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FGF1 nuclear translocation is required for both its neurotrophic activity and its p53-dependent apoptosis protection

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ABSTRACT

Fibroblast growth factor 1 (FGF1) is a differentiation and survival factor for neuronal cells both *in vitro* and *in vivo*. FGF1 activities can be mediated not only by paracrine and autocrine pathways involving FGF receptors but also by an intracrine pathway, which is an underestimated mode of action. Indeed, FGF1 lacks a secretion signal peptide and contains a nuclear localization sequence (NLS), which is consistent with its usual intracellular and nuclear localization. To progress in the comprehension of the FGF1 intracrine pathway in neuronal cells, we examined the role of the nuclear translocation of FGF1 for its neurotrophic activity as well as for its protective activity against p53-dependent apoptosis. Thus, we have transfected PC12 cells with different FGF1 expression vectors encoding wild type or mutant (Δ NLS) FGF1. This deletion inhibited both FGF1 nuclear translocation and FGF1 neurotrophic activity (including differentiation and serum-free cell survival). We also show that endogenous FGF1 protection of PC12 cells against p53-dependent cell death requires FGF1 nuclear translocation. Strikingly, wild type FGF1 is found interacting with p53, in contrast to the mutant FGF1 deleted of its NLS, suggesting the presence of direct and/or indirect interactions between FGF1 and p53 pathways. Thus, we present evidences that FGF1 may act by a nuclear pathway to induce neuronal differentiation and to protect the cells from apoptosis whether cell death is induced by serum depletion or p53 activation.

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1. Introduction

Fibroblast growth factor 1 (FGF1), one of the 22 members of the FGF family, is a multipotent factor involved in proliferation, differentiation and cell survival [1–3]. It is widely distributed in the adult peripheral and central nervous systems. FGF1 expression increase during neuronal development and a high level of FGF1 is detected in various adult neuronal cell types [4–6]. For example, all neuronal layers of the retina express FGF1 and this expression matches the sequential differentiation of the different layers [6–8] suggesting a direct role of FGF1 in the retina development. Indeed, suppression of FGF1 expression by antisense oligonucleotides in embryonic chick retinal cells inhibits neuronal differentiation and survival [9]. FGF1 is a differentiation and survival factor for various neuronal cells both *in vitro* and *in vivo* [9–14].

FGF activities are usually mediated in cells by autocrine and/or paracrine pathways involving FGF receptors (FGF-R) with tyrosine kinase activities. Exogenous FGF (externally added FGF) binds to FGF-R, thus promoting the phosphorylation and activation of these receptors, which initiates various intracellular signaling pathways, such as Ras/MAP kinases, PI3K/AKT and PLC-γ pathways [15]. However, FGF1 lacks a secretion signal peptide and contains a nuclear localization sequence (NLS), consistent with being an intracellular and a nuclear localization in most of the cell-types tested [16]. Further studies of endogenous FGF1 activities have revealed the presence of an FGF1 intracrine pathway involved in the proliferation, differentiation and survival of different cell-types [3,9,12,13,16–19].

In PC12 cells, we have previously shown that during the differentiation process induced by exogenous FGF, endogenous FGF1 and FGF2 expression increases [12]. We also demonstrated that overexpression of FGF1 by transfection is sufficient to induce differentiation and serum-free survival of these cells by a mechanism independent of the MAP kinases pathway. Recently, we have shown that exogenous FGF1 protected PC12 cells from p53-dependent apoptosis [14]. FGF1 decreased phosphorylation, nuclear translocation and transcriptional activities of the tumor suppressor p53 following DNA damage induced by etoposide treatment. In the present study, we examined the role of the nuclear translocation of

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FGF1 for its neurotrophic activity as well as for its protective activity against p53-dependent apoptosis. For that purpose, we stably transfected PC12 cells with different FGF1 expression vectors encoding wild type FGF1 or mutant FGF1 deleted for the nuclear translocation sequence KKPK. In PC12 cells, we showed that this deletion inhibits FGF1 nuclear translocation and FGF1 neurotrophic activity including differentiation and cell survival in the absence of serum. We also showed that protection of PC12 cells against p53-dependent cell death by endogenous FGF1 requires nuclear translocation of this neuroprotective protein. The deletion of the NLS sequence dramatically decreases wild type FGF1 effects, namely a decrease of p53 stabilization and phosphorylation, an inhibition of both p53-dependent trans-activation of *puma* and caspase activation and, the interaction of FGF1 with p53. This study suggests that FGF1 may act through a nuclear pathway both to induce neuronal differentiation and to protect cells from apoptosis, whether the cell death signal is serum depletion or p53 activation.

2. Materials and methods

2.1. Cell cultures and drugs

PC12 cells, a rat pheochromocytoma derived cell line [20], were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 5% horse serum (HS), 100 μg/ml penicillin, 100 U/ml streptomycin and 1% Glutamax at 37 °C in a humidified atmosphere of 5% CO_2 as previously described [12,14]. PC12 cells transfected with inducible FGF1 expression vectors (pLK-FGF1-140, pLK-FGF1-134 and pLK-FGF1-127) were maintained in DMEM supplemented with 10% fetal calf serum and 5% horse serum which were both depleted in glucocorticoid by fixation onto 1% charcoal vegetal Norit pulver (Prolabo) and 0.1% dextran T70 (Pharmacia). In transfected cells, FGF1 expression was induced by 5×10^{-7} M dexamethasone (Tebu). Etoposide (50 μg/ml, Sigma, E1383) was used to induce p53-dependent apoptosis.

2.2. FGF1 eukaryotic expression vectors

Wild type and mutant FGF1 coding regions were subcloned into a glucocorticoid-responsive vector (pLK), which contained a MMTV-LTR promoter variant and the neomycin resistance gene [21]. We had previously cloned the sequence coding for human FGF1 (aa 21 to 154) in this vector (pLK-FGF1-134) and isolated stable transfected PC12 cells with this vector [12]. We also cloned the sequence coding for human FGF1 (aa 15 to 154) in the pLK vector (pLK-FGF1-140), as a control. To examine the effect of FGF1 nuclear translocation, we cloned the sequence coding for a mutant human FGF1 (aa 28 to 154) deleted of its putative nuclear localization signal (KKPK, aa 23 to 26) in the pLK vector (pLK-FGF1-127).

