# Ligand-binding specificity of human fibroblast growth factor receptor-3 IIIc

Hsien-Yi Lin[1,a], June Kaplow[c], Michael Jaye[c], Michael J. Hayman[b,*]

[a] *Graduate Program in Molecular Biology and Biochemistry, State University of New York at Stony Brook, Stony Brook, NY 11794-5222, USA*
[b] *Department of Molecular Genetics and Microbiology, State University of New York at Stony Brook, Stony Brook, NY 11794-5222, USA*
[c] *Rhône-Poulenc Rorer Central Research, 500 Arcola Road NW14, Collegeville, PA 19426, USA*

Received 1 May 1997; revised version received 6 June 1997

**Abstract Earlier studies indicated that human fibroblast growth factor receptor (FGFR)-3 IIIc was activated equally well by both FGF-1 and FGF-2. In contrast, murine FGFR-3 IIIc was preferentially activated by FGF-1. To address this issue, we determined the ligand-binding specificity of human FGFR-3 IIIc in comparison with human FGFR-1 IIIc. By equilibrium binding human FGFR-3 IIIc preferentially bound FGF-1 with high affinity, whereas FGFR-1 IIIc bound both FGF-1 and -2 with high affinity. By competition binding using FGF-1, -2, -4, or -6, FGF-1 competed more efficiently than the other FGFs. These results suggest that like the murine FGFR-3 III, FGF-1 is a preferred ligand for human FGFR-3 IIIc.**



*Key words:* Ligand binding; Fibroblast growth factor; Fibroblast growth factor receptor

## 1. Introduction

Fibroblast growth factors (FGF) are a family of polypeptide mitogens consisting of at least nine members (FGF-1–9) [1,2]. FGFs are expressed in specific temporal and spatial patterns and are involved in developmental processes, angiogenesis, wound healing, and tumorigenicity. FGFs bind to high-affinity receptors on cell surface and activate signal transduction pathways. The cloning of FGF receptors identified four different genes encoding the FGFR family of receptor tyrosine kinases (FGFR-1–4) [3–5]. Alternatively spliced forms of FGFR-1, -2, and -3 have been identified. The alternative splicing may affect the ligand-binding specificity, biological activity, or localization of the receptors. Heparin or cell-surface heparan sulfate proteoglycans (HSPG) serve as low-affinity receptors and potentiate the binding and function of FGFs [6–8].

Signals from ligand-activated FGFRs appear to control differentiation as well as proliferation. Mutations in these receptors have indicated that they may control the differentiation of specific cell types during development. Point mutations of the genes encoding human FGFR-1, -2, or -3 have been shown to cause different syndromes that involve defects in bone development [9,10]. In particular, point mutations that activate FGFR-3 [11–13] may cause dwarfism such as achondroplasia [14,15], hypochondroplasia [16,17] or thanatophoric dysplasia [18,19]. To address the underlying mechanism behind these syndromes, it is necessary to understand the role that FGFR-3 can play in normal human development. In order to do this, it is necessary to identify which of the FGF family members can serve as physiologically relevant ligands for this receptor.

Using a calcium efflux assay performed with microinjected *Xenopus* oocytes, our previous studies showed that human FGFR-3 IIIc can be activated with similar efficiencies by both FGF-1 and -2 [20]. However, it was subsequently reported that mouse FGFR-3 IIIc preferentially binds FGF-1 over FGF-2 [21]. There are three possibilities for the discrepancy between these two studies. The first possibility is that the calcium efflux assay may not be able to distinguish the difference in affinities of FGF-1 and -2 to human FGFR-3 IIIc. The second possibility is that these results may reflect species-specific difference between human and mouse FGFR-3 IIIc. The third possibility is that in different cell types, different composition of cell surface HSPGs may affect the binding affinity of FGF-2 to FGFR-3 IIIc. To distinguish between these possibilities, we have determined the ligand-binding specificity of human FGFR-3 IIIc in comparison with FGFR-1 IIIc using L6 cells engineered to express FGFR-3 IIIc or -1 IIIc. We found that, like mouse FGFR-3 IIIc, human FGFR-3 IIIc also preferentially bound FGF-1 with a high affinity.

