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Phosphoinositide 3-Kinase Inhibition Enables Retinoic Acid-Induced Neurogenesis in Monolayer Culture of Embryonic Stem Cells

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ABSTRACT

Retinoic acid (RA) is able to induce the differentiation of embryonic stem cells into neuronal lineages. The mechanism of this effect is unknown but it has been evidenced to be dependent on the formation of floating spheroids called embryoid bodies. Results presented here show that the inhibition of phosphoinositide 3-kinase signaling pre-determines mouse embryonic stem cells to RA induced neurogenesis in monolayer culture with no need of embryoid bodies formation. J. Cell. Biochem. 113: 563–570, 2012.

KEY WORDS: EMBRYONIC STEM CELL; RETIONIC ACID; NEUROGENESIS; DIFFERENTIATION

Embryonic stem (ES) cells are derived from inner cell mass in the blastocyst stage of mammalian development, and they are able to generate all cell types under relevant conditions [Keller, 2005]. Among others, ES cells can be differentiated into neuronal lineages and this can be induced by two approaches. Firstly, the default pathway is induced by growth factor deprivation [Pachernik et al., 2002]. Secondly, ES cell neurogenesis is induced by retinoic acid (RA) [Bain et al., 1995; Fraichard et al., 1995; Tonge and Andrews, 2010]. In contrast to default neurogenesis, the RA effect is fast and robust, but cells are preferentially driven towards motoneurons [Maden, 2007]. Further, RA has a pleiotropic effect on cells and thus its effect is dose and cell type dependent [Rohwedel et al., 1999; Pachernik et al., 2002, 2005]. Moreover, although RA induced neuronal differentiation of pluripotent cells has been intensively studied, its precise mechanism is still largely unknown.

The most efficient RA-induced neurogenesis of ES cells was described by Bain et al. [1995]. Bain's protocol relies on the formation of embryoid bodies (EBs, floating spheroids formed from ES cells that simulate the early embryogenesis) for 4 days, followed by RA treatment. It seems that pre-differentiation of ES cells by EBs formation is indispensable for highly efficient RA-induced neurogenesis [Bain et al., 1995]. This contrasts with RA induced neurogenesis of embryonal carcinoma (EC) P19 cells. P19 are pluripotent cells frequently employed in studies of the early differentiation processes, similarly to ES cells [McBurney, 1993]. P19 cells are also prone to RA-induced neurogenesis with high efficiency. However, in the case of P19 cells, the cells were exposed to RA during the whole period of EBs formation [Bain et al., 1994]. Later, we showed that very effective and highly pure neurogenesis may be induced in P19 cells by RA also in a better defined

Hana Kotasová and Iva Veselá contributed equally to this work.

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monolayer culture [Pachernik et al., 2005], but this was not possible in ES cells [Bain et al., 1995; Pachernik et al., 2002].

Cell–cell contacts, para- and autocrine factors, and specific signaling pathways play key roles in stem cell fate decision. Self-renewal and maintenance of pluripotency in ES cells are dominantly mediated by STAT3 and Smads signaling pathways activities [Silva and Smith, 2008]. Recently, the necessity of a phosphoinositide 3-kinase (PI3K)-signaling pathway for ES cells self-renewal was also recognized [Storm et al., 2007; Xu et al., 2008]. In principal, the above-mentioned signaling pathways block the default differentiation processes and thus mediate ES cells maintenance. Inhibition of one of these pathways releases the differentiation block and thus enables the driving of the cells to the relevant fate [Silva and Smith, 2008].

In this study regarding the mechanism of RA-induced neurogenesis in ES cells, we observed that the inhibition of PI3K signaling leads to a pre-disposition to the neural differentiation of ES cells triggered by RA. Results presented here clearly show that inhibition of PI3K by LY29400 is sufficient to make ES cells prone to RA induced neurogenesis in monolayer culture without any need of EBs formation.