2.3. PC12 cells transfection

PC12 cells were transfected with 10 μg of the different vectors (pLK-Neo, pLK-FGF1-140, pLK-FGF1-134, pLK-FGF1-127) and 60 μl of Lipofectin reagent (Invitrogen) in 100-mm diameter Petri dishes as previously described [12]. Two days after transfection, the cells were trypsinized and replated in four 100-mm diameter Petri dishes with selection medium (0.5 mg/ml geneticin in culture medium). After 15 days of selection, geneticin resistant colonies appeared whatever the expression vector used. For each transfection, two plates were maintained in selection medium to isolate stable clones for further analyses. The other two plates were treated with 5×0^{-7} M dexamethasone in selection medium to induce FGF1 expression. After 10 days of treatment, cell morphology was observed by phase contrast microscopy. The number of non-differentiated clones (composed of cells with no neuritis extension or extensions smaller than the size of the soma) and differentiated clones (composed of cells extending neuritis longer than the size of the cell body) were quantified for each expression vector. This experiment was performed twice with similar results. To simplify the text and figures, PC12 cells transfected by pLK-Neo, pLK-FGF1-140, pLK-FGF1-134 and pLK-FGF1-127 were named Neo, WTα, WTβ and TM, respectively.

2.4. Serum-free cell survival analysis

PC12 cell lines (PC12, Neo, WTα, WTβ and TM) were plated on poly-L-lysine-coated 12-multiwell plates at a density of 10^5 cells/ml in low serum medium (0.8 % FCS, 0.4% HS) with 5×10^{-7} M dexamethasone. After 4 days, the cells were cultured in serum-free medium in presence of 5×10^{-7} M dexamethasone for 1–2 weeks. Cell viability was estimated using the crystal violet method (0.1% crystal violet, 0.1 M citric acid) after different times of treatment (from 0 to 15 days). The percentage of surviving cells, corresponding to the proportion of viable attached cells (with intact nuclear staining), was expressed as a percentage of the initial population (present at the beginning of the serum-free culture). Cell morphology was observed by phase contrast microscopy and pictures were taken after different times of serum-free culture.

2.5. p53-dependent apoptosis analysis

PC12 cell lines (PC12, Neo, WTα, WTβ and TM) were plated in 12-multiwell plates at a density of 7×10^4 cells/ml with 5×10^{-7} M dexamethasone. When the cells reached 70% confluence, 50 μg/ml etoposide was added to the medium to induce p53-dependent apoptosis (as previously described [14]). Cell viability was estimated using the crystal violet method (0.1% crystal violet, 0.1 M citric acid) after 24 to 48 h of etoposide treatment. The percentage of surviving cells, corresponding to the proportion of viable attached cells (with intact nuclear staining), was expressed as a percentage of the initial population (present at the beginning of etoposide treatment).

2.6. Immunocytochemistry

Transfected PC12 cells (Neo, WTα, WTβ and TM) were plated onto poly-L-lysine-coated Lab-Tek chamber slides (Nunc) at a density of 5×10^4 cells/ml in the presence or absence of 5×10^{-7} M dexamethasone. After seven days of treatment, cells were fixed with 4% paraformaldehyde, incubated with anti-FGF1 (1:100, polyclonal anti-human FGF1, serum I4, gift from Lisa Oliver [22]) or with anti-NF160 (1:200, monoclonal anti-Neurofilament 160 kDa, clone NN18, Sigma) in 0.3% Triton X-100, 1% milk PBS for 1 h. The antigen-antibody complexes were amplified with anti-rabbit or anti-mouse Ig biotinylated antibodies (1:100, Amersham) and then revealed with Extravidin-TRITC conjugate (1:100, Sigma). Immunoreactive cells were observed with a Leitz's microscope and photographed.

2.7. Nuclear and cytoplasmic fractions extraction

PC12 cell lines (Neo, WTα, WTβ and TM) were cultured for 3–5 days in the presence of 5×10^{-7} M dexamethasone. At 80% confluence, cells were lysed in 400 μl of cold buffer (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) in the presence of proteases inhibitors (1 mM AEBSF-Boehringer). After 15 minutes of incubation on ice, 25 μl of 10 % NP-40 were added to the samples. After 10-second vortexing, samples were centrifuged for 30 s at 2000 g. The supernatant (cytoplasmic fraction) was then harvested. The pellet (nuclear fraction) was rinsed with 1 ml of PBS and resuspended in 150–300 μl of PBS. Proteins (1–20 μg) of cytoplasmic and nuclear fractions were analyzed by Western blotting.

2.8. Western blot analysis

PC12 cell lines (PC12, WTα and TM) were plated in 60-mm dishes in the presence of 5×10^{-7} M dexamethasone. At 70% confluence, cells were incubated with 50 μg/ml etoposide and dexamethasone. After different treatment times (0, 8 and 16 h), cells were harvested, lysed and frozen at −20 °C. Proteins (20–40 μg) from etoposide-treated cell lysates or from nuclear and cytoplasmic fractions (see previous paragraph) were electrophoresed in NuPAGE 4-12 % Bis-Tris polyacrylamide gels (Invitrogen) and transferred onto PVDF membranes (Millipore). Blots were incubated with the primary antibody (see below) overnight at 4 °C, and further incubated for 1 h with peroxidase-conjugated antibodies (Biosystem). The primary antibodies used were rabbit polyclonal anti-FGF1 (1:500, AB-32-NA, R&D Systems), mouse monoclonal anti-p53 (1:100, Pab 122, gift from Dr. E. May, IRSC, Villejuif, France), rabbit polyclonal anti-p53-P (Ser-15) (1:200, Santa Cruz), goat polyclonal anti-PUMAα (1:200, N-19, Santa Cruz), rabbit monoclonal anti-cleaved Caspase-3 (1:1000, Asp175, Cell Signaling), rat monoclonal anti-Tubulin (1:500, MAS078, Sera-Lab) and rabbit polyclonal anti-Enolase (1:500, Santa Cruz). IgG peroxidase-conjugated secondary antibodies were detected by ECL or ECLplus kits depending on signal intensity (Amersham).