## 2. Materials and methods

### 2.1. Cell culture and transfection of L6 cells

Rat L6 skeletal muscle myoblasts and L6-derived transfectant lines were grown in DMEM (Life Technologies, Grand Island, NY) with 8% fetal calf serum. Before subcloning of the human FGFR-3 IIIc cDNA [20] into an expression vector, the sequences upstream of the start codon were removed to increase the efficiency of translation. Full-length FGFR-3 IIIc cDNA was subcloned into the *Hin*dIII site of the retroviral vector pLNCX [22]. This placed the receptor directly downstream of cytomegalovirus immediate-early promoter element. The constructed plasmid was named pLhR3-5.

Transfection of L6 cells was carried out by using the calcium phosphate method. The cells were seeded at $3\times10^6$ cells per 10-cm dish 1 day before transfection. On the day of transfection, the DNA solution containing 20 μg DNA, 125 mM calcium chloride, and 1× BBS (BES-buffered saline, 50 mM BES, pH 6.95, 280 mM NaCl, and 1.5 mM Na2HPO4) was added slowly to the cells dropwise. The cells were incubated with the DNA overnight at 37°C in a 3% $CO_2$ incubator. Two days after transfection, we split the cells and selected G418-resistant clones with complete media containing 500 μg/ml of Geneticin (Life Technologies). Two weeks after transfection, G418-resistant colonies were picked with cloning cylinders. Expression of FGFR-3 IIIc in individual clones was determined by Western blot with the anti-FGFR-3 monoclonal antibody 8.34.

*Corresponding author. Fax: (516) 632-8891.
E-mail: hayman@asterix.bio.sunysb.edu

[1] *Present address:* Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014, USA.


*PII* S0014-5793(97)00739-4

*2.2. Antibodies*

The anti-FGFR-1 antisera flg-2B were raised against the peptide SSGEDSVFSHEPLPEE, the human FGFR-1 C-terminal penultimate sequence. The anti-FGFR-3 monoclonal antibody 8.34 was raised against bacterially expressed polypeptides encompassing amino acids 94–255 of the extracellular domain of human FGFR-3.

*2.3. Western blot*

For Western blot, the cells were lysed in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 50 mM NaF, 1 mM $Na_3VO_4$, 5 mM benzamidine, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40), clarified, and the protein concentrations in the lysates were determined by BCA method (Pierce, Rockford, IL). Aliquots of the lysates (75 μg) of each sample were mixed with sample buffer, boiled, and fractionated on an SDS-7.5% polyacrylamide gel. Protein was transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by electroblotting. Non-specific binding sites were blocked with blocking buffer (3% BSA in PBS, 1 mM EDTA, 1 mM sodium vanadate) for 1 h at room temperature. Membranes were then incubated with the appropriate anti-FGFR antiserum diluted in blocking buffer for 1 h at room temperature. Membranes were washed with PBST (PBS, 0.1% Tween 20) and subsequently incubated with anti-mouse Ig- or anti-rabbit Ig- horseradish peroxidase-linked whole antibody (Amersham, Arlington Heights, IL) at a dilution of 1:2500 in PBS plus 5% non-fat dry milk, and reactivity was determined by the ECL chemiluminescence reaction (Amersham).

*2.4. Growth factors and radio-iodination*

Recombinant human FGF-2 was a kind gift of D. Moscatelli (New York University, NY) or purchased from Promega (Madison, WI). Recombinant human FGF-4 was kindly provided by C. Basilico (New York University, NY). Recombinant human FGF-6 was kindly provided by F. Coulier (Institut National de la Sante et de la Recherche Medicale, Marseille, France). Recombinant human FGF-1 and FGF-2 were radio-iodinated using the chloramine T method. The specific activity of the radiolabeled ligand was determined by radio-isotope dilution using the respective unlabeled competitor. The specific activity of [$^{125}$I]FGF-1 was 750 000 cpm/ng and the specific activity of [$^{125}$I]FGF-2 was 130 000 cpm/ng.

*2.5. Binding of FGF*

The binding assay was performed as follows. Fibronectin-coated 24-well plates (Collaborative Research) containing $1\times10^5$ cells/well were placed on ice, and the wells were rinsed twice with cold binding buffer. Plates were incubated on ice with 1 ml of ice-cold binding buffer for 20 min, followed by 2 h at 4°C with serial dilutions of the [$^{125}$I]FGF in cold binding buffer. Non-specific binding was obtained using the same serial dilutions but in the presence of 20 μg/ml of non-radioactive FGF-1. After incubation, the cells were placed on ice, rinsed once with ice-cold binding buffer, twice with ice-cold PBS, and then solubilized in 0.25 ml of 1 N NaOH at 37°C for 15 min. When [$^{125}$I]FGF-2 was used, the cells were placed on ice after incubation, rinsed once with binding buffer, once with PBS, once with 2 M NaCl in 20 mM HEPES, pH 7.5, and then solubilized in 0.25 ml of 1 N NaOH at 37°C for 15 min. The lysates were counted in a γ-counter.

