Down-regulation of protein kinase Cα and γ and enhanced TPA-induced neurite formation in *DAN*-transfected neuroblastoma cells

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Abstract ***DAN*** **gene was first isolated by differential screening between rat 3Y1 and v-*src*-transformed 3Y1 cells and showed a tumor-suppressive activity toward v-*src*-transformed 3Y1 cells. When *DAN*-transfected neuroblastoma cells were treated with a tumor promoter phorbol ester, TPA, neurite-like processes appeared within 2 h whereas no apparent change was observed in the parent and vector-transfected cells up to 8 h. This suggests some difference in TPA-receptor, protein kinase C (PKC), between *DAN*-transfectants and the control cells. *DAN*-transfected SH-SY5Y cells showed complete loss in PKCα and a large decrease in PKCγ. Similar down-regulation in PKCα and PKCγ was also observed in *DAN*-transfected Ha-*ras*-transformed NIH 3T3 cells. The decreased level of PKCα was partially recovered after treatment with a calpain inhibitor, ZLLH. A 150-kDa proteolytic product of a calpain-specific substrate, non-erythroid α-spectrin, was detectable in *DAN*-transfected SH-SY5Y cells but not in the parent or vector-transfected control cells. This suggests that *DAN*-transfected cells contain activated calpain which may cause down-regulation of PKC and hence induce the altered TPA response.**

Key words: DAN; Tumor suppressor; Protein kinase C; Calpain; Neuroblastoma

1. Introduction

Malignant transformation is frequently associated with loss of expression of tumor-suppressor genes. To identify the candidates of tumor-suppressive genes, differential screening between rat 3Y1 and its v-*src*-transformed cells was performed [1]. Among a number of identified differentially expressed genes, *DAN* gene was of much interest since its expression was completely suppressed in v-*src*-, v-*mos*- and SV40-transformed 3Y1 cells [1]. Transfection of *DAN* into v-*src*-transformed 3Y1 cells resulted in morphological reversion as well as loss of tumorigenicity [2]. These results suggested that *DAN* possesses a tumor-suppressive activity. Overexpression of *DAN* in 3Y1 cells caused retardation of entry into the S phase in normal cells [3]. Ectopic expression of *DAN* enhances retinoic acid-induced neuronal differentiation in human neuroblastoma cell lines [4]. *DAN* gene product (DAN) is composed of 178 amino acid residues with a possible signal peptide at its amino-terminus and is secreted into culture media although a substantial amount still remains in the cell [5]. In addition, DAN has a $(HX)_n$ repeat motif and, therefore, an affinity for Ni^{2+} [6].

Recently, Hsu et al. have identified a new gene, designated *Gremlin*, whose gene product can induce a secondary axis or hyperdorsalization when it was injected into ventral blastomeres of *Xenopus* embryos [7]. Interestingly, *Gremlin* contains a similar structural motif to *DAN* and *Cerberus*, which has an ability to induce an ectopic head in *Xenopus* embryos [7,8]. Functional analysis has demonstrated that all of these proteins (DAN family) [9] are secreted and can associate with BMP2 (bone morphogenetic protein) to prevent its signaling [7]. It was thus suggested that DAN family members can control diverse processes in cell growth and differentiation.

In the present study, further investigation on the biological function of DAN was carried out by transfection of *DAN* cDNA into human neuroblastoma and mouse fibroblast cells. The results showed a remarkable down-regulation of protein kinase C (PKC) as well as calpain activation in *DAN*-transfected cells.

2. Materials and methods

2.1. Cell culture

SH-SY5Y neuroblastoma cell line [9] was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and OPI (Sigma). Activated Ha-*ras*-transformed NIH 3T3 cells (ras-NIH, clone F25) [10] were cultured in Dulbecco's modified medium supplemented with 5% calf serum.

2.2. Transfection

Rat *DAN* cDNA was inserted into eukaryotic expression vectors, pRc/CMV (Invitrogen) and transfected into SH-SY5Y cells using lipofectin (Gibco-BRL). Transfected cells were selected in the presence of G418 (400 μg/ml) and two clones designated D1 and D4 were obtained as described [6]. Rat *DAN* cDNA was also inserted into the pMAMneo vector (Clontech) and transfected into ras-NIH cells. FMD-1 and FMD-6 were G418-selected *DAN*-expressing clones and FVE-5 and FVE-8 were the vector-transfected control clones.