MATERIALS AND METHODS

Undifferentiated mouse ES cells lines D3 and R1 were adapted to feeder-free culture and routinely cultivated as we described previously [Krejci et al., 2008; Vesela et al., 2010]. The differentiation of ES cells was performed by their seeding in serum-containing (15%) LIF free-media DMEM (Gibco–Invitrogen) at a density of 3,000 cells/cm^2 on gelatinized tissue culture plastic. Cells were treated as presented in Figure 1. DMEM-F12 supplemented with insulin, transferrin, selenium (ITS, Gibco–Invitrogen), and antibiotics was used as serum-free medium. For comparison was used the original Bain's protocol for RA-induced neurogenesis [Bain et al., 1995; Bryja et al., 2005]. Culture medium was changed each 2 days. The specific PI3K inhibitor LY29400 (Sigma–Aldrich) at a concentration 10 μM was employed for inhibition of the PI3K > Akt signaling pathways. Smads signaling pathway was blocked by Dorsomorphin (0.5 μM, inhibitor of bone morphogenic protein type I receptors), ERK signaling pathway by UO126 (5 μM, inhibitor of MEK), and STAT3 signaling pathway by StatticV (2 μM) [Pachernik et al., 2007; Vesela et al., 2010]. RA (Sigma–Aldrich) was used in 0.5 μM concentration.

WESTERN BLOT ANALYSIS

The immunoblot analysis and harvesting of cell samples were performed as we presented previously [Pachernik et al., 2005; Vesela et al., 2010]. The used antibodies were: rabbit polyclonal antibodies against ERK (Santa Cruz Biotechnology), Akt (Cell Signaling Technology), phospho-Akt (Cell Signaling Technology), phospho-STAT3 (Cell Signaling Technology), phospho-ERK (Cell Signaling Technology), GAPDH (Cell Signaling Technology), mouse monoclonal antibody against N-CAM (Sigma–Aldrich), E-cadherin (BD Transduction Laboratories), STAT3 (Santa Cruz Biotechnology), and cytokeratin Endo-A (the hybridoma TROMA-I developed by Dr. P. Brulet and Dr. R. Kemler was obtained from the Developmental Studies Hybridoma Bank and developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, Iowa. Department of Biological Sciences—http://www.uiowa.edu/~dshbwww). After immunodetection, each membrane was stained by amidoblack to confirm the equal protein loading.

qRT-PCR ANALYSIS

Total RNA was extracted by the RNeasy Mini Kit (QIAGEN, Valencia, CA). Complementary DNA was synthesized according to the manufacturer's instructions for M-MLV reverse transcriptase kit (Sigma–Aldrich). qRT-PCR was performed in Light-cycler Roche using the following program: initial activation step at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, annealing temperature (see below) for 10 s, and 72°C for 10 s. Gene expression of each sample was expressed in terms of the threshold cycle normalized to glyceraldehyde-3-phosphate dehydrogenase expression (*Gapdh*) as we described previously [Bryja et al., 2008; Vesela et al., 2010]. Primers sequence and annealing temperature follow:

Oct-4(*Pou5f1*)	5′-CGTTCTCTTTGGAAAGGTGTTC-3′ 5′-GAACCATACTCGAACCACATCC-3′/59°C
Nanog	5′-AAGCAGAAGATGCGGACTGT-3′ 5′-GTGCTGAGCCCTTCTGAATC-3′/59°C
Fgf-5	5′-CTGTACTGCAGAGTGGGCATCGG-3′ 5′-GACTTCTGCGAGGCTGCGACAGG-3′/57°C
Pax-6	5′-TGCCCTTCCATCTTTGCTTG-3′ 5′-TCTGCCCGTTCAACATCCTTAG-3′/57°C
Gapdh	5′-AAGGGCTCATGACCACAGTC-3′ 5′-CATACTTGGCAGGTTTCTCCA-3′/61°C
Nestin	5′-CATACAGGACTCTGCTGGAGG-3′ 5′-GAGAAGGATGTTGGGCTGAG-3′/61°C
SOX-1	5′-CCAGCCTCCAGAGCCCGACT-3′ 5′-GGCATCGCCTCGCTGGGTTT-3′/62°C
RARβ	5′-AGGGGTGCAAGGGCTTTTTC-3′ 5′-AGTGAGGTCGTCTAGCTCCG-3′/58°C
Cyp26a1	5′-GTGAAGGCGCGGAACCTTAT-3′ 5′-TCTTGCAAAGTAAGCCCTTG-3′/60°C

LUCIFERASE REPORTER ASSAY

Transient transfections of ES cells were performed by polyethylenimine (PEI, Sigma–Aldrich; stock solution 1.5 mg/ml, pH 7.2). Exponentially growing cells were re-suspended in serum-containing culture media and supplemented with the mix of PEI and luciferase reporter pRAREβ2-TK-luc plasmid (provided by Christopher Glass, University of California, San Diego, La Jolla, CA, transfection mix = 4 μl of PEI + 2 μg DNA in 1 ml of media). Cells suspension and transfection mix were seeded to 12-wells plate for overnight. Next day, medium was changed to serum-containing medium and cells were appropriately treated. Twelve hours after RA treatment, the cells were washed with phosphate-buffered saline and lysed by 200 μl of lysis buffer for luciferase/ATP assay. Luciferase activity was measured according to manufacturer's instructions (Luciferase Assay System, Promega). ATP activity assay is described in Konopka et al. [2010].

