FEBS Letters 457 (1999) 478–482 FEBS 22497

A zinc-finger transcription factor induced by TGF-β promotes apoptotic cell death in epithelial Mv1Lu cells

Elisabet Chalaux[a], Teresa López-Rovira[a], Jose Luis Rosa[a], Gabriel Pons[a], Linda M. Boxer[b], Ramon Bartrons[a], Francesc Ventura[a,*]

[a] *Departament Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, C/ Feixa Llarga s/n., 08907 Hospitalet de Llobregat, Spain*
[b] *Department of Medicine, University of Stanford, Stanford, CA 94305, USA*

Received 13 July 1999

Abstract Transforming growth factor-β (TGF-β) superfamily members constitute a group of multifunctional factors that are able to stimulate apoptotic cell death in a variety of cells. In this report, we show that a zinc-finger transcription factor (TIEG) is an immediate early gene transcriptionally induced by TGF-β in the epithelial Mv1Lu cell line. We also demonstrate that, mimicking TGF-β effects, ectopic overexpression of TIEG is sufficient to trigger the apoptotic cell program in these cells, which is preceded by a decrease of Bcl-2 protein levels. Finally, apoptotic events elicited by TIEG overexpression can be effectively prevented by ectopic co-expression of Bcl-2. On the basis of these results we suggest that induction of TIEG expression has a role in the pro-apoptotic properties of TGF-β.

Key words: Transforming growth factor-β; Apoptosis; Transcriptional regulation

1. Introduction

Several lines of evidence indicate that apoptosis, or programmed cell death, plays an essential role during embryonic development and later in the maintenance of homeostasis in multicellular organisms [1,2]. In addition, when dysregulated, its effects contribute to pathogenesis of many diseases, including cancer, autoimmunity and neurodegenerative disorders. Apoptosis constitutes an active and gene-directed event with a well characterized morphological and biochemical set of cellular alterations [2–4].

Among the cytokines with the ability to trigger the onset of apoptosis there are several transforming growth factor-β (TGF-β) superfamily members. TGF-β mediates induction of apoptosis in several cell types, including hepatocytes [5], B-cell precursors [6], endothelial and epithelial cell lines [7]. Bone morphogenetic protein (BMP) 2/4 signalling may also induce apoptosis of neural crest cells (reviewed in [8]), interdigital regions of developing limbs [9] or the epithelial enamel knot during tooth formation [10].

Members of the TGF-β superfamily interact with two types of related transmembrane serine/threonine kinase receptors. Downstream signalling from receptor to the nucleus appears to be mediated by the family of Smad transcriptional activators (reviewed in [11,12]). Consistent with this function, DNA binding and transcriptional activity of various Smads have been shown [11,12]. In addition, interaction of Smads with additional DNA binding partners, such as FAST, AP-1 or vitamin D receptor, are required for efficient transcriptional activation of certain target genes [13].

Although the molecular mechanisms involved in TGF-β signalling are beginning to emerge, the mechanisms leading to the onset of apoptosis in response to this cytokine are still poorly understood. In this respect, recent evidence suggests that induction of the homeobox-gene Msx-2 by BMP4 mediates cell death during limb and neural crest development [14] and that induction of a zinc-finger transcription factor (TIEG) by TGF-β mediates exocrine pancreatic apoptosis [15]. TIEG was first identified as a TGF-β inducible gene in human osteoblasts [16]. In addition, EGR-α, which corresponds to a putative alternative spliced version of TIEG lacking the first 12 amino acids, was shown to have cell-cycle regulated expression in prostate cells [17]. Close homologs, named mGIF and TIEG2 (with 85% and 91% amino acid homology to TIEG, respectively), have recently been cloned defining a new expanding subfamily of Sp1-like transcription factors involved in growth regulation [15,18]. TIEG family members are widely expressed during development in several tissues, including brain, bone and differentiating mesenchyme, skin and kidney [18].

In this report, we have characterized the regulation of TIEG expression by TGF-β in epithelial cells. TIEG is transcriptionally induced by TGF-β without requirement of protein synthesis. Furthermore, ectopic overexpression of TIEG is sufficient to induce apoptotic cell death in Mv1Lu cells.

