrate that inhibition of ERK activity after RA treatment does not affect terminal adipocyte differentiation (Figure 5). Conflicting results concerning the role of the ERK pathway have been described (see the Introduction section). It is noteworthy that these studies concern terminal stages of adipogenesis and were performed in cell lines predetermined to undergo the adipocyte differentiation programme. Conversely, RA treatment between day 2 and day 5 corresponds with an earlier event when mesodermal specification takes place [45,46].

It is known that, during the early stages of ES cell commitment, RA displays a dual role, inhibiting myogenesis and cardiomyogenesis and promoting the commitment of ES cells into adipocyte and neuronal lineages. We found that the blockade of ERK activation inhibits RA-induced adipocyte differentiation, but does not interfere with the other lineages (Figure 6). Therefore, although RA promotes neurogenesis, the underlying molecular mechanism does not involve the ERK pathway. Moreover, since the ERK blockade does not reverse the inhibitory effect of RA on cardiomyocytes or skeletal muscle formation, these RA-inhibited differentiations are not mediated by activation of the ERK pathway. Taken together, these results indicate that RA exerts a pleiotropic effect on ES cell commitment involving distinct molecular mechanisms. Further investigations are now required to understand the molecular mechanism involved in RA activation of ERK, and to identify genes regulated by the ERK pathway during this period, and specifically involved in adipogenesis.

In conclusion, our results strongly suggest an essential and specific role for the ERK pathway in the early proliferative stage of adipogenesis. To our knowledge, this study provides the first evidence that adipogenesis can be regulated from ES cells by interfering with a signal transduction pathway, such as the ERK pathway. Inhibition of an early proliferative stage of adipogenesis *in vivo* could potentially limit the development of adipose tissue in adult animals. Whether this development can be modulated in animals by interfering with the ERK pathway at the corresponding specific stage without affecting myogenesis and neurogenesis needs to be determined. Furthermore, the absence of any effect on other mesenchymal lineages, such as osteogenesis and chondrogenesis, also has to be verified both *in vitro* and *in vivo*, before any potential therapeutic application in pathological states, such as obesity, can be envisioned.

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