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. Statistical analysis was assessed by one-way ANOVA. The values of $P < 0.05$ were considered to be statistically significant.

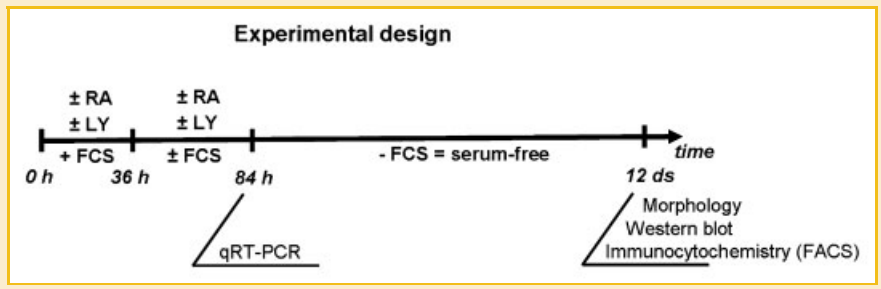

Fig. 1. Scheme of the experimental design of our study. All possible tested combinations of LY (LY24900), RA, and serum (FCS) treatments are depicted. Harvesting and appropriate method of analysis was performed within the indicated time period. In all the presented figures treatments are designated according to the following key: pre-treatment/treatment. For example, LY/RA means—pre-treatment with LY, followed by treatment with RA; "—" means that cells were (pre)treated with neither LY nor RA.

RESULTS

EXPERIMENTAL DESIGN

Cells seeded in serum-containing LIF-free media for 36 h (24, 36, and 48 h were tested in preliminary experiments; 36 h seemed to give the best result—this comparison is not shown) were treated with LY29400 (LY), RA and their combinations. Subsequently, the serum-containing LIF-free media were replaced by serum-free LIF-free media with RA, LY, and their combination for the next 48 h (84 h overall). Parallel experiments were performed also in serum-containing media. Cells underwent an additional differentiation always in serum-free media. The experimental design is shown in Figure 1.

We confirmed the LY effect on PI3-kinase activity through the detection of phosphorylation-active Akt kinase—the down-stream PI3-kinase target. For comparison, the LY effects on STAT3 and ERK phosphorylation were also determined. Figure 2A illustrates that 10 μM LY clearly inhibited Akt phosphorylation, but did not modify ERK and STAT3 phosphorylation. Since 10 μM LY did not significantly affect cell proliferation and viability (not shown), we used this selected concentration of PI3K inhibitor in all the following cell differentiation experiments.

We also analyzed Akt phosphorylation in differentiating ES cells in the form of EBs for 4 days. We observed only a slight increase in phosphorylation of Akt in EBs in comparison with ES cells. The differentiation process in this case was determined as down-regulation of pluripotency marker genes such as Oct-4 and Nanog (Fig. 2B).

INHIBITION OF PI3K ENABLES RA-INDUCED NEUROGENESIS IN MONOLAYER CULTURE

The effect of LY combined with RA treatment and the presence/absence of serum on the neural differentiation of ES cells is shown in Figure 3A. LY treatment of differentiating ES cells in serum-containing LIF-free media in monolayer culture clearly potentiated the ability of these cells to adopt markers of neural differentiation after RA induction. These cells strongly expressed neural specific markers such as N-CAM and β III-tubulin (Fig. 3A), and gained characteristic features of neural morphology. Cells formed three-dimensional colonies with expanded axons and neurites (Fig. 3C,D). We did not observe these features using LY as a single treatment. Such samples contained populations of flat cells with some interstitial colonies (not shown). Although RA alone partially induced detectable N-CAM and β III-tubulin expression in some experiments, we did not observe the typical neural morphology characterized by many protrusions. The presence of serum during RA treatment significantly annulled its pro-neural differentiating effects. In all the experiments RA also induced expression of cytokeratin Endo A, which is potentiated in the presence of serum and reduced in serum-free conditions or in samples treated with LY (excluding LY alone). In contrast to Endo A, RA treatment under serum-free conditions led to decreased expression of E-cadherin (Fig. 3A). The observed phenomena were also analyzed by means of