2. Materials and methods

2.1. TIEG cDNA, plasmids, cell lines and transfections

A cDNA encoding the full coding sequence of TIEG described in [16] was amplified by PCR from a mouse brain cDNA library using oligonucleotides corresponding to its amino and carboxy terminus and analyzed by automatic sequencing. $TIEG^{HA}$ construct containing the hemagglutinin (HA) epitope at the amino terminus was generated by PCR and subcloned into a pCDNA3 vector (Invitrogen) for mammalian expression. The pEGFP vector encoding the green fluorescence protein (GFP) was purchased from Clontech. COS-1, Mv1Lu and R1B/L17 cell lines were cultured and transiently transfected with the indicated vectors as described in [19].

2.2. GFP/Hoechst staining

Transfected cells were fixed in PBS containing 4% paraformaldehyde, stained with 4 μg/ml Hoechst 33342 and mounted. Green fluorescent protein and stained nuclei were visualized under a fluorescence microscope.

*Corresponding author. Fax: (34) (93) 4024213.
E-mail: fventura@bellvitge.bvg.ub.es

Abbreviations: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; TIEG, TGF-β induced transcription factor; GIF, GDNF induced factor; PCR, polymerase chain reaction; GFP, green fluorescent protein

PII: S0014-5793(99)01051-0

2.3. Analysis of DNA fragmentation

Cells were washed in PBS, lysed with 400 μl of 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% Triton X-100 and centrifuged. Cleared supernatants were collected and incubated at 37°C in a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 8, 40 mM EDTA, 1% SDS and 200 μg/ml of proteinase K during 6–12 h. After two extractions of phenol/chloroform/isoamyl alcohol (25:24:1), DNA was precipitated and washed with 70% ethanol. Resuspended DNA was treated with 25–50 mg/ml of RNase A at 37°C for 1 h. DNA was separated in 2% agarose gel and stained with ethidium bromide.

2.4. Western analysis

Equal amounts of protein were loaded onto 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Detection was performed using a mouse monoclonal antibody against Bcl-2 (Dako) and HA (Babco), followed by a horseradish peroxidase-conjugated anti-mouse antibody from sheep (Amersham) and enhanced chemiluminescence detection (Amersham). Blots were normalized using a monoclonal anti α-tubulin antibody (Calbiochem).

2.5. Cell viability assay

Cells were transiently transfected with the indicated plasmids together with a plasmid containing the β-galactosidase gene. The cells that showed positive blue staining were counted under a microscope every day after transfection and compared to their relative controls.

2.6. RNA preparation and Northern blots

Total RNA was extracted from Mv1Lu cells according to the Chomczynski method. Northern analysis was done as described in [20].

3. Results

Regulation of TIEG mRNA expression by TGF-β was analyzed in the epithelial cell line Mv1Lu, which is highly responsive for TGF-β [21,22]. RNAs from Mv1Lu cells treated with 1 nM TGF-β were analyzed by Northern blotting. Induction of TIEG mRNA was detected as early as 30 min after the addition of TGF-β, reached maximal levels after 30–60 min, and decreased thereafter (Fig. 1A).