2.9. Immunoprecipitation analysis

PC12 cell lines (PC12, WTα and TM) were plated in 100-mm dishes in the presence of 5×10^{-7} M dexamethasone. At 70% confluence, cells were incubated with 50 μg/ml etoposide and dexamethasone. After 16 h treatment, cells were harvested, pelleted and resuspended in lysis/immunoprecipitation buffer (250 mM NaCl, 1% NP40, 50 mM Hepes [pH 7], 5 mM EDTA, 0.5 mM DTT) supplemented with Protease Inhibitor Cocktail (AEBSF, Roche). Cells were incubated for 30 min on ice prior homogenization. Prior to immunoprecipitation, 1.2 μg anti-p53 (Pab 122, gift from Dr. E. May, IRSC, Villejuif, France) or anti-GFP (clones 7.1 and 13.1, Roche) were coupled with protein G magnetic beads O/N at 4 °C. Protein G

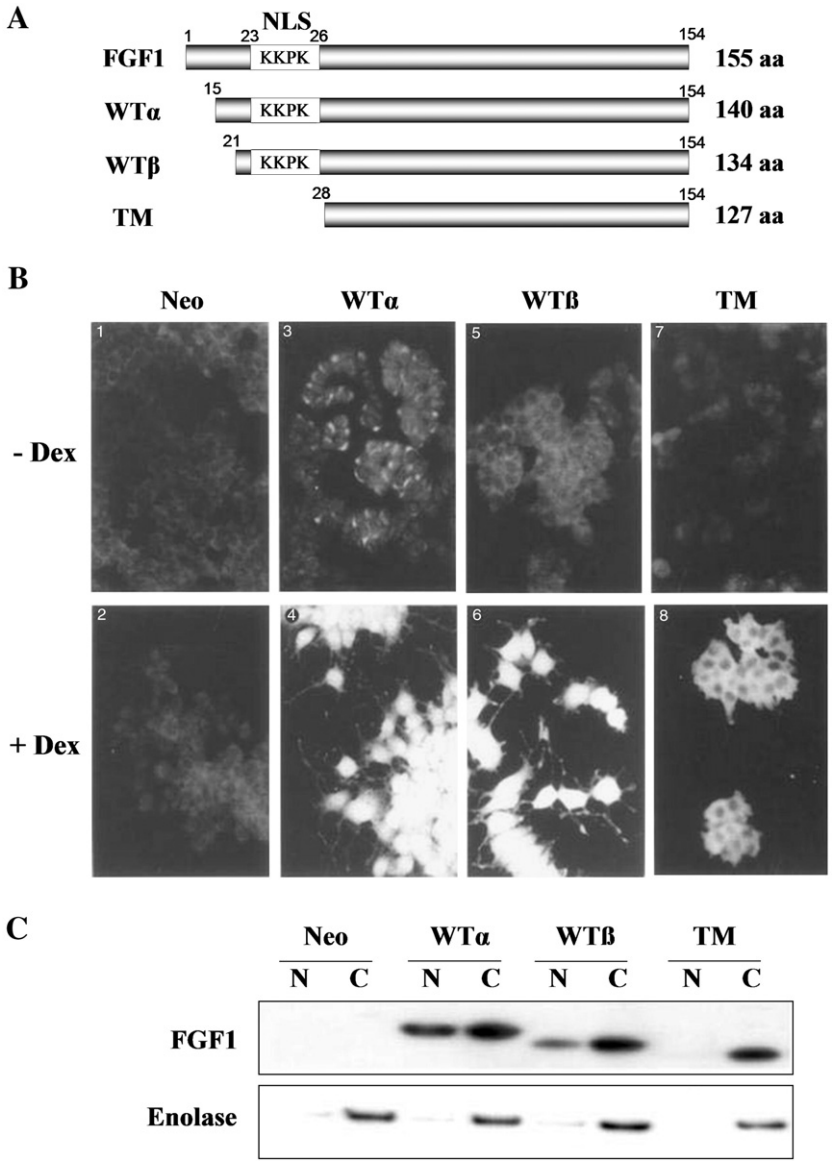

Fig. 1. Subcellular localization of FGF1. (A) PC12 cells were transfected with glucocorticoid-responsive vectors pLK-Neo, pLK-FGF1-140, pLK-FGF1-134, pLK-FGF1-127 and stable clones were isolated and named Neo, WTα, WTβ and TM, respectively. (B) Transfected PC12 cell lines were cultured in the absence or presence of 5×10^{-7} M dexamethasone for seven days. FGF1 expression and localization was analyzed by immunocytochemistry using a polyclonal anti-FGF1 antibody (I4). (C) Transfected PC12 cell lines were cultured in the presence of 5×10^{-7} M dexamethasone for 3–5 days. Nuclear and cytoplasmic proteins were extracted and analyzed by Western blot for FGF1 and Enolase detection in the different fractions of the different cell lines.

magnetic beads coupled with anti-GFP were used as a control to assess the background of aspecific binding proteins. Then, cell extracts were incubated with protein G magnetic beads (pre-coupled with anti-p53 or anti-GFP) for 4 h at 4 °C. After 5 washes with 0.1% PBS-Tween, the immunoprecipitated proteins were eluted from the magnetic beads by boiling in loading buffer (0.1 M DTT, 25% NuPage LDS Sample Buffer, Invitrogen, diluted in PBS) for 5 min before Western blot analysis.

3. Results

To determine the role of the nuclear localization of FGF1 for its neurotrophic activity, we transfected PC12 cells with different FGF1 expression vectors encoding wild type FGF1 or a mutant FGF1 that is deleted for the nuclear localization signal KKPK (aa 23 to 26). For that purpose, we subcloned into a glucocorticoid-responsive vector (pLK) the coding sequence of wild type FGF1s (aa 15 to 154 and aa 21 to 154) and of the nuclear translocation mutant FGF1 (aa 28 to 154) to isolate PC12 stable transfected cell lines respectively named WTα, WTβ and TM (Fig. 1A). The two wild type forms (WTα and β) correspond to the two major forms of FGF1 (140 and 134 aa) which can be extracted from tissues and which are currently used as recombinant factors for *in vitro* experiments. As a control, the pLK-Neo vector was transfected to isolate PC12 Neo cell lines. Different stable clones were isolated for each vector. The expression level and subcellular localization of FGF1 were analyzed by immuno-cytochemistry (Fig. 1B) and by Western blot after nuclear