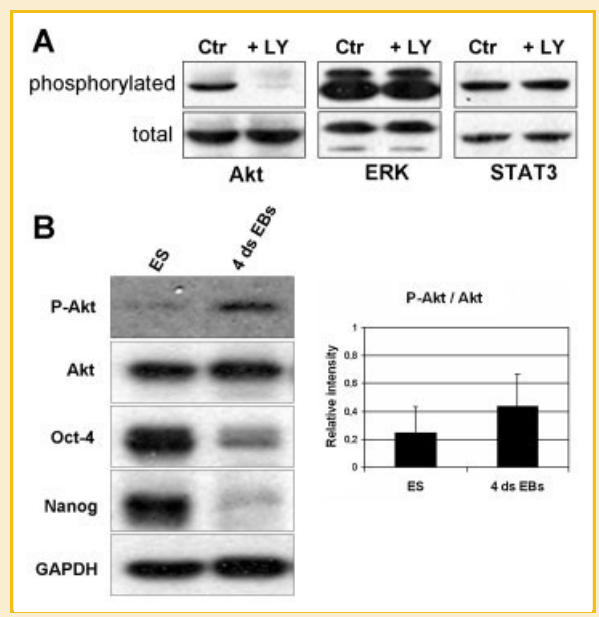

Fig. 2. Effect of the PI3-kinase inhibitor LY24900 on intracellular signaling in ES cells, and Akt phosphorylation in ES and in form of EBs differentiated ES cells. A: ES cells were treated with LY24900 (10 μM) for 6 h. LY24900 inhibited phosphorylation of Akt kinase, but did not modify phosphorylation of ERK and STAT3 proteins. B: Phosphorylation of Akt in ES cells and their differentiated progeny for 4 days in form of EBs. Expression of pluripotency markers (Oct-4, Nanog) determining the differentiating status of cells is also shown. GAPDH is employed as a loading control.