For competition of the binding of [$^{125}$I]FGF-1 with non-radioactive FGFs, we used 10 ng/ml of [$^{125}$I]FGF-1 with serial dilutions of non-radioactive FGFs, and followed the procedure as described above.

## 3. Results

*3.1. Expression of FGFRs in L6 cells*

To determine the ligand binding specificity of human FGFR-3 IIIc, we transfected cDNAs encoding either FGFR-3 IIIc or -1 IIIc into L6 cells. We used human FGFR-1 IIIc as a control since its ligand binding characteristics are well known. L6 cells were chosen because they do not express any endogenous FGFRs. Cell lines stably expressed FGFR-3 or -1 were selected as described in Section 2. The selected cloned were screened by Western blot analysis with receptor-specific antibodies for FGFR-1 and -3. Fig. 1 shows the expression of FGFR-1 and -3 in two cell clones, Lflg17 and LR3-19. The receptors are detected as a 150-(FGFR-1) and a 130-kDa protein (FGFR-3). Since these two cell clones expressed roughly equivalent receptor numbers as judged by scatchard analysis (see below), they were chosen for the experiments described in this study.

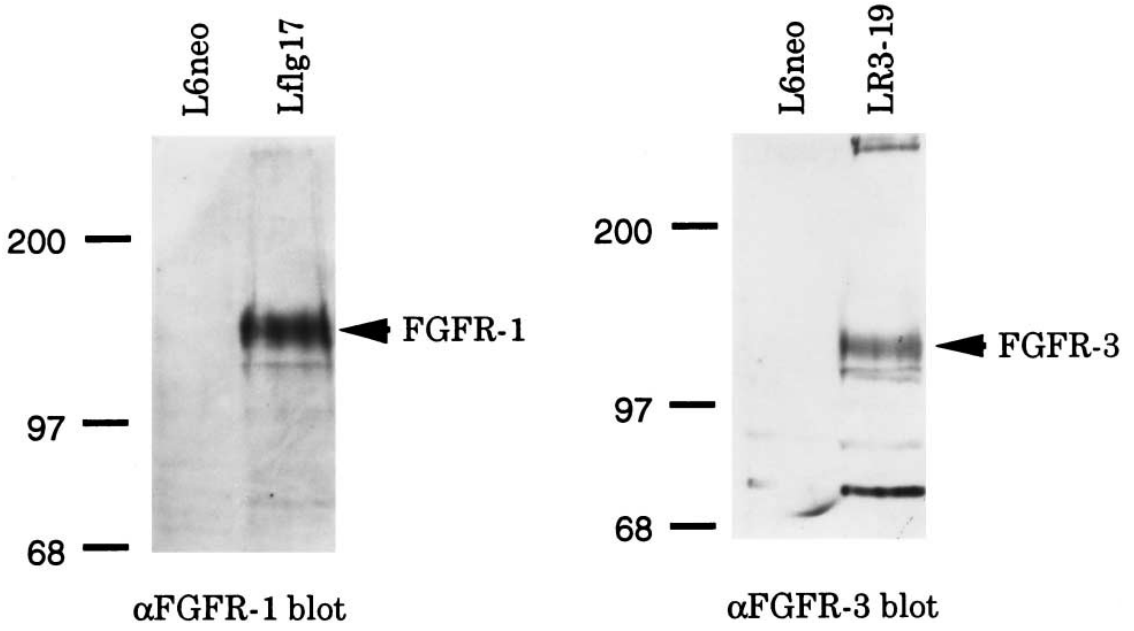


Fig. 1. Western blot analysis of FGFR-1 and -3 expression. L6neo represents L6 cells which was transfected with the expression vector alone. Lflg17 represents a clone of L6 cells which stably express human FGFR-1 IIIc. LR3-19 represents a clone of L6 cells which stably express human FGFR-3 IIIc. These three different cells were lysed. The lysates were resolved by SDS-PAGE and blotted with receptor-specific antibodies as indicated in the figure. The bands of mature forms of FGFR-1 and -3 are indicated with arrows. The lower molecular weight species are likely the immature forms of the receptors with incomplete glycosylation.