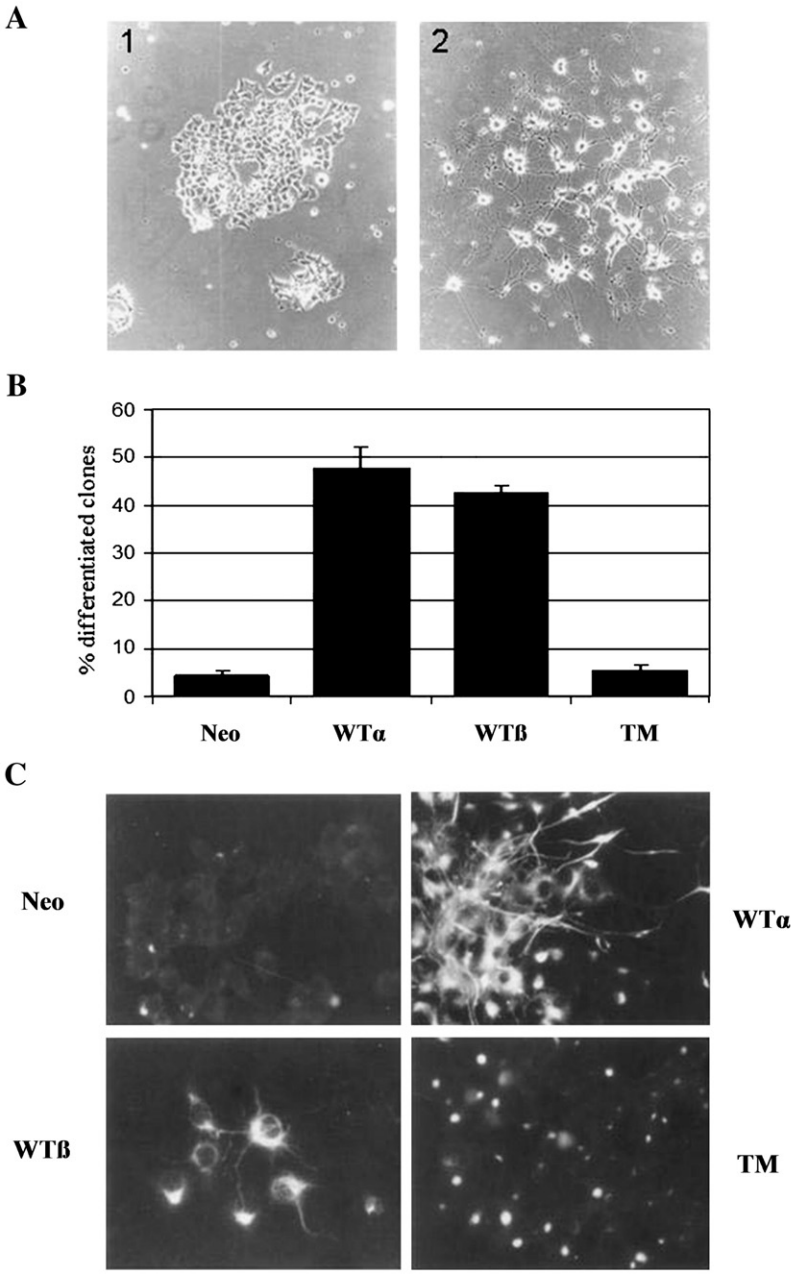

Fig. 2. PC12 cell differentiation. (A, B) Neo, WTα, WTβ and TM transfected PC12 cells were treated with 5×10^{-7} M dexamethasone in selection medium to induce FGF1 expression. After 10 days of dexamethasone treatment, cell morphology was observed and the number of non differentiated clones (A,1) and differentiated clones (A,2) were quantified for each expression vector (B). This experiment was performed twice with similar results. (C) Stable transfected PC12 cell lines (Neo, WTα, WTβ and TM) were cultured in the presence of 5×10^{-7} M dexamethasone for 7 days. The presence of Neurofilament 160 kDa, a differentiation marker, was analyzed by immunocytochemistry using a monoclonal anti-NF160 (clone NN18).

and cytoplasmic fractionation (Fig. 1C). In control cells (Neo), as previously described for native PC12 cells, the level of rat endogenous FGF1 was too low to be detected by immunocytochemistry or western blotting without heparin sepharose purification [12]. In WTα, WTβ and TM transfected PC12 cells, FGF1 (wild type and mutant) was only detected in presence of dexamethasone (which induced MMTV-LTR promoter of pLK vectors). It can be noticed that wild type and mutant FGF1 are never detected in the conditioned medium of unstressed or stressed PC12 cells even after heparin sepharose concentration suggesting that these factors were actually not secreted (data not shown). In WT (α or β) cells, FGF1 presented both nuclear and cytoplasmic localization (Fig. 1B, C). In contrast, in TM cells mutated FGF1 was principally detected in the cytoplasmic compartment. This showed that deletion of the NLS (KKPK) significantly decreases FGF1 nuclear translocation.

We then tested the differentiation activities of FGF1 (WT and mutant) on a large population of PC12 transfected clones (about 200 clones per expression vector) after induced FGF1 expression by the addition of 5×10^{-7} M dexamethasone for 10 days. Cellular morphology was observed by phase contrast microscopy (Fig. 2A). The number of non-differentiated clones (containing cells with no neuritis extension or extensions smaller than cell size) and differentiated clones (containing cells with neurites longer than cell size) were quantified for each transfected expression vector. The expression of wild type FGF1 (WTα or β) induced the differentiation process in 40 to 50% of the transfected clones, in contrast to mutant FGF1 (TM), which presented a basal level of differentiation similar to control PC12 cells (Neo) (Fig. 2B). This result was confirmed by analysis of stable transfected cell lines. Only wild type FGF1 (WT α or β) induced the differentiation of PC12 cells as detected by cell morphology analysis and by immunocytochemistry detection of a neuronal marker, expression of Neurofilament 160 kDa (NF160) (Fig. 2C). FGF1 mutant transfected cell lines (TM), similarly to control cell lines (Neo), presented neither a differentiated phenotype nor NF160 expression. Therefore, deletion of the nuclear translocation signal significantly decreases the differentiation activity of FGF1.

In an attempt to determine the pro-survival activity of nuclear FGF1 in serum-free PC12 cell cultures, the different transfected cell lines were cultured without serum (Fig. 3). After 11 days, PC12 cells overexpressing the wild type FGF1 (WTα, WTβ) still survived in serum-free media and presented a differentiated morphology, in contrast to mutant-FGF1-transfected cells (TM) and control cells (Neo). Thus, in the absence of serum wild type FGF1 (WTα and WTβ) protects PC12 cells from cell death and the deletion of nuclear localization signal strongly decreases this protection, suggesting that FGF1 nuclear translocation is required for its neurotrophic activity.