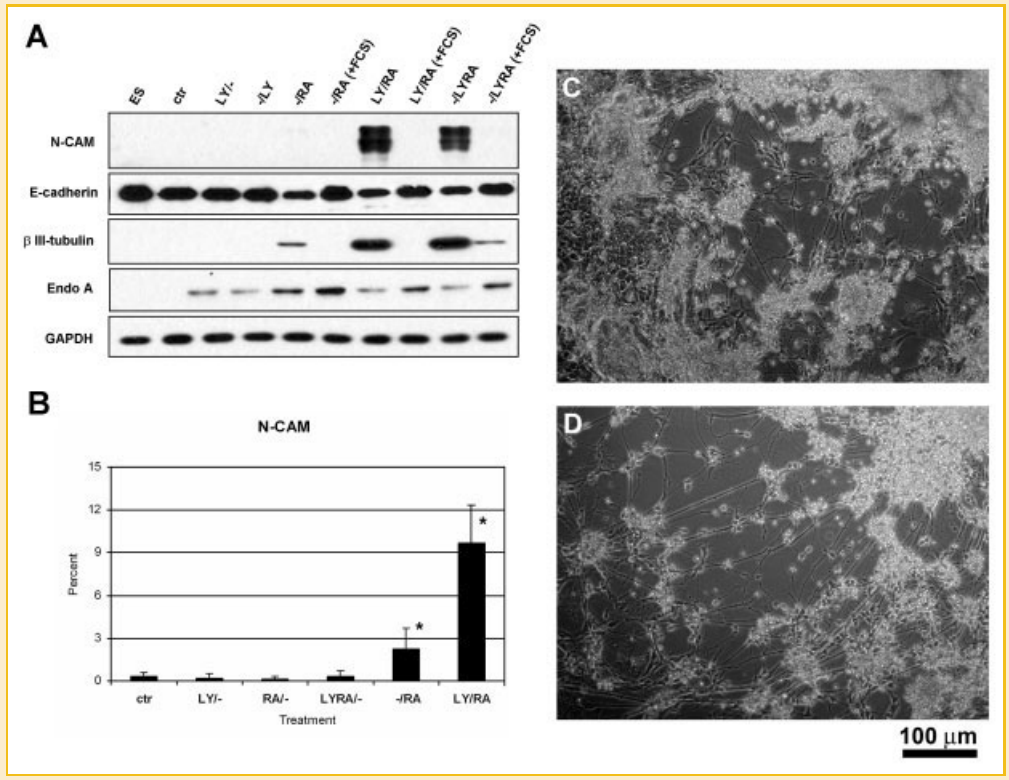

Fig. 3. Analysis of expression of differentiation-specific proteins and neural morphology of ES cells differentiated by LY/RA. A: Western blot analysis of expression of neural (N-CAM, β III-tubulin) and non-neural (Endo A, E-cadherin) protein markers in non-differentiating cells (ES) and in EBs differentiated by means of various 12-day treatments. GAPDH is employed as a loading control. A representative result of at least three independent experiments is presented. B: FACS analysis of N-CAM expression in 12-day-differentiated ES cells under various conditions. Results are presented as means and standard error means of three independent experiments. *Significant difference from the control (ctr) ($P < 0.05$). Morphology of 8- (C) and 12-(D) day-old differentiating ES cells, which were pre-treated by LY (36 h) followed by induction of neurogenesis with RA in serum-free media for 48 h and then cultured in serum-free media.

immunocytochemical determination of N-CAM positive cells using flow-cytometry. This experiment confirmed our results from the Western blotting analysis. Pre-treatment with LY and subsequent treatment with RA under serum-free conditions led to a highly increased number of N-CAM positive cells—the hallmark of neural differentiation. In the presence of serum, the RA pro-neural effect was not observed (Fig. 3B). Pre-treatment of ES cells with inhibitors of the other pluripotency-maintaining signaling pathways (Smads, STAT3, and ERK; details above in the Materials and Methods Section) did not lead to RA-induced neurogenesis (Fig. 4A).

To sum up, the combination of LY pre-treatment followed by RA treatment in serum-free media had a major effect on the induction of neural differentiation of ES cells in our experiments. Our observation was also compared with the original Bain's protocol of RA-induced neurogenesis (for details see the Materials and Methods Section). The initial 4-day EBs were cultured with or without LY, following next 4 days with RA. Samples were collected at the same overall time of differentiation as in monolayer-differentiated ES cells. We obtained more differentiating cells with our protocol of RA-induced neurogenesis in monolayer culture which is presented here than with Bain's EBs protocol. Interestingly, if initial EBs were cultured in the presence of LY, the RA-induced neurogenesis was also significantly improved (Fig. 4B).

INHIBITION OF PI3K WITH LY29400 DID NOT DIRECTLY AFFECT THE RA RESPONSE

To exclude potential interaction between PI3K inhibition and RA-regulated signaling and transcription we analyzed the effect of the PI3K inhibitor LY on RA-induced transcription. We tested these interactions by means of the RA-sensitive luciferase reporter assay (Fig. 5A). Non-differentiated cells and cells differentiating spontaneously, with or without LY treatment, showed a luciferase-mediated signal near to the background. RA induction (12 h) strongly increased the reporter signal independent of pre- or co-treatment with LY. If we determined the expression of the RA-sensitive genes RARβ and Cyp26a1 we obtained similar results (Fig. 5B). Pre-treatment with LY had no effect on their RA-induced expression. Such an analysis thus confirmed that the PI3K inhibitor LY did not affect RA-induced transcription.

THE CELL'S RESPONSE TO LY29400, RA, AND THEIR COMBINATION—EARLY DEVELOPMENTAL TRANSCRIPTS ANALYSIS

To better understand the processes mediated by LY and RA in neural differentiating cells we analyzed the expressions of various transcripts on day 4 of differentiation. Changes in the expression of pluripotent (Oct-4, Nanog), early differentiation (FGF-5), and early neural differentiation (Nestin, Pax-6, and Sox-1) marker genes