2.3. Treatment of transfected cells with Dex and ZLLH

To induce the expression of *DAN*, the transfected NIH 3T3 cells were treated with dexamethasone (Dex, 1 μM) or the solvent dimethylsulfoxide (DMSO, 0.1%) for 2 days. To inhibit the calpain activity, cells were treated with ZLLH (50 μM) (Peptide Institute, Osaka, Japan) for 24 h.

2.4. Preparation of cell extract and Western blot analysis

Cells were lysed in 0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 50 μM *N*-acetyl-leucyl-leucyl-norleucinal as described, incubated at 0°C for 10 min and centrifuged at $13\,000 \times g$ for 10 min. The supernatant was then electrophoresed through an SDS-polyacrylamide gel

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Abbreviations: BMP, bone morphogenetic protein; Dex, dexamethasone; DMSO, dimethylsulfoxide; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ZLLH, carbobenzoxy-leucyl-leucinal

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followed by immunoblot analysis using affinity-purified anti-rat DAN polyclonal antibody [3,4], anti-PKC antibody (Amersham), anti-PKCα, -β, -γ, -δ, -ε, -ι, -μ, -θ, -λ and -ζ antibodies (Transduction Laboratories), and anti-150-kDa-non-erythroid α-spectrin (fodrin) antibody [11], as described previously [12].

3. Results

The expression of *DAN* is reduced in several human neuroblastoma cell lines including SH-SY5Y. *DAN* gene is mapped to chromosome 1p36.11-p36.13, which is close to the commonly deleted chromosome region in neuroblastomas [10]. To assess the tumor-suppressive activity of DAN against neuroblastoma cells, rat *DAN* cDNA was inserted in a constitutive expression vector, pRc/CMV, and transfected into SH-SY5Y cells [6]. Several G418-resistant clones were isolated and the expression of DAN was examined by immunoblot analysis. DAN was highly expressed in clones D1 and D4 (Fig. 1A). Anti-rat DAN antibody used in the present study could not recognize endogenous human DAN [6], and hence we were unable to estimate the increase in the amount of DAN in transfected cells.

When the transfected cells were treated with a tumor promoter phorbol ester, TPA, a marked increase in neurite formation was observed in *DAN*- but not in vector-transfected clones. Within 2 h of the addition of TPA, many neurite-like processes were formed in D4 cells (Fig. 2C and D), whereas vector-control cells did not show any morphological change at this time interval (Fig. 2A and B) or even 12 h after the addition of TPA (data not shown). The response to TPA in parent SH-SY5Y cells was similar to that of vector-transfected cells (data not shown).

The large difference in response to TPA between *DAN*-overexpressors and control cells suggested a possible alteration in TPA-receptor, PKC, in *DAN*-transfected cells. Thus, in the next step, we analyzed both the types and amounts of PKC by immunoblot. The results showed a complete loss of PKCα in two *DAN*-transfectants (Fig. 1B). The parent and vector-control cells contained high levels of PKCα. PKCγ, PKCδ, PKCλ and PKCθ decreased but did not completely disappear in *DAN*-transfected D4 cells (Fig. 1D). Notably, the molecular size of PKCδ was reduced in D4 cells, suggesting that the decrease in PKCs was due to increased degradation. On the other hand, the expression of PKCμ and -ζ was slightly increased in *DAN*-transfected cells (Fig. 1D). The expression of PKCβ and PKCι was below the detection level (data not shown).

