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# Modifications of the Fibroblast Growth Factor-2 Gene Led to a Marked Enhancement in Secretion and Stability of the Recombinant Fibroblast Growth Factor-2 Protein

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**Abstract** Progress in FGF-2 gene therapy has been hampered by the difficulty in achieving therapeutic levels of FGF-2 secretion. This study tested whether the addition of BMP2/4 hybrid secretion signal to the FGF-2 gene and mutation of cys-70 and cys-88 to serine and asparagine, respectively, would increase the stability and secretion of active FGF-2 protein in mammalian cells using MLV-based vectors. Single or double mutations of cys-70 and cys-88 to ser-70 and asp-88, respectively, markedly increased the amounts of FGF-2 protein in conditioned media and cell lysates, which may be due to glycosylation, particularly at the mutated asp-88 residue. Addition of BMP2/4 secretion signal increased FGF-2 secretion, but also suppressed FGF-2 biosynthesis. The combination of BMP2/4 secretion signal and double cys-70 and cys-88 mutations increased the total amount of secreted FGF-2 protein >60-fold. The modifications did not alter its ability to stimulate cell proliferation and Erk1/2 phosphorylation in marrow stromal cells or its ability to bind heparin in vitro, suggesting that the modified FGF-2 protein was functionally as effective as the unmodified FGF-2. An ex vivo application of rat skin fibroblasts (RSF) transduced with the modified FGF-2 vector in a subcutaneous implant model showed that rats with implants containing cells transduced with the modified FGF-2 vector increased serum FGF-2 level >15-fold, increased growth of the implant, and increased vascularization within the implant, compared to rats that received implants containing β-galactosidase- or wild-type FGF-2-transduced control cells. This modified vector may be useful in FGF-2 gene therapy investigations. J. Cell. Biochem. 100: 1493–1508, 2007. 

**Key words:** FGF-2, secretion; signal sequence; stability; chimera; glycosylation

Basic fibroblast growth factor (bFGF or FGF-2) is a pleiotropic regulator of the proliferation, differentiation, migration, and survival in a variety of cell types [D'Amore and Smith, 1993; Friesel and Maciag, 1995; Bikfalvi et al., 1997; Moyamoto et al., 1998]. It is also a potent stimulator of angiogenesis [D'Amore and Smith, 1993; Moyamoto et al., 1998] and hematopoiesis in vivo [Allouche and Bikfalvi, 1995]. FGF-2 is involved in organogenesis [Martin, 1998], vascularization [D'Amore and Smith, 1993], and wound healing [Ortega et al., 1998], and plays an important role in the differentiation and/or function of various organs, including the nervous system [Ortega et al., 1998], the skeleton [Montero et al., 2000], and several other organs [Bikfalvi et al., 1997]. Thus, FGF-2 has received considerable attention for potential clinical applications, including wound healing and tissue repair.

The therapeutic utility of the FGF-2 protein has been assessed in various animal models with promising results. Administration of recombinant FGF-2 protein improved the healing of ischemic wounds in rats [Quirinia and Viidik, 1998], promoted scar-less healing of skin incisional wounds in normal rats [Spyrou and Naylor, 2002; Akasaka et al., 2004], enhanced wound healing in healing-impaired diabetic rats [Takeuchi et al., 1997], and accelerated the wound healing of chick

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embryo chorioallantoic membrane [Ribatti et al., 1999]. Administration of FGF-2 protein also promoted fracture healing in the monkey [Kawaguchi et al., 2001], improved cartilage repair in the rabbit [Tanaka et al., 2004], stimulated early stages of tendon healing in the rat [Chan et al., 2000], and formed new trabeculae that physically connect with pre-existing trabeculae in osteopenic rats [Lane et al., 2003a,b]. Subcutaneous implantation of controlled-release FGF-2 protein into the back of mice resulted in de novo formation of adipose tissue [Tabata et al., 2000]. However, the results of FGF-2 therapy via gene transfer have been mixed. While ex vivo FGF-2 gene therapy promoted collateral vessel development in a rabbit hind limb ischemia model [Ishii et al., 2004] and improved blood flow and cardiac function in a swine myocardial ischemia model [Ninomiya et al., 2003], in vivo FGF-2 gene therapy did not improve survival of rat ischemic myocutaneous flaps [Hijjawi et al., 2004] or preserve functional responses to photoreceptor in a rat retinal degeneration model [Spencer et al., 2001].