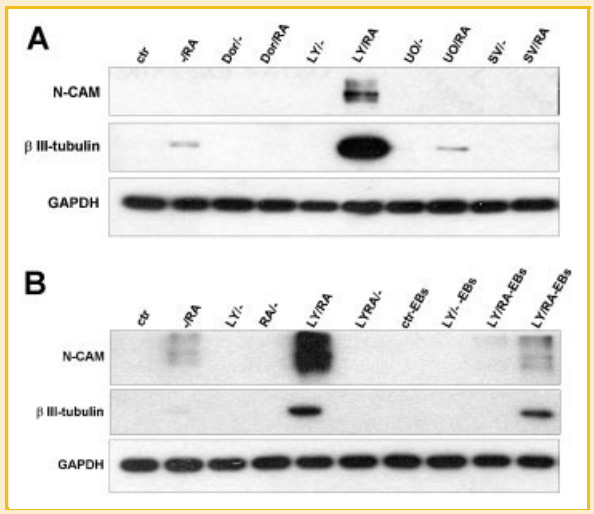

Fig. 4. Effect of selected signaling inhibitors on RA-induced neurogenesis in ES cells. A: Pre-treatment with inhibitors of BMP/Smads signaling (Dorsomorphin—Dor), MEK/ERK signaling (UO126—UO), and JAK/STAT3 signaling (Stattic V—SV) did not lead to RA-induced neurogenesis compared to pre-treatment with inhibitor of PI3K signaling (LY29400—LY). B: Comparison of RA-induced neurogenesis after LY pre-treatment in monolayer and according to the original Bain's 4/4 protocol (here designed as EBs). EBs were treated with LY during the first 4 days of differentiation. In both cases (A,B), neurogenesis was assessed after 11 days of differentiation.

were analyzed (Fig. 6). All the differentiation conditions led to a significant down-regulation of Oct-4 and Nanog transcript. The combination of LY and RA treatment had a more powerful effect. The expression of FGF-5 was also significantly increased under all differentiation conditions. The highest up-regulation (20-fold) of FGF-5 transcript was in spontaneously differentiating cells. The presence of LY, RA, and/or their combination had a significantly weaker effect. Nestin expression was also significantly increased under all differentiation conditions. Treatment by LY had a stronger effect independent of treatment by RA. When cells were treated by RA alone, Nestin expression was significantly lower and similar to that found in spontaneously differentiating cells. On the other hand, in RA-treated cells the expression of Pax-6 and Sox-1 was significantly increased (up to 30-fold). Treatment by LY alone did not increase the expression of these transcripts, and the LY pre-treatment effect in RA-induced cells is only marginal. In contrast to LY-treated cells in spontaneously differentiating cells, both Pax-6 and Sox-1 expressions were significantly increased (3- and 5-fold, respectively), but almost 10-fold lower compared to RA-treated cells (Fig. 6).

DISCUSSION

Phosphoinositide kinase (PI3K) activation of Akt (also protein kinase B, PKB) represents an important signaling pathway with pleiotropic

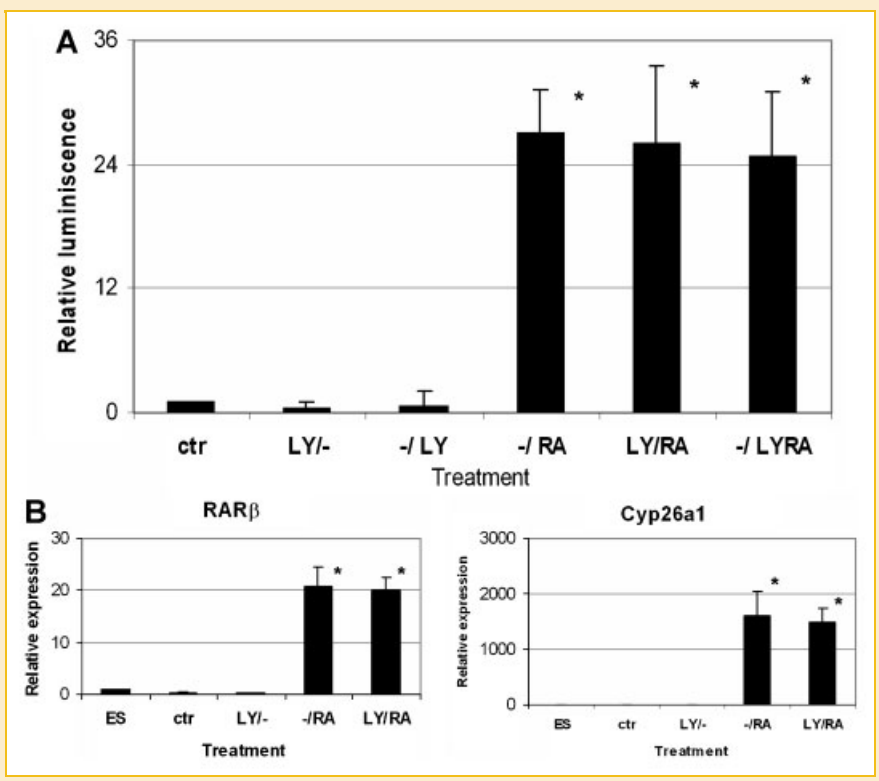

Fig. 5. Activity of RA signaling determined by RA-sensitive luciferase reporter and RA-responsive genes. Effect of LY, RA, and their combinations on RA-induced transcription in differentiating ES cells determined by RA-sensitive luciferase reporter (A), and effects of LY on RA-induced transcription of RA-responsive genes such as RARβ and Cyp26a1 (B). Results from three independent experiments presented as means and standard error means. *Significant difference from the control (ctr), $P < 0.05$.