It is still unclear whether down-regulation of PKC can be induced directly by DAN itself. Then, we transfected *DAN* gene with an inducible expression vector, pMSG, into ras-NIH cells. Among the transfectants, FMD1, FMD6 and FMD7, showed a high expression of DAN after treatment with dexamethasone (Dex) and a low leaked expression without treatment (Fig. 3A, data not shown). Another vector-transfected clone, FVE-5, did not express a detectable level of DAN irrespective of treatment with Dex. The level of PKCα correlated inversely with the expression of DAN, i.e. PKCα decreased after induction of DAN expression by Dex

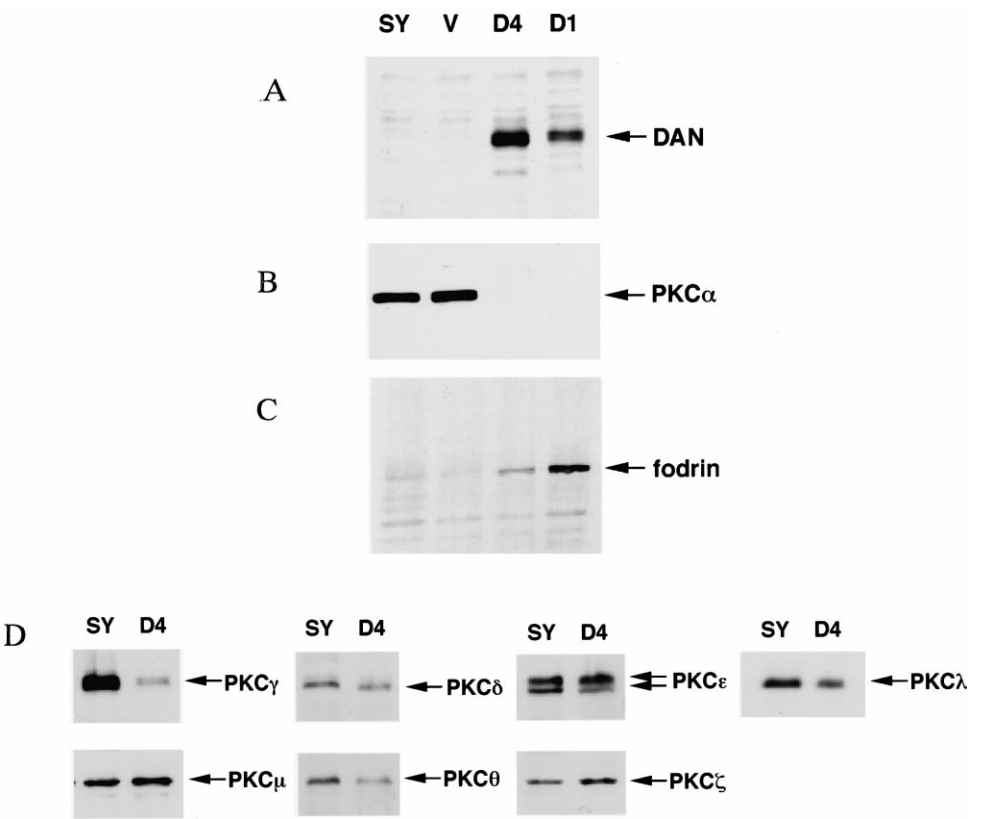

Fig. 1. Expression of DAN, PKCα and fodrin in *DAN*-transfected SH-SY5Y neuroblastoma cells. Cell extracts of parent SH-SY5Y cells (SY), vector-transfected control clone (V) and two *DAN*-transfected clones (D4 and D1) were analyzed by immunoblot using anti-DAN (A), anti-PKCα (B), anti-150-kDa fodrin (C) and anti-PKC subtype-specific antibodies (D). Anti-fodrin antibody used in C was 150-kDa-form-specific and therefore the 240-kDa precursor form was not seen.