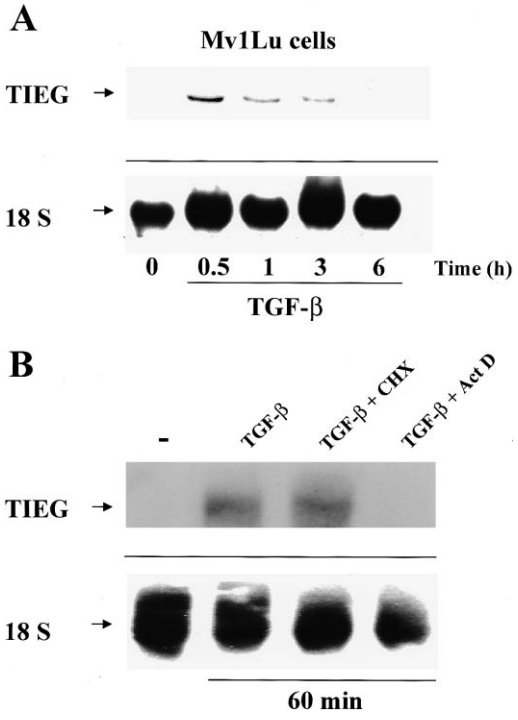

Fig. 1. Effect of TGF-β on TIEG m RNA levels. A: Epithelial Mv1L1 cells were treated with TGF-β (1 nM). Total RNA was extracted at different time points after cytokine addition and analyzed by Northern analysis with the mouse TIEG cDNA probe. B: Mv1Lu were treated for 60 min with 1 nM of TGF-β, and some cultures also received cycloheximide (CHX) (10 μg/ml) or actinomycin D (Act D) (1 μg/ml). Total RNA was extracted and TIEG expression was measured by Northern blot analysis.

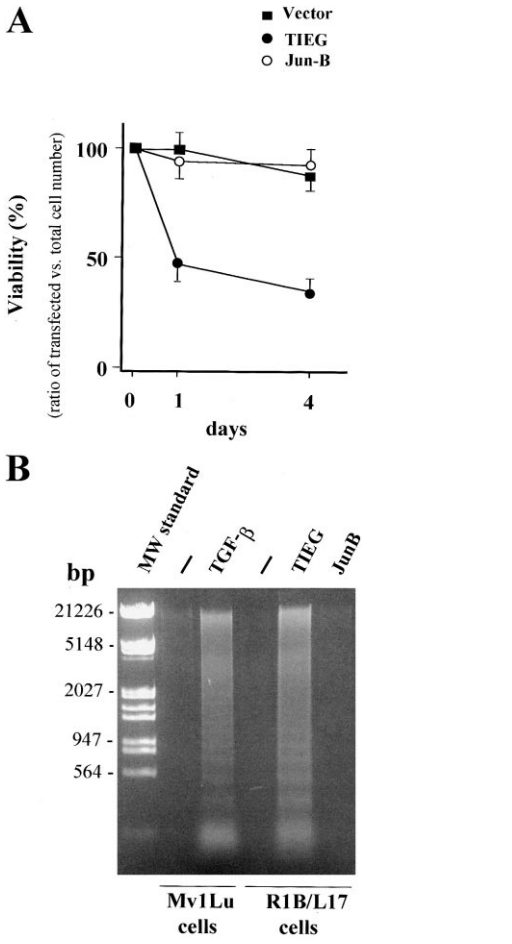

Fig. 2. Overexpression of TIEG induces apoptosis in Mv1Lu cells. A: R1B/L17 cells were transfected with TIEG, JunB or empty vector together with a plasmid expressing the β-galactosidase gene. The viability of blue-stained cells were determined microscopically every day after transfection and compared to their relative controls. Results are expressed as means ± S.E.M. B: Internucleosomal fragmentation was analyzed using the DNA ladder assay. Mv1Lu cells were treated with 1 nM TGF-β during 24–48 h. R1B/L17 cells transfected with TIEG, JunB or empty vector were analyzed 2 days after transfection.

We next assessed whether induction of TIEG mRNA was dependent on protein synthesis. Mv1Lu cells were treated with TGF-β for 60 min together with the protein synthesis inhibitor cycloheximide (10 μg/ml) or the RNA synthesis inhibitor actinomycin D (1 μg/ml). Northern analysis revealed a similar induction of TIEG mRNA in the presence of cycloheximide and a complete block of induction when actinomycin D was added (Fig. 1B). These results indicate that TGF-β induces TIEG by a transcriptional mechanism that does not require protein synthesis.

Previous data showed cell-cycle regulated expression and involvement of TIEG in the regulation of pancreatic cell growth and apoptosis [15,17]. To pursue our examination on the effect of TIEG on cell growth, we overexpressed