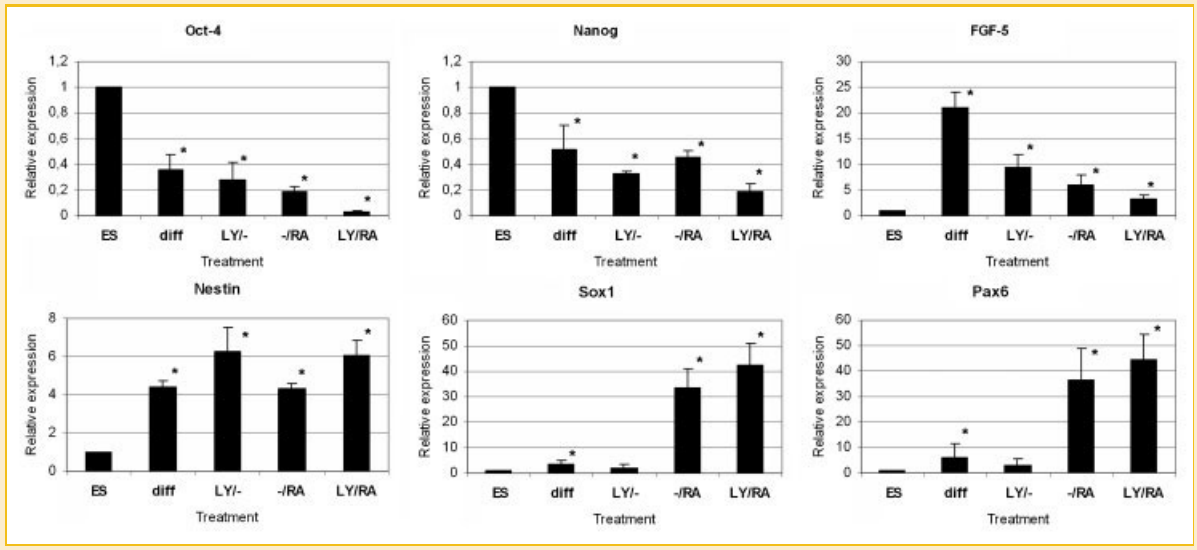

Fig. 6. qRT-PCR analysis of differentiation-specific transcripts. qRT-PCR analysis of ES specific (Oct-4, Nanog), early differentiation-specific (FGF-5), and pro-neural-specific transcripts in non-differentiated (ES) and differentiating ES cells under various conditions. Results from three independent experiments presented as means and standard error means. *Significant difference from the control (ES), $P < 0.05$.

effects on the fate of the cell. Its activity, induced by a broad spectrum of growth factors, regulates cell proliferation and differentiation as well as apoptosis. This variability is mediated by its abundant interactions with other signaling pathways and by a number of down-stream substrates of Akt. The majority of experiments showed the dominant pro-survival role of this signaling in the fate of the cell [Hanada et al., 2004; Liao and Hung, 2010]. PI3K activity is also indispensable for the maintenance of ES cells. In ES cells, inhibition of PI3K by LY29400 leads to the down-regulation of the pluripotent phenotype and to less specified differentiation [Storm et al., 2007]. The mechanisms of this effect are largely unknown, owing, among other reasons, to a broad spectrum of down-stream substrates [Hanada et al., 2004; Liao and Hung, 2010].

Here, we present the pro-neural pre-determination of ES cells to RA-induced neurogenesis triggered by the inhibition of PI3K signaling. Moreover, we proved EB formation to be inessential for RA-induced neurogenesis in ES [Bain et al., 1995; Pachernik et al., 2002]. We also thoroughly tested the differentiation conditions to find out the best combination: the depletion of LIF and serum with the inhibition of PI3K by LY pre-determined ES cells to undergo the most efficient RA-induced neurogenesis. Co-treatment of differentiating cells with LY and RA on day 3 brought about a similar but not so potent effect. The rest of the tested conditions (Fig. 1) did not lead to neurogenesis or were not compatible with cell survival. The mechanism of the aforementioned pre-determination is unknown, but it seems to be PI3K-specific, since employment of STAT3, ERK, or Smads signaling inhibitors had no neurogenic effect (Fig. 4A).