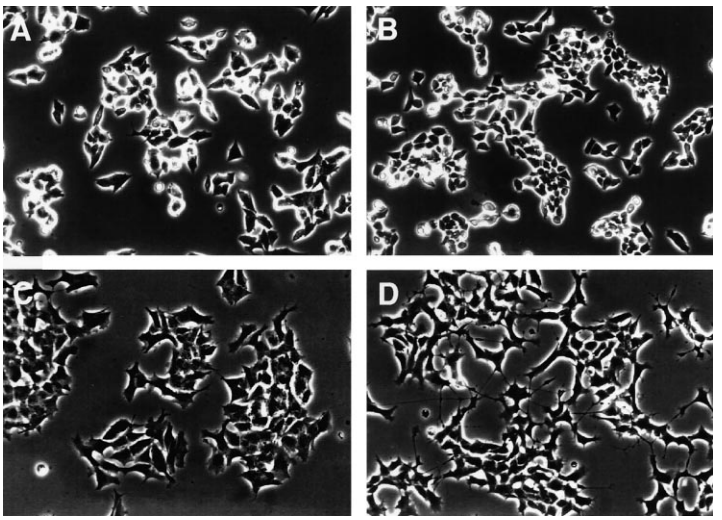

Fig. 2. Phase morphology of *DAN*-transfected SH-SY5Y cells before or after treatment with TPA. Shown are phase micrographs of vector-transfected SH-SY5Y cells (A and B) and *DAN*-transfected D4 cells (C and D). A and C show the cells without any treatment, and B and D show cells after treatment with 100 nM TPA for 2 h. Original magnification, ×100.

(Fig. 3B). A slightly higher mobility of PKCα in FMD1 compared with that in FVE5 suggested that truncating degradation of PKCα might occur in the *DAN*-transfectant. Similar differences in the level and molecular size of PKCα were also observed between other vector-transfected FVE1 cells and *DAN*-transfected FMD7 cell (Fig. 3C). The level of PKCγ, PKCθ and PKCζ also decreased in FMD7 cells compared to those in FVE1 cells. The expression of PKCβ, PKCδ and PKCι was undetectable in these cells (data not shown).

The decrease in both the protein amount and molecular size of multiple subtypes of PKC suggested that these changes were induced by enhanced degradation rather than reduced