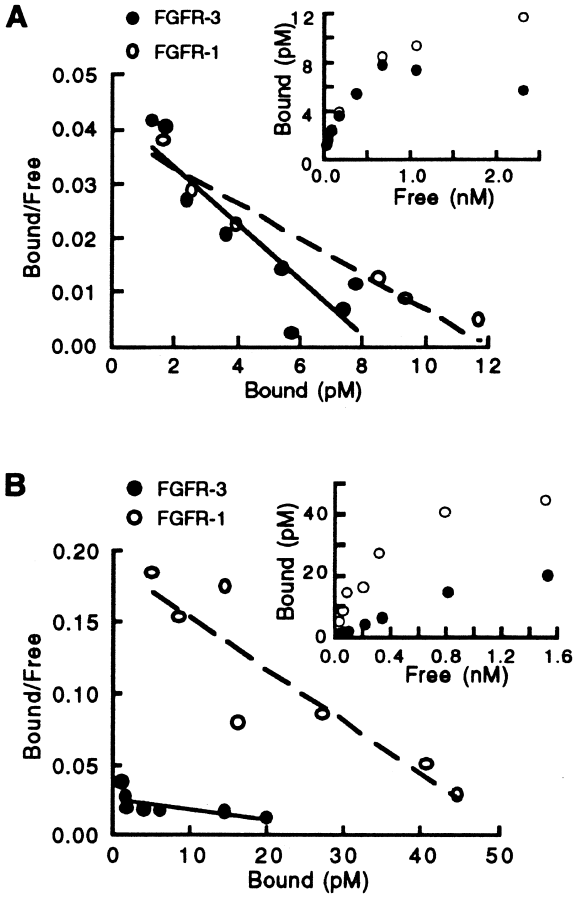


Fig. 2. Scatchard analysis of binding of [$^{125}$I]FGF-1 and [$^{125}$I]FGF-2 to L6 cells expressing FGFR-3 IIIc or -1 IIIc. This figure shows Scatchard analysis for the binding of (A) [$^{125}$I]FGF-1 and (B) [$^{125}$I]FGF-2 to FGFR-3 (●) and FGFR-1 (○) expressed in LR3-19 and Lflg22 cells, respectively.

### 3.2. FGF-1, but not FGF-2, binds to FGFR-3 IIIc with a high affinity

To determine whether FGF-1 and -2 bind to FGFR-3 IIIc with similar affinity, we performed equilibrium binding of [$^{125}$I]FGF-1 and [$^{125}$I]FGF-2 on the FGFR-3 expressing LR3-19 cells and the FGFR-1 expressing Lflg17 cells. The binding of $^{125}$I-labeled FGFs to these cells was saturatable, except for the low level binding of [$^{125}$I]FGF-2 to LR3-19 (Fig. 2, insets). The results were subsequently analyzed by Scatchard analysis (Fig. 2). The results show that FGF-1 binds to FGFR-3 with a $K_d$ of 190 pM, in contrast FGF-2 binds with very low affinity with a $K_d$ of at least 1300 pM. In contrast, control experiments demonstrated that FGF-1 and FGF-2 bound to FGFR-1 with very similar $K_d$s of 310 and 280 pM, respectively. The receptor numbers per cell on LR3-19 and Lflg22 are 39 000 and 53 000, respectively. These results suggest that FGF-1, but not FGF-2, binds to FGFR-3 IIIc with a high affinity, whereas both FGF-1 and -2 binds to FGFR-1 IIIc with high affinities.

### 3.3. FGF-1 binds to FGFR-3 IIIc with a higher affinity than FGF-2, -4, and -6

To extend this analysis we determined the relative binding affinity of different FGF family members to human FGFR-3 IIIc, using competition experiments. By competing the binding of [$^{125}$I]FGF-1 to LR3-19 with different concentrations of unlabeled FGF-1, -2, -4, or -6, we found that unlabeled FGF-1 competed for the binding most efficiently among these four FGFs, unlabeled FGF-2 and -4 competed the binding less efficiently than unlabeled FGF-1, and unlabeled FGF-6 had very little effect on competition of the binding (Fig. 3). Unlabeled FGF-1 can compete 50% of [$^{125}$I]FGF-1 binding at a concentration of 3 nM, whereas unlabeled FGF-2, -4, or -6 could not compete 50% of [$^{125}$I]FGF-1 binding at concentrations up to 100 nM (Fig. 3). These results suggest that FGF-1 binds to FGFR-3 IIIc with a higher affinity than FGF-2, -4, and -6. Thus among these FGF family members, FGF-1 was the only ligand found which bound to human FGFR-3 IIIc with a high affinity.