We recently showed that exogenous FGF1 (added to the culture medium) protects PC12 cells from p53-dependent apoptosis [14]. In the present study, we examined the activity of endogenous FGF1 (wild type and translocation mutant) in this cell death process. p53-dependent apoptosis was induced by etoposide, a DNA damaging agent as previously described [14] in both native and stably transfected PC12 cell lines (Neo, WTα, WTβ and TM). After 28 h of etoposide treatment, cell survival was analyzed (Fig. 4A). Overexpression of wild type FGF1 (WTα and WTβ) protected PC12 cells from p53-dependent apoptosis, in contrast to mutant FGF1 (TM). Therefore, nuclear localization of FGF1 is important for the protection activity of FGF1 against p53-dependent apoptosis.

We further examined the status of p53 (accumulation and phosphorylation) in the different cell lines (PC12, WTα and TM). Etoposide led to the increase of p53 protein level in PC12 cells and to p53 phosphorylation on serine 15 (Fig. 4B), a post-translational modification that was previously correlated with an increase of p53 stability and transcriptional activity [14,23]. Wild type FGF1 (WTα) prevented p53 accumulation and phosphorylation induced by

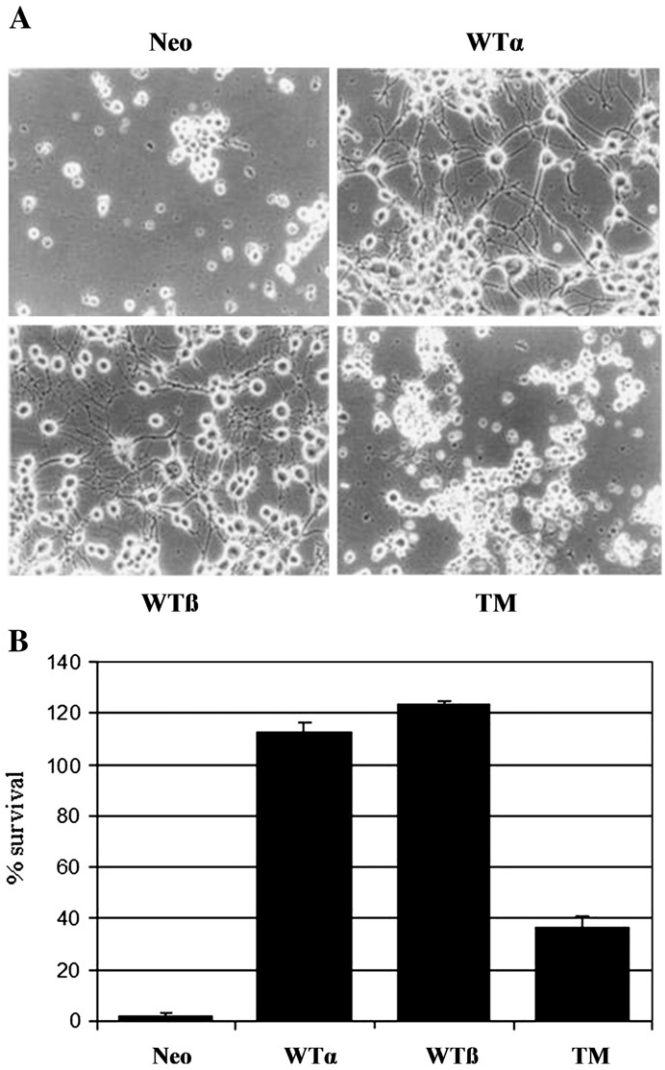

Fig. 3. Serum-free cell survival. Stable transfected PC12 cell lines (Neo, WTα, WTβ and TM) were cultured in presence of 5×10^{-7} M dexamethasone in serum-free medium for eleven days. Cell morphology was observed by phase contrast microscopy (A) and cell survival was quantified by crystal violet nuclei staining (B).

etoposide. In the mutant-FGF1-transfected PC12 cells (TM), etoposide induced intermediate levels of p53 and p53-P, suggesting that the deletion of the nuclear localization sequence partially inhibits FGF1 activity on p53 accumulation and phosphorylation. In these cell lines, we then examined p53-dependent transactivation of *puma*, which encodes a pro-apoptotic BH3-only member of the Bcl-2 family. In mutant-FGF1-transfected cells (TM), like in native PC12 cells, etoposide treatment induced an increase of the levels of Puma (Fig. 4C). However this increase was significantly reduced in wild type-FGF1-transfected cells (WTα). This result suggests that FGF1 nuclear localization is required for this factor to inhibit p53-dependent trans-activation of *puma*. Then, we examined by Western blot the cleavage of caspase-3, as a marker of the activation of the cascade of caspases, in the different cell lines after etoposide treatment (Fig. 4C). In mutant-FGF1-transfected cells (TM), like in native PC12 cells, etoposide treatment induced the cleavage and activation of caspase-3, however no cleaved caspase-3 can be detected in wild type-FGF1-transfected cells (WTα). Thus, wild type FGF1 inhibited p53-dependent activation of caspases and protected the cells from apoptosis, while mutant FGF1 deleted for its nuclear translocation sequence did not. Last, we explored whether p53 and FGF1 could interact by immunoprecipitation