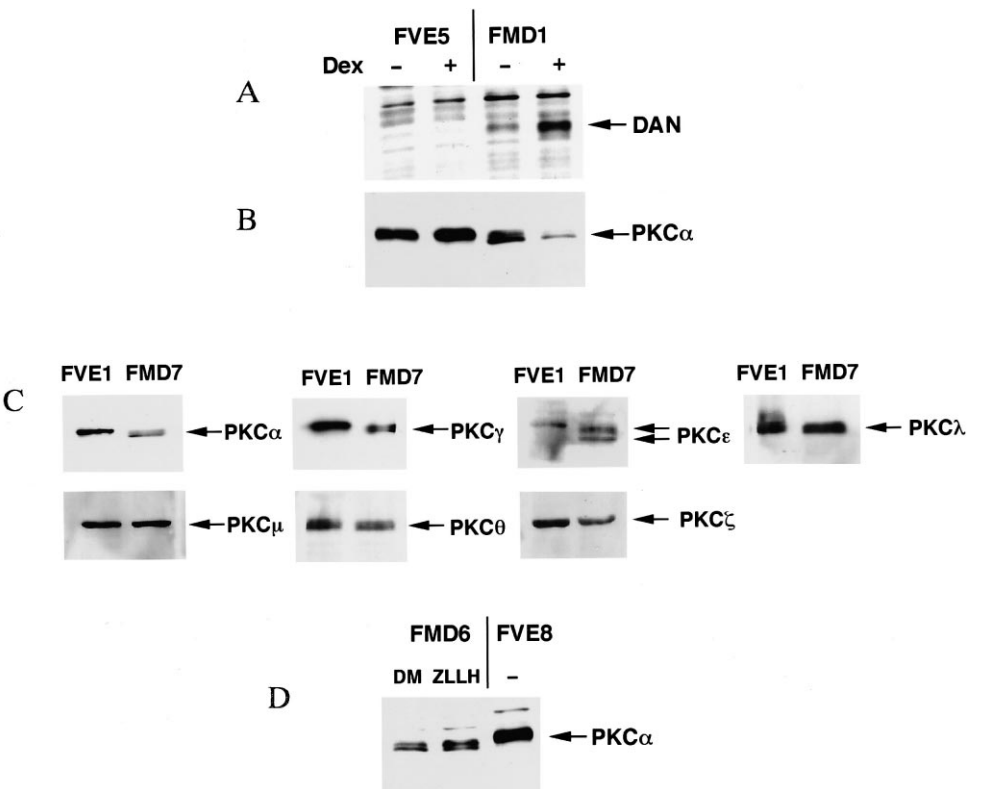

Fig. 3. Expression of DAN and PKCα in *DAN*-transfected ras-NIH cells. A and B: *DAN*-transfected ras-NIH (FMD1) and the vector-control clone (FVE5) were treated with (+) or without (−) Dex at a concentration of 1 μM for 2 days. The cell extract was analyzed by immunoblot using anti-DAN (A) and anti-PKCα antibodies (B). C: Vector-transfected FVE1 and *DAN*-transfected FMD7 were treated with Dex for 2 days and the cell extract was analyzed by immunoblot using anti-PKC subtype-specific antibodies. D: FMD6 was treated with a calpain inhibitor, ZLLH, at a concentration of 50 μM for 24 h. For control, the cells were treated with the solvent DMSO (DM). Results of immunoblot using anti-PKCα are shown. Cell extract of a vector-transfected FVE8 without any treatment was also examined.

synthesis. PKC can be degraded by a calcium-dependent neutral proteinase, calpain [11]. If the decrease in PKC is caused by calpain, the level of PKC might increase by a calpain inhibitor. FMD6, another *DAN*-transfected ras-NIH clone, was treated with a calpain inhibitor, ZLLH [12], for 24 h and PKC was examined by immunoblot. FMD6 showed a similar decrease in PKCα without Dex treatment compared with a vector control, FVE8, probably due to a leaked expression of DAN (Fig. 3D). Treatment with ZLLH significantly increased the level of PKCα although the increased level was still lower than that in untreated FVE8. These results suggest that calpain activity is enhanced in *DAN*-transfected cells. The intracellular activity of calpain can be estimated by investigating the degradation of the specific substrate, non-erythroid α-spectrin (fodrin) [13]. Immunoblot analysis showed the presence of a 150-kDa degradation product of fodrin only in *DAN*-transfected clones but not in the parent and vector-control cells (Fig. 1C). This implies that calpain is activated in *DAN*-transfectants.

4. Discussion

DAN gene product has a tumor-suppressive activity in v-*src*-transformed 3Y1 cells [2]. On the other hand, other members of DAN family possess different biological activities, i.e. *Gremlin* induces a secondary axis in *Xenopus* embryo [8] and *Cerberus* promotes the formation of head structures such as cement gland, olfactory placodes, eyes and forebrain [7]. All three members are secreted and bind BMP2 [8]. Thus, we postulated that DAN family was probably involved in controlling cell growth and differentiation by affecting signaling of TGFβ family [8]. However, the precise mechanism of action of DAN family remains to be confirmed.

In the present study, we found marked differences in TPA-induced neurite formation between *DAN*-transfected and control cells (Fig. 2). This suggested that certain changes were induced in the receptor, PKC, or its downstream pathway. Immunoblot analysis showed a complete loss of PKCα irrespective of the expression level of DAN (Fig. 1B). Some of other PKC subtypes also decreased not only in *DAN*-transfected neuroblastoma cells but also in *DAN*-transfected ras-NIH cells (Figs. 1D and 3C). Consequently, down-regulation of PKC can be one of the intrinsic changes caused by DAN.

A dramatic induction of neurite formation after treatment with TPA was observed only in *DAN*-transfected SH-SY5Y cells despite the undetectable expression level of PKCα. A possible explanation is that the loss of PKCα could result in binding of TPA to another PKC subtype, which effectively induces neurite formation. It is important to identify the PKC subtype mainly involved in neuronal differentiation. A recent report has shown that PKCα, -γ, -ε and -θ are down-regulated after treatment with TPA in lymphokine-activated killer cells [16]. Thus, PKCε, which was not reduced in *DAN*-transfected cells, might be a possible candidate. The implication of PKCε in neurite outgrowth in SH-SY5Y cells has also been suggested by Fagerstrom et al. [17].

It is conceivable that the decrease in PKCs was not induced by a reduced synthesis but by enhanced degradation since down-regulation was observed not only in PKCα but also in other PKC subtypes in *DAN*-transfected cells (Figs. 1D and 3C). It is well documented that PKC is degraded by a Ca^{2+}-activated neutral cysteine proteinase, calpain [14,15]. The involvement of calpain in PKC regulation was suggested by our results that the reduced expression level of PKCα in *DAN*-transfected cells was partly recovered by treatment with a calpain inhibitor, ZLLH (Fig. 3D). The appearance of a 150-kDa degradation product of fodrin strongly suggests that calpain was activated in *DAN*-transfected cells.