## 4. Discussion

The current study was undertaken to address the issue of the binding of FGF-2 to human and murine FGFR-3 IIIc. Analysis of ligand binding using L6 cells engineered to express human FGFR-3 IIIc indicated that this receptor preferentially binds FGF-1 over FGF-2. These results are in agreement with the results of ligand binding of soluble mouse FGFR-3 IIIc performed with an in vitro system [21]. Previous reports had indicated that human FGFR-3 can be activated similarly by FGF-1 and -2 in a calcium efflux assay performed with *Xenopus* oocytes microinjected with mRNA encoding the receptor [20]. The discrepancy we have noted between activation [20] and binding specificity is similar to that recently described for mouse FGFR-3 IIIc [23]. It is presently unclear why this discrepancy exists. It may reflect effects due to the expression of different heparan sulfate proteoglycans on different cell types which may influence ligand binding and receptor signaling. Although the composition of cell surface HSPGs of *Xenopus* oocytes may influence the binding affinities of FGF-1 and -2 to human FGFR-3, we feel it is more likely that this assay amy have been unable to distinguish the difference in binding affinities between FGF-1 and -2 at the high concentrations of FGF that were used in this assay in comparison to the expression levels of the receptors [20] The studies described in this report, using cell lines with defined receptor numbers, allowed a more accurate determination of ligand-binding af-

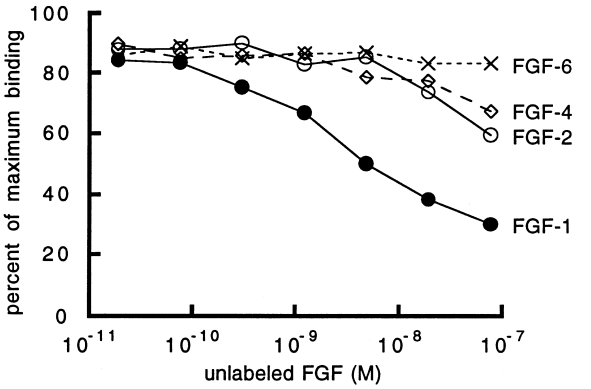


Fig. 3. Competition of Binding of [$^{125}$I]FGF-1 to FGFR-3 IIIc L6 Cells. LR3-19 cells were incubated with 0.625 nM of [$^{125}$I]FGF-1 in the presence of different concentrations of unlabeled FGF-1, -2, -4, or -6. The percent of maximum binding was calculated by dividing the counts of radioactivity measured in the presence of unlabeled ligands by the counts measured in the absence of unlabeled ligands.

finity and demonstrated that like the murine FGFR-3 IIIc receptor the human FGFR-3 IIIc receptor preferentially binds FGF-1 over FGF-2.

The results from the competition experiments showed that among unlabeled FGF-1, -2, -4, and -6, only unlabeled FGF-1 can efficiently compete the binding of [$^{125}$I]FGF-1 to FGFR-3 IIIc expressed in L6 cells. Although there are no other comprehensive studies on ligand binding by human FGFR-3, other reports have addressed some aspects of the activation of FGFR-3. For example, FGF-3 was found not to bind FGFR-3 [24,25] BaF3 cells engineered to express different splicing variants of murine FGF receptors have been used to address activation of the receptors by FGF-1–9, [23]. These studies demonstrated that the recently described FGF-8 and -9 can significantly activate mouse FGFR-3 IIIc. This raises the possibility that, in addition to FGF-1, -8 and -9 may also be physiologically relevant ligands for human FGFR-3 IIIc. This possibility can be tested when the human FGF-8 and -9 proteins become available.

FGFRs have been implicated in several human diseases, in particular those genetic disorders that effect bone development. For example, three different mutations in FGFR-3 have been described that effect dwarfism to varying degrees. All three mutations are caused by point mutations within the FGFR-3 protein which give rise to constitutively activated receptors. The severity of the disease was found to reflect the degree of activation of the receptor. In the most common form of the disease, namely achondroplasia, the receptor is only partially activated and it can still be activated further by the addition of ligand. Hence in developing a full understanding of the role of FGFR-3 in these human diseases, as well as during normal development, it is essential to identify the physiologically relevant ligands. This study was undertaken to resolve the apparent discrepancy between the binding of murine and human FGFR-3 IIIc receptors to FGF-2. The results demonstrate that like the murine FGFR-3 IIIc receptor, FGF-1 binds to human FGFR-3 IIIc receptor with a high affinity whereas FGF-2 does not. This indicates that there is no species difference between the binding abilities of the human and murine receptors and thus it is likely that studies on murine receptors can be extrapolated to the human situation. This is an important consideration in the development of murine models for the human diseases.