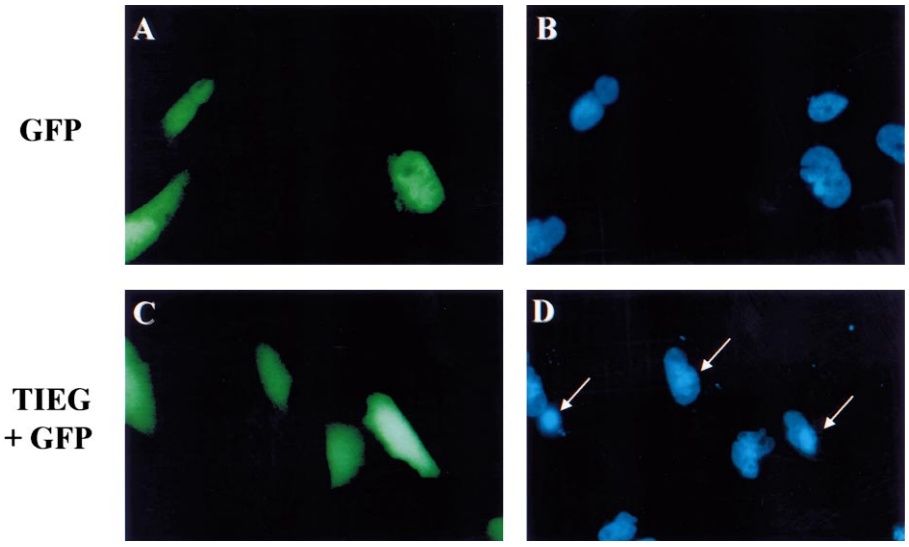

Fig. 3. Chromatin analysis after overexpression of TIEG. R1B/L17 cells were transfected with a green fluorescent protein expression vector alone (A and B) or together with a TIEG expression vector (C and D). 48 h after transfection cells were fixed and stained with Hoechst to visualize nuclear chromatin (B and D).

TIEG in a clone of Mv1Lu cells (R1B/L17). This clone lacks TGF-β receptors and was selected for its high transfectability (up to 80% efficiency of transfection) [19]. Analysis of viability of transfected and β-galactoside-positive cells showed that ectopic expression of TIEG not only blocked proliferation but also resulted in a significant increase in cell death, compared to vector or junB (previously reported as a TGF-β and BMP-2 induced gene [22]) transfected cells (Fig. 2A). These results raised the hypothesis that TIEG induces apoptotic cell death rather than blocking cell cycle progression. To analyze this possibility, we performed DNA fragmentation assays to determine whether these cells become apoptotic. Mv1Lu cells showed internucleosomal cleavage of DNA when analyzed 24–48 h after TGF-β (Fig. 2B). Similarly, transient overexpression of TIEG results in the formation of a DNA ladder as a result of internucleosomal fragmentation, even without the addition of TGF-β (Fig. 2B). Transfection of the vector alone did not affect apoptosis, nor the overexpression of junB or β-galactosidase, used as controls. To confirm these effects at cellular level, we also analyzed the morphological apoptotic feature of chromatin condensation. Individual transfected cells were visualized by cotransfection of a green fluorescent protein expression vector. DNA staining with Hoechst 33258 revealed that only TIEG-transfected cells (green cells) showed chromatin margination (Fig. 3). This feature of apoptotic nuclei was absent in cells transfected with control vector.

Several lines of evidence suggest that different stimuli are able to modulate the expression of Bcl-2 both transcriptionally and post-transcriptionally [23]. First, we analyzed whether TGF-β had any effect on Bcl-2 levels by Western blotting.