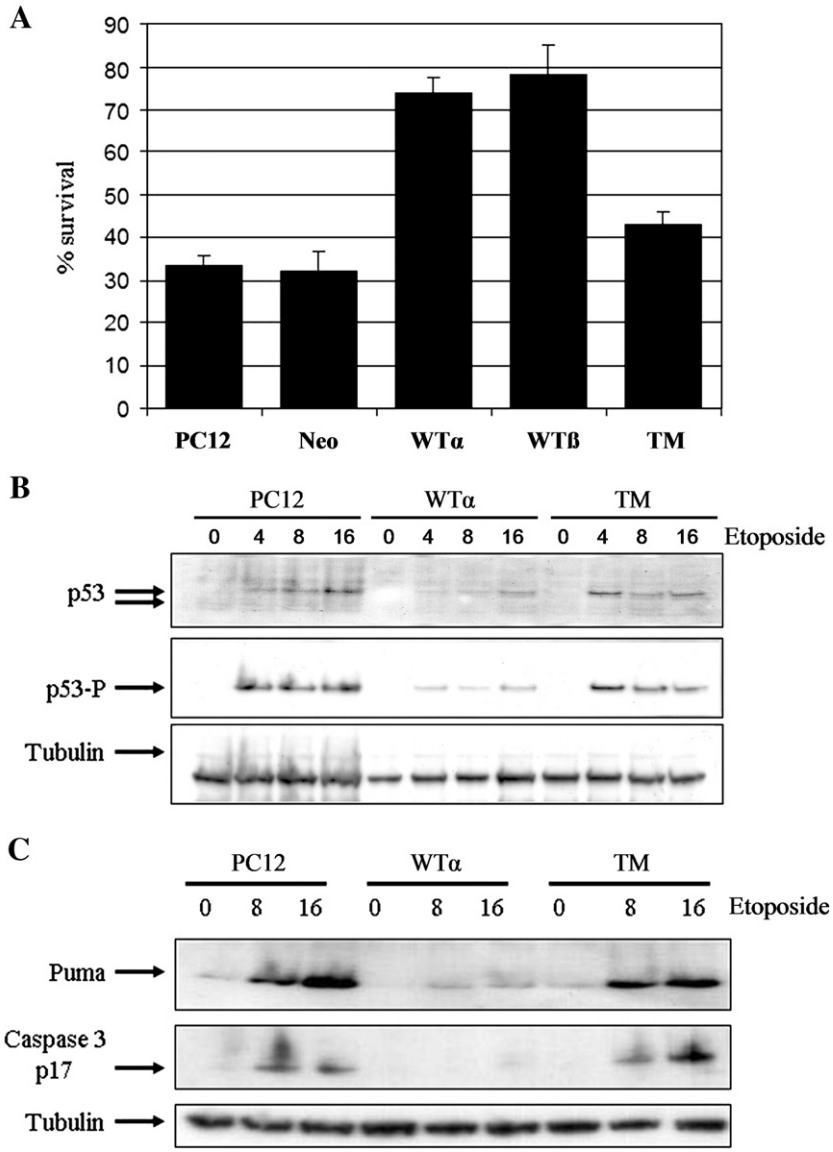

Fig. 4. p53-dependent apoptosis. Native PC12 cells and stable transfected PC12 cell lines (Neo, WTα, WTβ and TM) were cultured in the presence of 5×10^{-7} M dexamethasone. Subconfluent cultures were treated with 50 μg/ml etoposide to induce p53-dependent apoptosis. (A) Cell viability after 28 h of etoposide treatment was quantified using the crystal violet method. (B) Western blot analysis of p53 and p53-P (Ser-15) protein levels in PC12, WTα and TM cells treated with etoposide for 0, 8 and 16 h. (C) Western blot analysis of Puma and cleaved caspase-3 (p17) protein levels in PC12, WTα and TM cells treated with etoposide for 0, 8 and 16 h. Tubulin was used as a control. Each result is representative of three independent experiments.

experiments (Fig. 5). In native PC12 cells and both mutant and wild type FGF1 transfected cell lines in presence of etoposide, p53 was immunoprecipitated with anti-p53 antibody but not with anti-GFP antibody which was used as a negative control. In absence of etoposide, the level of p53 is very low and the presence of the IgG just upstream p53 hardens the detection of the oncosuppressor. We then tested whether FGF1 co-immunoprecipates with p53 in the three cell lines. As shown in Fig. 5, wild type FGF1 co-immunoprecipitated with p53 in the presence and in the absence (to a lesser extend) of etoposide in contrast to mutant FGF1. In native PC12 cells, the level of endogenous FGF1 is too low to detect any interaction between p53 and FGF1. These data suggest that p53 interacts with FGF1 only in the nuclear compartment. However, we cannot exclude that the deletion of the NLS could modify the structure or conformation of FGF1, thus affecting its ability to interact with p53. All our data strongly suggest that nuclear localization of FGF1 is required to inhibit the p53-dependent apoptotic process by direct or indirect interactions.

4. Discussion

We previously reported that endogenous FGF1 is a neurotrophic factor for PC12 cells which induces both neuritis extension and survival to serum starvation [12]. Recently, we showed that exogenous FGF1 (added in the culture medium) protects PC12 cells from p53-dependent apoptosis [14]. In the present study, we present evidence that endogenous FGF1, which is localized in both nuclear and cytoplasmic compartments, protects PC12 cells from p53-dependent apoptosis. These data suggest that FGF1 induces PC12 cells differentiation as well as survival after serum depletion or p53 activation by an intracrine pathway. To progress in the comprehension of this pathway and to determine the role of FGF1 nuclear localization, we tested different truncated forms of FGF1 (with or without NLS). WTα and β correspond to the two major forms of FGF1 (140 and 134 aa) which can be extracted from tissues and which are currently used as recombinant factor for *in vitro* experiments. Both wild type forms presented the same nuclear and cytoplasmic

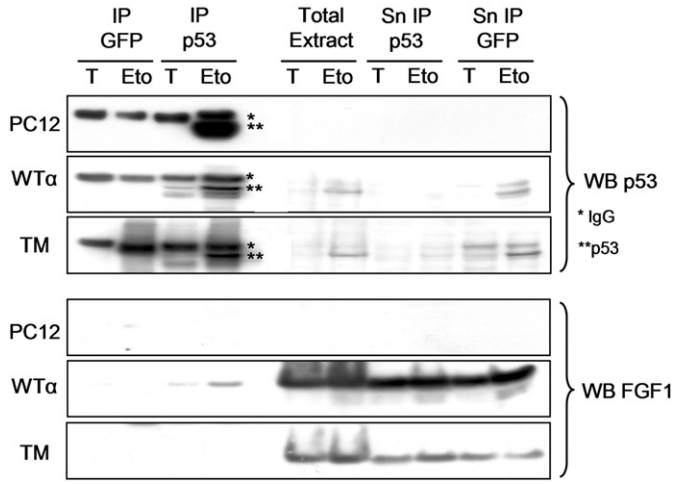

Fig. 5. Wild type FGF1 interacts with p53. Native and stably transfected PC12 cell lines (WTα and TM) were cultured in the presence of 5×10^{-7} M dexamethasone and in absence or presence of etoposide (16 h). Total cell extracts were subjected to immunoprecipitation using anti-p53 or anti-GFP precoupled with Protein G Magnetic Beads. Immunoprecipitated complexes (IP p53) were analyzed for the presence of p53 and FGF1 by immunoblotting. Equivalent amounts of anti-GFP immunoprecipitated complexes (IP GFP), the IP supernatants (Sn IP p53 and Sn IP GFP) and the total cell extracts were loaded as controls.