*Acknowledgements:* We thank Drs. C. Basilico, F. Coulier, and D. Moscatelli for their generous gifts of FGFs. This work was supported in part by Public Service Grants CA28146 and CA42573 to M.J.H.

## References

[1] Basilico, C. and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115–165.
[2] Mason, I.J. (1994) Cell 78, 547–552.
[3] Jaye, M., Schlessinger, J. and Dionne, C.A. (1992) Biochim. Biophys. Acta 1135, 185–199.
[4] Partanen, J., Vainikka, S., Korhonen, J., Armstrong, E. and Alitalo, K. (1992) Prog. Growth Factor Res. 4, 69–83.
[5] Johnson, D.E. and Williams, L.T. (1993) Adv. Cancer Res. 60, 1–41.
[6] Bashkin, P., Doctrow, S., Klagsbrun, M., Svahn, C.M., Folkman, J. and Vlodavsky, I. (1989) Biochemistry 28, 1737–1743.
[7] Moscatelli, D. (1987) J. Cell Physiol. 131, 123–130.
[8] Moscatelli, D. (1988) J. Cell Biol. 107, 753–759.
[9] Muenke, M. and Schell, U. (1995) Trend Genet. 11, 308–313.
[10] Wilkie, A.O., Morriss-Kay, G.M., Jones, E.Y. and Heath, J.K. (1995) Curr. Biol. 5, 500–507.
[11] Naski, M.C., Wang, Q., Xu, J. and Ornitz, D.M. (1996) Nature Genet. 13, 233–237.
[12] Webster, M.K. and Donoghue, D.J. (1996) EMBO J. 15, 520–527.
[13] Webster, M.K., D'Avis, P.Y., Robertson, S.C. and Donoghue, D.J. (1996) Mol. Cell Biol. 16, 4081–4087.
[14] Rousseau, F., Bonaventure, J., Legeai-Mallet, L., Pelet, A., Rozet, J.M., Maroteaux, P., Le Merrer, M. and Munnich, A. (1994) Nature 371, 252–254.
[15] Shiang, R., Thompson, L.M., Zhu, Y.Z., Church, D.M., Fielder, T.J., Bocian, M., Winokur, S.T. and Wasmuth, J.J. (1994) Cell 78, 335–342.
[16] Bellus, G.A., McIntosh, I., Smith, E.A., Aylsworth, A.S., Kaitila, I., Horton, W.A., Greenhaw, G.A., Hecht, J.T. and Francomano, C.A. (1995) Nature Genet. 10, 357–359.
[17] Prinos, P., Costa, T., Sommer, A., Kilpatrick, M.W. and Tsipouras, P. (1995) Hum. Mol. Genet. 4, 2097–2101.
[18] Tavormina, P.L., Rimoin, D.L., Cohn, D.H., Zhu, Y.Z., Shiang, R. and Wasmuth, J.J. (1995) Hum. Mol. Genet. 4, 2175–2177.
[19] Tavormina, P.L., Shiang, R., Thompson, L.M., Zhu, Y.Z., Wilkin, D.J., Lachman, R.S., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Wasmuth, J.J. (1995) Nature Genet. 9, 321–328.
[20] Keegan, K., Johnson, D.E., Williams, L.T. and Hayman, M.J. (1991) Proc. Natl. Acad. Sci. USA 88, 1095–1099.
[21] Ornitz, D.M. and Leder, P. (1992) J. Biol. Chem. 267, 16305–16311.
[22] Miller, A.D. and Rosman, G.J. (1989) Biotechniques 7, 980–982.
[23] Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G. and Goldfarb, M. (1996) J. Biol. Chem. 271, 15292–15296.
[24] Mathieu, M., Chatelain, E., Ornitz, D., Bresnick, J., Mason, I., Kiefer, P. and Dickson, C. (1995) J. Biol. Chem. 270, 24197–24203.
[25] Mathieu, M., Kiefer, P., Mason, I. and Dickson, C. (1995) J. Biol. Chem. 270, 6779–6787.