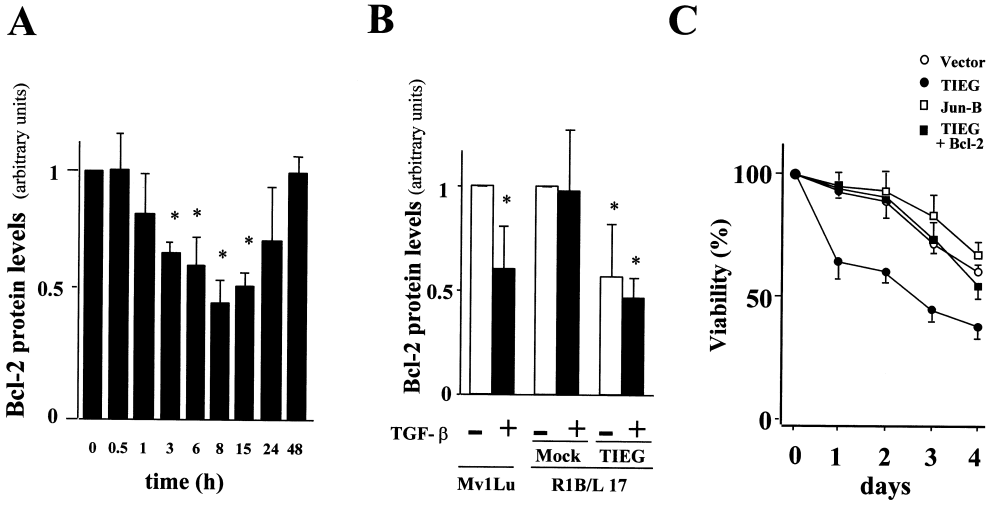

Fig. 4. Overexpression of TIEG decreases Bcl-2 protein levels. A: Mv1Lu cells were incubated with 1 nM TGF-β for different times. B: R1B/L17 cells were transfected with empty or TIEG encoding vectors. Cells were lysed and Bcl-2 levels analyzed by Western blotting. Results are expressed as means ± S.E.M. of five separate experiments. Asterisks denote significant differences ($P < 0.05$). C: R1B/L17 cells were transfected with TIEG, TIEG and Bcl-2, Jun-B or empty plasmids. Cellular viability (transfected plus untransfected cells) were determined using a cell counter every day after transfection and compared to their relative controls for 4 days.

Bcl-2 protein levels fell to a half 6 to 8 h after TGF-β treatment and recovered thereafter (Fig. 4A). This evidence together with the fact that TIEG is a transcription factor that belongs to a family of Sp1-like zinc finger proteins suggested a possible relationship between TIEG and Bcl-2 expression levels. To analyze this hypothesis, we overexpressed TIEG in R1B/L17 cells. Results in Fig. 4B show that cells transfected with TIEG had lower levels of Bcl-2 than those transfected with the vector alone. We also measured caspase-3 activity using a fluorogenic caspase-3 subfamily substrate. Neither Mv1Lu cells treated with TGF-β nor R1B/L17 cells overexpressing TIEG showed significant differences in caspase-3 activity compared to their respective controls (data not shown). We cannot rule out that technical limitations may be responsible for the lack of caspase-3 activation or, alternatively, that other caspases subfamilies with distinct substrate specificity are involved in TGF-β induced apoptosis.

To examine whether Bcl-2 is able to interfere with apoptosis induced by TIEG, we ectopically expressed Bcl-2 together with TIEG in R1B/L17 cells and measured their viability over time. Immunoblot assays showed only a small decrease in TIEG expression levels in cells coexpressing Bcl-2 (data not shown). As shown in Fig. 4C, there was a progressive loss of transfected cells that is highly enhanced by overexpression of TIEG. However, ectopic Bcl-2 coexpression provided almost complete protection of those cells from death, for up to 4 days. In addition, we also found that TIEG-induced apoptotic DNA fragmentation was effectively prevented by Bcl-2 coexpression (data not shown).

Taken together, these data show that TGF-β, in addition to growth inhibition, is able to induce apoptosis in epithelial cells and that overexpression of TIEG is sufficient to mimic this apoptotic effect of TGF-β.

4. Discussion

TGF-β and GDNF regulation of the expression of the TIEG subfamily of transcription factors have been described in osteoblasts and neuroblastoma cell lines [16,18]. Here, we show that TIEG mRNA is highly induced soon after TGF-β addition in epithelial cells (Fig. 1). Regulation of the steady-state levels of TIEG may be due to regulation of its transcription rate since addition of actinomycin D completely blocked induction of its mRNA by TGF-β. In addition, induction of TIEG was not affected by the protein synthesis inhibitor cycloheximide, suggesting that *trans*-acting factors required for transcription pre-exist in the cell. The Smad family of proteins are known transcriptional mediators of the TGF-β superfamily signalling. The time course assays showed that the profile of TIEG mRNA induction is consistent with the activation of Smads [24] suggesting that the Smad family may mediate this TIEG transcriptional activation.