localization and the same activity in all experiments performed in this study. The first 14 residues (propeptide deleted in FGF1-140, WTα) or 20 residues (deleted in FGF1-134, WTβ) of the coding sequence of FGF1 are not required for FGF1 neurotrophic activity and FGF1 protection against p53-dependent apoptosis. By contrast, deletion of the first 27 residues (FGF1-127, TM) modified both the subcellular localization and the activity of this factor. The sequence deleted in FGF1 TM by comparison to FGF1 WTβ (only seven residues) contains the NLS (KKPK, aa 23 to 26) previously characterized by Imamura et al. [16]. In PC12 cells, FGF1 TM was principally detected in the cytoplasmic compartment and was unable to induce differentiation of the PC12 cells as well as to protect them from serum depletion or p53-dependent apoptosis, in contrast to wild-type forms of FGF1. Deletion of the KKPK NLS inhibits FGF1 nuclear translocation both in PC12 cells (of neuroendocrine cell type) and in 3T3 cells (murine fibroblasts, [16]) suggesting that KKPK-dependent FGF1 nuclear translocation process is neither species nor tissue specific. Wesche and collaborators [24] characterized a second putative NLS in the FGF1 coding sequence located near its C-terminus. This second NLS is a bipartite NLS consisting of clusters of lysines separated by a spacer of 10 amino acids. Both NLSs (N-terminal NLS1 and C-terminal bipartite NLS2) are able to target GFP to the nucleus when expressed in HeLa cells. Mutation in either of the NLS impaired nuclear translocation of exogenously added FGF1 in NIH3T3 cells. However, only the double mutation (NLS1 and NLS2) decreases FGF1 efficiency in stimulating DNA synthesis. In PC12 cells, deletion of only NLS1 impaired FGF1 nuclear translocation as well as its neurotrophic and p53-dependent protection activities. We cannot exclude that NLS2 could participate to FGF1 localization and activity in these cells. However, our results suggest that NLS1 is required to FGF1 nuclear translocation and activities in PC12 cells while NLS2 is not sufficient for a nuclear localization in theses cells.

The activity of this mutant FGF1 was initially examined by Imamura et al. [16]. In endothelial and fibroblast cells, addition of exogenous FGF1 TM (named HBGF-1U in [16]) induced neither DNA synthesis nor cell growth in contrast to FGF1 WTβ (named HBGF-1α), suggesting that FGF1 nuclear translocation is required for both activities. However, both FGF1 TM and WTβ could induce the phosphorylation of FGF1 receptors and stimulate *c-fos* expression, suggesting that these two activities were insufficient for the stimulation of DNA synthesis. In murine 3T3 cells, overexpression of FGF1 TM, but not FGF1 WTβ overexpression, failed to trigger translocation of FGF1 to the nuclear compartment and to induce a transformed phenotype [16]. More recently, two laboratories examined the activity of this mutant FGF1 *in vitro* and *in vivo* [25,26]. *In vitro*, mutant FGF1 (named aFGF$^{28\text{-}154}$ in [25]) added in the culture medium did not induce NIH3T3 or PC12 cell proliferation, as assessed by MTT experiments, in contrast to wild type FGF1. With dexamethasone-induced mouse thymocyte apoptosis models developed *in vitro* and *in vivo*, the recombinant mutant FGF1 conserved its anti-apoptotic activity, which was slightly increased compared to wild type FGF1. *In vivo*, this mutant FGF1 reduced apoptosis in rat gut epithelium after ischemia-reperfusion injury [25] and partially protected from N-methyl-N-nitrosourea (MNU)-induced rat retinal degeneration [26]. For both studies, *Escherichia coli* recombinant wild type or mutant FGF1 were added in the culture medium for *in vitro* studies or were injected for *in vivo* studies involving the receptor-dependent pathway and/or internalization by endocytosis process before intracrine and/or nuclear pathway implication. In PC12 cells, we have previously shown that overexpression of endogenous wild type FGF1 induced differentiation and serum-free survival *via* an intracrine pathway independent of FGF receptors and the MAP kinase cascade [12]. Thus, in the present study, we focused our research on the potential alternative nuclear pathway and compared activity of endogenously expressed wild type and mutant FGF1. We showed that overexpression of FGF1 TM in PC12 cells failed to translocate to the nuclear compartment and to induce differentiation, serum-free survival and protection against p53-dependent apoptosis in contrast to both WTα and WTβ FGF1s. It suggests that FGF1 nuclear translocation is required for its neurotrophic activity and to inhibit p53-dependent apoptosis. In transfected PC12 cells, whatever the form (wild type or mutant), whatever the conditions of cultures (control or etoposide treated), we never detected FGF1 in the culture medium even after heparin sepharose concentration suggesting that this factor (WT or mutant) is not secreted and did not act via a receptor-dependent process. It has been reported that the structure of TM FGF1 could be less stable than WT FGF1. However, a study of the Guimenez-Gallego laboratory examined the three dimensional structure of this mutant (named FGF1$^{127\text{-}154}$) and concluded that the structure of this mutant could be even more stable than the wild type protein and is similar to that of the wild type except at site 1 of interaction with the cell membrane receptor [27]. In PC12 cells, we never detected any significant difference in expression or stability of TM FGF1 compared to WT FGF1. In serum-free media, TM FGF1 slightly increased PC12 cell survival. This result led us to the hypothesis that either FGF1 could partially act in the cytoplasmic compartment or that a small part of FGF1 can passively diffuse in the nuclear compartment to inhibit serum-free apoptosis, even in absence of its NLS domain. We have previously shown by Enzyme Immuno-Assay [12] that a four fold increase of endogenous FGF1 is enough to induce serum-free survival. In PC12 cells transfected with pLK expression vector, the level of FGF1 is about ten to hundred fold increased when compared to native endogenous FGF1 levels. Thus, if only a small part of TM FGF1 could passively enter the nucleus, it would be enough to partially protect the cells. All these data suggest that the difference of activity of TM FGF1 for cell survival between our study and the Fu et al. report [25] could result not only from differences in cell type (PC12 / thymocytes) but more probably from different modes of action of exogenously added FGF1 and endogenously translated FGF1.