Unlike most extracellular growth factors, FGF-2 lacks a classical secretion signal sequence and its extracellular secretion is mediated by an energy-dependent, non-ER/golgi pathway [Mignatti et al., 1992; Florkiewicz et al., 1995; Dahl et al., 2000]. This export mechanism is inefficient and the amount of FGF-2 released into the extracellular fluid is extremely low and highly inconsistent [Moscatelli et al., 1986]. In addition, the FGF-2 protein exists in various molecular forms through intra- and/or intermolecular disulfide formation. The disulfide formation causes conformational changes, significant loss of biological activities, and protein instability [Iwane et al., 1987]. The inefficient FGF-2 secretion, along with the formation of FGF-2 disulfide complexes, may explain the inconsistent biological effects of FGF-2 gene therapies. Previous attempts to increase FGF-2 secretion by adding to the FGF-2 gene a classical secretion signal sequence of FGF-4 [Sohn et al., 2001], IL-2 [Sasada et al., 1991], or growth hormone [Blam et al., 1988], each only slightly improved the secretion of FGF-2 chimeric proteins in COS-7 cells. Mutation of the second and third of the four cysteines (i.e., cys-70 and cys-88 (which are referred to herein as C2 and C3, respectively)) to serine and asparagine, respectively, markedly enhanced the stability of the recombinant protein without affecting its biological activity [Seno et al., 1988; Sasada et al., 1991]. The cysteine mutations did not appear to enhance FGF-2 secretion [Seno et al., 1988; Sasada et al., 1991]. An earlier attempt to combine the IL-2 secretion signal sequence with mutation of the key cysteines also led only to a four- to fivefold increase in secretion [Seno et al., 1988].

Our past studies have suggested that certain classical secretion signal sequences are more effective than others in directing protein secretion. For example, the replacement of the signal sequence of BMP-4 with the BMP-2/4 hybrid secretion signal sequence enhanced the secretion of the BMP-4 chimera in normal and transformed cells by >150-fold [Peng et al., 2001]. Accordingly, this study sought to evaluate the hypothesis that addition of the BMP2/4 secretion signal sequence along with the C2 and C3 mutations would increase the total amounts of FGF-2 protein secreted by mammalian cells through gene transfer approach.

## MATERIALS AND METHODS

### Human FGF-2 Expression Plasmids

The full-length human FGF-2 cDNA was cloned by PCR amplification using purified RNA of normal human skin fibroblasts as the template. Briefly, sense and antisense primers were designed to obtain the entire open reading frame: sense primer: 5′-gcg cgc aag ctt G*TG GCA GCC GGG AGC ATC AC-3′; antisense primer: 5′-gcg gct gacGGC CAT TAA AAT CAG CTC TT-3′. *Hind*III and *Sal*I restriction sites (underlined) were added to the sense and antisense primer, respectively, to facilitate its cloning into pFLAGCMV-1 cloning vector. The sense primer contains the first six codons of the human FGF-2 gene (identified by capital letters) with the start codon for methionine (AUG) mutated to valine (GUG) (indicated by the asterisk). This mutation was introduced so that a secretion signal sequence could be added. The antisense primer corresponds to the 3′-end of the open reading frame, including the termination codon (identified by capital letters). A single, 500-bp PCR product was obtained and its identity was confirmed by restriction mappings. The PCR product containing the full-length FGF-2 cDNA was digested with *Hind*III/*Sal*I, and subcloned into the pFLAGCMV-1