TGF-β and BMP-2 triggers apoptosis in a wide range of cell types [5–10]. Interestingly, addition of cycloheximide blocks TGF-β but not staurosporine-induced apoptosis in hepatocyte cultures, suggesting a requirement of protein synthesis in the early events induced by this cytokine [25]. In addition, ectopic overexpression of components of both BMP and TGF-β signal transduction and transcriptional machinery, such as Smad4 and TAK1, result in apoptotic cell death both in vitro and in vivo [26,27]. This could also be the case in the model presented here since ectopic overexpression of TIEG is sufficient to induce cellular death with the morphological and molecular features of apoptosis (Figs. 2 and 3). TIEG might therefore mediate some of the effects of TGF-β on apoptosis by acting as a transcriptional repressor/activator on promoters containing Sp1-like sites.

The results presented here suggest that both TGF-β and TIEG-induced apoptosis are preceded by down-regulation of Bcl-2 protein levels (Fig. 4), whereas no significant increases in activity of the caspase-3 subfamily were detected. Decrease of Bcl-2 levels becomes significant after 3 h of addition of TGF-β, time which follows the peak of TIEG induction. To determine whether TGF-β and/or TIEG regulate Bcl-2 at the transcriptional level, we analyzed a Bcl-2 promoter construct coupled to luciferase reporter activity. Overexpression of TIEG, or TGF-β addition, failed to repress the Bcl-2 promoter in luciferase reporter assays (data not shown). We thus suggest that TIEG may have translational or posttranslational effects on Bcl-2 expression, rather than a direct transcriptional effect. In this respect, Bcl-2 function has been shown to be inactivated at posttranslational level by either phosphorylation on serine residues or by both trypsin-like and caspase-mediated proteolysis in the linker region [23,28,29].

The apoptosis described herein was prevented by Bcl-2 overexpression. Bcl-2 can protect against both caspase-dependent and independent apoptosis through prevention of executioner caspase activation and/or stabilization of the mitochondrial permeability transition pore [23,30,31]. Thus, among the potential explanations for the role of Bcl-2 in TGF-β-induced apoptosis is the prevention of both formation of oxygen free radicals and reduction in mitochondrial membrane potential. In fact, TGF-β action has been related to the induction of cellular oxidative stress [32]. Moreover, TGF-β induction of reactive oxygen species preceding the onset of apoptosis has been described in hepatocytes, where radical scavengers partially blocked these apoptotic effects without altering TGF-β growth-inhibitory properties [33]. In this context, it has been described that TGF-β down-regulates expression of cytochrome P450 family members and that TIEG is able to bind to the Sp1-like sites on promoters of cytochrome *P*450 members [18,34].

In conclusion, the present study provides evidence of transcriptional regulation of an Sp1-like transcription factor (TIEG) by TGF-β in epithelial cells. Results also suggest that ectopic overexpression of TIEG is sufficient both to decrease endogenous Bcl-2 levels and trigger the apoptotic cell program, mimicking TGF-β effects. These apoptotic events elicited by TIEG can be prevented by co-expression of Bcl-2. Taken together, these data suggest that induction of TIEG has a role in the pro-apoptotic properties of TGF-β.

Acknowledgements: We thank Esther Adanero, Mireia Dalmau and Mònica Vallés for technical assistance, Robin Rycroft for English language revision and Drs. S. Ambrosio and J. Gil for discussion, gifts of plasmids, antibodies and reagents. This work was supported by Grants from the Ministerio de Educación y Ciencia (PM98-0183) and Generalitat de Catalunya (97SGR-0074). Fellowships were provided to E.C. by Fundació Pi i Sunyer and to T.L. by Ministerio de Educación y Ciencia (F.P.I.).