How calpain was activated in *DAN*-transfected cells remains unknown at present. Calpain activity is regulated by Ca^{2+}, dissociation of a small subunit, a specific inhibitor, calpastatin [18] and calpain activator proteins [19–21]. It is also possible that the activation of calpain was not the cause but the result of activation of PKCs. Further investigation is necessary to elucidate the mechanism of calpain activation and PKC down-regulation in *DAN*-transfected cells as well as their possible involvement in neuronal differentiation and malignant transformation.

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References

[1] Ozaki, T. and Sakiyama, S. (1993) Proc. Natl. Acad. Sci. USA 90, 2593–2597.
[2] Ozaki, T. and Sakiyama, S. (1994) Cancer Res. 54, 646–648.
[3] Ozaki, T., Nakamura, Y., Enomoto, H., Hirose, M. and Sakiyama, S. (1995) Cancer Res. 55, 895–900.
[4] Nakamura, Y., Ozaki, T., Nakagawara, A. and Sakiyama, S. (1997) Eur. J. Cancer 33, 1986–1990.
[5] Kondo, K., Ozaki, T., Nakamura, Y. and Sakiyama, S. (1995) Biochem. Biophys. Res. Commun. 216, 209–215.
[6] Nakamura, Y., Ozaki, T., Ichimiya, S., Nakagawara, A. and Sakiyama, S. (1998) Biochem. Biophys. Res. Commun. 243, 722–726.
[7] Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E.M. (1996) Curr. Opin. Genet. Dev. 3, 641–647.
[8] Hsu, D.R., Economides, A.N., Wang, X., Eimon, P.M. and Harland, R.M. (1998) Mol. Cell 1, 673–683.
[9] Ciccarone, V., Spengler, B.A., Meyers, M.B., Biedler, J.L. and Ross, R.A. (1989) Cancer Res. 49, 219–225.
[10] Sekiya, T., Fushimi, M., Hori, H., Hirohashi, S., Nishimura, S. and Sugimura, T. (1984) Proc. Natl. Acad. Sci. USA 81, 4771–4775.
[11] Saido, T.C., Yokota, M., Nagao, S., Yamaura, I., Tani, E., Tsuchiya, T., Suzuki, K. and Kawashima, S. (1993) J. Biol. Chem. 268, 25239–25243.
[12] Hiwasa, T., Ma, J., Ike, Y., Katunuma, N. and Sakiyama, S. (1995) Cell Biochem. Funct. 13, 293–296.
[13] Enomoto, H., Ozaki, T., Takahashi, E., Nomura, N., Tabata, S., Yakahashi, H., Ohnuma, N., Tanabe, M., Iwai, J., Yoshida, H., Matsunaga, T. and Sakiyama, S. (1994) Oncogene 9, 2785–2791.
[14] Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T. and Nishizuka, Y. (1989) J. Biol. Chem. 264, 4088–4092.
[15] Tsubuki, S., Saito, Y., Tomioka, M., Ito, H. and Kawashima, S. (1996) J. Biochem. 119, 572–576.
[16] Ohmi, Y., Ohta, A., Sasakuma, Y., Sato, N., Yahata, T., Santa, K., Habu, S. and Nishimura, T. (1997) Biochem. Biophys. Res. Commun. 235, 461–464.
[17] Fagerstrom, S., Pahlman, S., Gestblom, S. and Nanberg, E. (1996) Cell Growth Differ. 7, 775–785.
[18] Saido, T.C., Sorimachi, H. and Suzuki, K. (1994) FASEB J. 8, 814–822.
[19] Yamaguchi, M. and Nishina, N. (1995) Mol. Cell. Biochem. 148, 67–72.
[20] Melloni, E., Michetti, M., Salamino, F. and Pontremoli, S. (1998) J. Biol. Chem. 273, 12827–12831.
[21] Dale, L., Howes, G., Price, B.M. and Simth, J.C. (1992) Development 115, 573–585.