We described similar results in pluripotent EC P19 cells. We could also observe direct RA-induced neurogenesis of P19 cells when cells were treated in monolayer culture and serum-free media [Pachernik et al., 2005]. Unfortunately, such an approach did not lead to neurogenesis in ES cells. Our unpublished results showed that although the first response of ES cells to RA in serum-free media was accompanied by increased survival, the final result was that the cells extensively died. This could be mediated by the RA-induced expression of some cytokines in the first response to RA treatment [McCaffery and Drager, 2000], but the condition does not favor survival of the cells for a prolonged time without the addition of serum or exogenous growth factors (not shown).

It seems that in ES cells inhibition of PI3K may cause a phenotype shift causing them to become closer to P19 cells, which are easily induced to undergo intensive neurogenesis by RA. This hypothesis is also supported by the fact that co- or pre-treatment of P19 cells with LY29400 did not modify the progress of RA-induced neurogenesis (our unpublished data). On the other hand, evidence that the non-specific pre-differentiation of ES cells leads to more massive neurogenesis triggered by RA was presented previously by Bain and co-workers. They showed the potentiation of neurogenesis in ES cells that were non-specifically differentiated by EBs formation for 4 days before RA treatment [Bain et al., 1995]. But our results also show that the original Bain's protocol may be improved with LY treatment (Fig. 4B). Interestingly, Bain's 4/4 protocol is more efficient than simple RA treatment of EBs during the first 4 days of differentiation described by Fraichard et al. [1995]. This implies the down-regulation of Akt phosphorylation during differentiation of ES cells in the form of EBs. But our results do not correspond with such a hypothesis. Akt phosphorylation in EBs is not decreased compared to ES cells (Fig. 2B). This indicates that not only inhibition of Akt phosphorylation but changes in the differentiation status due to the switched-off PI3K signaling lead to preferred induction of neural lineage by RA. To understand the principle of this mechanism further detailed studies will be required.

The presence of serum during induction aborted the pro-neural effect of RA. This involves an unknown mechanism, since the expression of both pro-neural and some other RA-sensitive genes

were induced independently of the presence of serum. However, the final phenotype is serum-born factor dependent [Pachernik et al., 2002, 2005]. We also observed the same response in this study. When the LY pre-treated cells were treated with RA in serum-containing media, neurogenesis was aborted. Cells did not adopt neural morphology (not shown) and the expression of neural specific genes was not detected (Fig. 3). On the other hand, these cells expressed a higher level of cytokeratin Endo A and did not down-regulate the expression of E-cadherin, a marker of primitive endoderm and generally of epithelia cells, respectively [Pachernik et al., 2005; Halbleib and Nelson, 2006].

The fact that the observed effect is not mediated by a direct interaction of PI3K inhibitory effect and RA-mediated signaling was further confirmed by analysis of the gene transcription regulation. Inhibition of PI3K did not significantly affect RA-induced transcription as we showed by analysis of both pro-neural genes (Sox-1 or Pax-6) [Aberdam et al., 2007] and luciferase reporter assay (Figs. 5 and 6). On the other hand, the combination of LY and RA intensified the down-regulation of pluripotent genes (Oct-4 and Nanog) [Silva and Smith, 2008] and thus clearly empowered differentiation processes (Fig. 6). This is also well documented by the high expression of FGF-5, which is characteristic for transient populations of primitive ectoderm cells [Pelton et al., 2002].

Surprisingly, LY treatment alone did not lead to neural differentiation, although Nestin expression was significantly increased. Cells did not adopt neural specific morphology or the expression of markers such as β III-tubulin or N-CAM. Moreover, spontaneously differentiated cells had a higher level of Sox-1 and Pax-6 transcript than cells treated with LY alone. Thus, we may presume that LY-established conditions are not strictly pro-neural. This may be affected by the presence of serum-born factors during LY treatment. However, when we directly transferred ES cells to serum-free media before any treatment, it lead to an extremely high frequency of cell death; therefore, the presence of serum during the initial differentiation step was required [data not shown, and Pachernik et al., 2002; Vesela et al., 2010].

In conclusion we presented a protocol for the RA-induced neurogenesis of ES cells independent of EBs formation. We also showed that the inhibition of PI3K by LY29400 pre-determined cells to neurogenesis induced by RA. To understand the precise mechanisms of this feature, further detailed study focused on the role of PI3K or Akt substrates in the self-renewal and lineage specification of pluripotent cells is required.

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