References

[1] Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Cell 88, 347–354.
[2] Green, D.R. (1998) Cell 94, 695–698.
[3] Vaux, D.L. and Strasser, A. (1996) Proc. Natl. Acad. Sci. USA 93, 2239–2244.
[4] Salvesen, G.S. and Dixit, V.M. (1997) Cell 91, 443–446.
[5] Oberhammer, F.A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A.F., Bursch, W. and Schulte-Hermann, R. (1992) Proc. Natl. Acad. Sci. USA 89, 5408–5412.
[6] Selvakumaran, M., Lin, H.K., Sjin, R.T.T., Reed, J.C., Liebermann, D.A. and Hoffman, B. (1994) Mol. Cell. Biol. 14, 2352–2360.
[7] Hsing, A.Y., Kadomatsu, K., Bonham, M.J. and Danielpour, D. (1996) Cancer Res. 56, 5146–5149.
[8] Mehler, M.F., Mabie, P.C., Zhang, D. and Kessler, J.A. (1997) Trends Neurosci. 20, 309–317.
[9] Zou, H. and Niswander, L. (1996) Science 272, 738–741.
[10] Jernvall, J., Aberg, T., Kettunen, P., Keränen, S. and Thesleff, I. (1998) Development 125, 161–169.
[11] Heldin, C.H., Miyazono, K. and ten Dijke, P. (1997) Nature 390, 465–471.
[12] Massagué, J. (1998) Annu. Rev. Biochem. 67, 753–791.
[13] Derynk, R., Zhang, Y. and Feng, X.-H. (1998) Cell 95, 737–740.
[14] Takahashi, K., Nuckolls, G.H., Tanaka, O., Semba, I., Takahashi, I., Dashner, R., Shum, L. and Slavkin, H.C. (1998) Development 125, 1627–1635.
[15] Tachibana, I., Imoto, M., Adjei, P.N., Gores, G.J., Subramaniam, M. and Spelsberg, T.C. (1997) J. Clin. Invest. 99, 2365–2374.
[16] Subramaniam, M., Harris, S.A., Oursler, M.J., Rasmussen, K., Riggs, S.L. and Spelsberg, T.C. (1995) Nucleic Acids Res. 23, 4907–4912.
[17] Blok, L.J., Grossmann, M.E., Perry, J.E. and Tindall, D.J. (1995) Mol. Endocrinol. 9, 1610–1620.
[18] Yajima, S., Lammers, C.H., Lee, S.H., Hara, Y., Mizuno, K. and Mouradian, M.M. (1997) J. Neurosci. 17, 8657–8666.
[19] Cárcamo, J., Weis, F.M.B., Ventura, F., Wieser, R., Wrana, J.L. and Massagué, J. (1994) Mol. Cell. Biol. 14, 3810–3821.
[20] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1992) Current Protocols in Molecular Biology, John Wiley and Sons, New York.
[21] ten Dijke, P., Yamashita, H., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C.H. and Miyazono, K. (1994) J. Biol. Chem. 269, 16985–16988.
[22] Chalaux, E., López-Rovira, T., Rosa, J.L., Bartrons, R. and Ventura, F. (1998) J. Biol. Chem. 273, 537–543.
[23] Chao, T.D. and Korsmeyer, S.J. (1998) Annu. Rev. Immunol. 16, 395–419.
[24] Hoodless, P.A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L. and Wrana, J.L. (1996) Cell 85, 489–500.
[25] Inayat-Hussain, S.H., Couet, C., Cohen, G.M. and Cain, K. (1997) Hepatology 25, 1516–1526.
[26] Atfi, A., Buisine, M., Mazars, A. and Gespach, C. (1997) J. Biol. Chem. 272, 24731–24734.
[27] Shibuya, H., Iwata, H., Masuyama, N., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E. and Ueno, N. (1998) EMBO J. 17, 1019–1028.
[28] Cheng, E.H.Y., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K. and Hardwick, J.M. (1997) Science 278, 1966–1968.
[29] Grandgirard, D., Studer, E., Monney, L., Belser, T., Fellay, I., Borner, C. and Michel, M.R. (1998) EMBO J. 17, 1268–1278.
[30] Green, D.R. and Reed, J.C. (1998) Science 281, 1309–1312.
[31] Monney, L., Otter, I., Olivier, R., Ozer, H., Haas, A.L., Omura, S. and Borner, C. (1998) J. Biol. Chem. 273, 6121–6131.
[32] Ohba, M., Shibanuma, M., Kuroki, T. and Nose, K. (1994) J. Cell Biol. 126, 1079–1088.
[33] Sanchez, A., Alvarez, A., Benito, M. and Fabregat, I. (1996) J. Biol. Chem. 271, 7416–7422.
[34] Abdel-Razzak, Z., Corcos, L., Fautrel, A., Campion, J.P. and Guillouzo, A. (1994) Mol. Pharmacol. 46, 1100–1110.