In rat embryonic fibroblasts, FGF1 inhibits both p53-dependent apoptosis and cell growth arrest through an intracrine pathway [18]. In these cells, overexpression of FGF1 WTβ blocks the p53 pathway at two levels: (i) FGF1 increases MDM2 expression, which is associated with an acceleration of p53 degradation; (ii) FGF1 decreases p53-dependent transactivation of the pro-apoptotic gene *bax*. In PC12 cells, addition of exogenous WTα FGF1 in presence of heparin (which

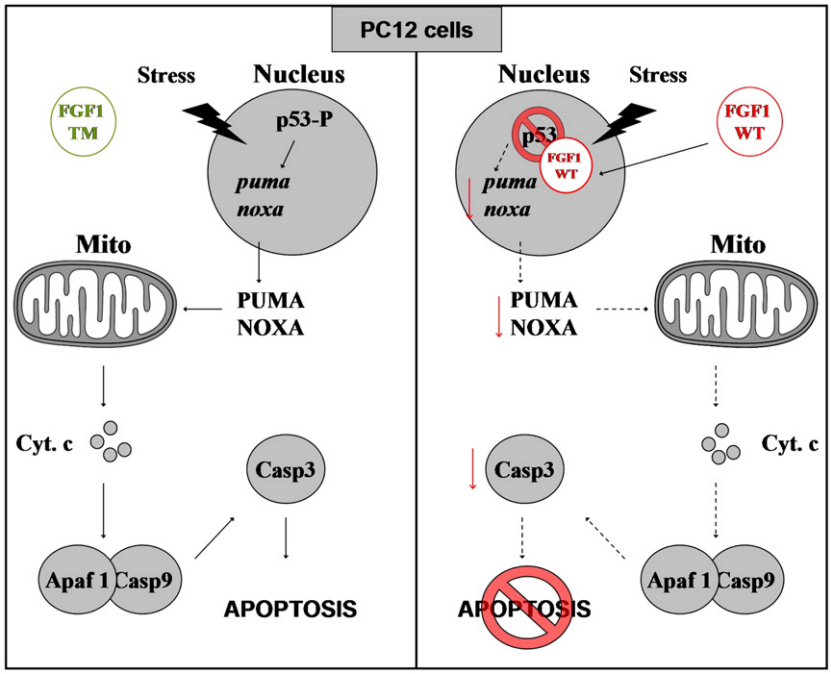

Fig. 6. Nuclear FGF1 inhibits p53-dependent apoptosis. Left panel: in native PC12 cells and in FGF1 TM transfected PC12 cell lines, DNA damages (stress) induce p53 phosphorylation and p53-dependent transactivation of proapoptotic genes like *puma* and *noxa*. Then, the BH3-only members of Bcl-2 family (Puma and Noxa) activate mitochondrial membranes depolarization, mitochondrial cytochrome *c* release in the cytosol and the formation of the apoptosome (cytochrome *c*, Apaf-1 and Procaspase-9). Finally, the apoptosome initiates the caspase cascade (caspase-9 and caspase-3 cleavage and activation) and cell death process. Right panel: in FGF1 WT (α and β) PC12 transfected cell lines, FGF1 WT can be translocated in the nuclear compartment and interacts with p53, in contrast to FGF1 TM (left panel). Nuclear FGF1 WT inhibits p53 phosphorylation and p53-dependent *puma* transactivation after DNA damages. This inhibition results in a decrease of the mitochondrial apoptotic pathway and in the absence of caspase-3 cleavage. Only nuclear FGF1 inhibits p53-dependent apoptosis.

activates endogenous FGF1 expression) or overexpression of either WTα or β FGF1s inhibits p53-dependent apoptosis at different levels by direct and/or indirect interactions ([14] and the present study, Fig. 6). In PC12 cells, we have shown that: (i) in presence of DNA damages, FGF1 decreases phosphorylation of p53 on its serine 15 (which is associated with p53 stabilization and trans-activation properties); (ii) FGF1 decreases p53-dependent trans-activation of the pro-apoptotic genes such as *puma* and *noxa*; (iii) FGF1 decreases mitochondrial depolarization and caspase cascade activation (caspase-9 and -3); (iv) FGF1 nuclear translocation is required for these different activities; and last (v) only wild type FGF1 co-immunoprecitates with p53. Our results on fibroblasts and PC12 cells suggest that FGF1 may block the p53 pathway at multiple levels in different cell types (fibroblasts versus neuroendocrine cells). Nevertheless, all of FGF1 actions lead to a decrease of both p53 stability and p53-dependent transactivation of pro-apoptotic genes. Our data also suggest that direct and/or indirect interactions between p53-dependent and FGF1-dependent pathways are taking place in the nuclear compartment. In this study, we present for the first time evidences that FGF1 nuclear translocation is required for its neurotrophic and survival activities. We also present the first evidence of interaction between p53 and FGF1 by immunoprecipitation experiment. Most of p53-interacting proteins previously characterized are involved in the regulation of the stability and/or the transcriptional activity of p53 (MDM2, kinases, acetylases, transcriptional factors, …) [28,29]. However, some p53-interacting proteins could be involved in transcriptional-independent activities of p53 (Bax, Bak, Bcl-2, Bcl-X_L, Puma, …) [30,31]. Only wild type FGF1 is found interacting with p53 suggesting that FGF1 interacts with p53 in the nuclear compartment to decrease its stability and its transcriptional activity.

This study must be pursued to progress in the comprehension of the nuclear activities of FGF1 and the relevance of the interaction between p53 and FGF1. In PC12 cells, FGF1 could be extracted with nucleoplasmic proteins associated with DNA (personal data) as the transcription factor p53 is. Is the interaction between FGF1 and p53 at the transcriptional level? Could FGF1 act as a transcription factor or co-factor? Wiedlochka and collaborators [19] presented evidences that FGF1 is phosphorylated by PKCδ in the nuclear compartment. Is this phosphorylation regulating the activity of this factor in the nucleus and/or its interaction with p53? In the next few years, answering these different questions will be required to progress in the characterization of the FGF1 intracrine pathway which is involved in major cellular events such as proliferation, differentiation and cell survival.

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