cloning plasmid (Eastman Kodak, Rochester, NY) to produce the pFC-FGF-2 plasmid vector. To enhance FGF-2 translation, an optimized Kozak sequence (italized letters) was added to the full-length human FGF-2 cDNA. The 27 nucleotide sequence corresponding to 5′-UTR region (bold letters) and a *Pst*I restriction site (underlined) were added to the 3′ end to facilitate subsequent cloning into the VR1012 cloning vector. This was accomplished by PCR amplification using the following sense primer: 5′-aaa *ctg cag* **GGG ATC CCG GCC GGG CCC CGC AGG** ATG GCA GCC GGG AGC ATC AC-3′ and antisense primer: 5′-gcg gct gac GGC CAT TAA AAT CAG CTC TT-3′. The purified PCR product was digested with *Pst*I and *Sal*I restriction enzymes and subcloned into *Pst*I/*Sal*I-digested VR1012 expression vector to generate the FGF-2 plasmid vector, VR1012-bFGF.

### MLV-Based FGF-2 Expression Vectors

To produce an MLV-based FGF-2 vector (pY-FGF), the FGF-2 gene from VR1012-bFGF was excised by *Not*I and *Xho*I digestion, blunt-ended, and subcloned into *Bam*HI-cleaved and blunt-ended MLV-based retroviral expression vector as described previously [Peng et al., 2001]. The secretion signal sequence of the BMP2/4 hybrid gene [Hammonds et al., 1990] was then subcloned into the 5′ end of the FGF-2 gene to produce the pY-BMP-FGF expression vector. The start codon for methionine was removed during the insertion of the BMP2/4 signal sequence. The amino acid sequence of the BMP-FGF junction is shown in Figure 1.

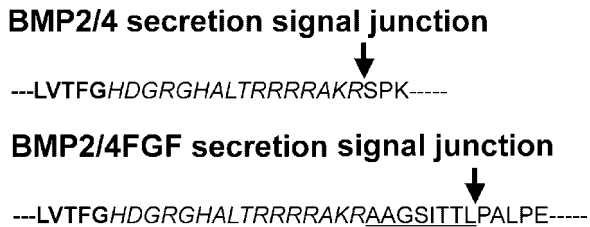


**Fig. 1.** Amino acid sequence surrounding the mature peptide cleavage site of the BMP2/4 chimera (**top**) and that surrounding the mature peptide cleavage site of the BMP2/4-FGF-2 chimera (**bottom**). The last five amino acid residues of the C-terminus of the 284-residue BMP-2 secretion signal sequence are shown in bold letters. The last 16 residues of the C-terminal end of the BMP-4 secretion signal sequence that had been incorporated in the BMP2/4 hybrid sequence are shown as italic letters. The entire 9-residue propeptide of FGF-2 (minus the starting methionine) was underlined. The respective arrow denotes the cleavage site for mature BMP-4 and FGF-2 protein, respectively. In the design of our BMP2/4-FGF-2 chimeric gene, the start codon for methionine of the FGF-2 gene was deleted and replaced with the BMP2/4 secretion signal sequence.

### PCR-Based Site-Directed Mutagenesis

The QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate C2 and C3 FGF-2 mutants. Briefly, five pairs of complementary primers (shown in Table I) were synthesized: the first pair was for the C2S mutation, the second pair for the C3S mutation, the third pair for the C3N mutation, and the fourth and fifth pairs were for the C2A and C3A mutations, respectively. The PCR-based, site-directed mutagenesis procedure was carried out according to the recommendation of the vendor using the pY-FGF or pY-BMPFGF

**TABLE I. The List of Primer Sets Used in the PCR-Based Site-Directed Mutation of the Two Key Cysteine Residues of Human FGF-2**

| Mutation | Primer | Sequence[a] |
|---|---|---|
| C2S mutation | Sense | 5′-TCTATCAAAGGAGTGTCTGCTAACCGTTACCTG-3′ |
| | Antisense | 5′-CGAGTAACGGTTAGCAGACACTCCTTTGATAGA-3′ |
| C3S mutation | Sense | 5′-TTACTGGCTTCTAAATCTGTTACGGATGAGTGT-3′ |
| | Antisense | 5′-ACACTCATCCGTAACAGATTTAGAAGCCAGTAA-3′ |
| C3N mutation | Sense | 5′-TTACTGGCTTCTAAAAATGTTACGGATGAGTGT-3′ |
| | Antisense | 5′-ACACTCATCCGTAACATTTTTAGAAGCCAGTAA-3′ |
| C2A mutation | Sense | 5′-TCTATCAAAGGAGTGGCTGCTAACCGTTACCTG-3′ |
| | Antisense | 5′-CGAGTAACGGTTAGCCGACACTCCTTTGATAGA-3′ |
| C3A mutation | Sense | 5′-TTACTGGCTTCTAAAGCTGTTACGGATGAGTGT-3′ |
| | Antisense | 5′-ACACTCATCCGTAACCGATTTAGAAGCCAGTAA-3′ |

[a]The underlined codon indicates the site of the mutation. For C2S mutation, the cys-70 codon (TGT) was mutated to a serine codon (TCT). For the C3S mutation, the cys-88 codon (TGT) was mutated to a serine codon (TCT). For the C3N mutation, the cys-88 codon was mutated to an asparagine codon (TCT). For C2A mutation, the cys-70 codon (TGT) was mutated to an alanine (GCT). For the C3A mutation, the cys-88 codon (TGT) was mutated to an alanine codon (GCT).

parental plasmids as the template. For double mutations, the mutation at the C2 and C3 was introduced sequentially. The resulting products were confirmed by DNA sequencing.

### MLV-Based Vector Production

The VSV-G pseudotyped MLV-based vectors were generated by transient transfection in 293T cells as described previously [Peng et al., 2001]. Briefly, a 10-cm plate of 293T cells was transfected with a mixture of 20 μg of retroviral expression vector (pY-FGF or its derivatives), 10 μg of MLV-GP expression vector, and 1 μg of VSV-G expression vector by $CaPO_4$ precipitation. The conditioned medium (CM) containing the viral vectors was collected 48 h after the transfection. The viral titer was determined by the end-point dilution assay for the marker gene (β-gal or eGFP) expression or by real-time PCR assay for FGF-2 transgene expression in HT1080 cells.

### FGF-2 Assays

Briefly, HT1080 cells or primary rat skin fibroblasts (RSF) in six-well plates were transduced with 200 μl of the test MLV-based vectors for 8 h and the transduction was repeated for two times. The transduced cells were then cultured in fresh medium for an additional 48–96 h. The CMs were collected and frozen immediately until assay. The cell layers were lysed with 500 μl of 1× Reporter lysis buffer (Promega, Madison, WI), and the cell lysates were kept frozen until assay. The amount of the FGF protein in cell lysates and CMs was determined with an ELISA kit (R&D systems, Minneapolis, MN). The amount of FGF-2 protein was normalized to ng of FGF-2 per $10^6$ cells.

The identity of FGF-2 proteins in CMs and cell lysates was also confirmed with Western immunoblot assays. Briefly, the CM and lysate proteins were resolved on 8% or 15% SDS–PAGE and transblotted onto PVDF membranes (Bio-Rad Labs., Hercules, CA). Known amounts of recombinant human FGF-2 standard (Sigma, St. Louis, MO) were included in each blot for comparison. To identify FGF-2 bands, the blots were incubated with a mouse anti-human FGF-2 monoclonal antibody (UpState Biotech., Lake Placid, NY) and FGF-2 bands were visualized with the goat-anti-mouse IgG antibody conjugated with horse radish peroxidase (Pierce, Rockford, IL) followed by the chemiluminescent assay (Pierce).

### Deglycosylation of FGF-2

Deglycosylation was performed with enzymes provided by the Enzymatic deglycosylation kit (Prozyme, San Leandro, CA) that contains PNGase F (which hydrolyzes core oligosaccharides of N-linked glycosylated proteins), endo-*O*-glycosidase (which hydrolyzes core oligosaccharides of O-linked glycosylated proteins, only after substitutions on the core oligosaccharide, such as sialic acid, galactose, fucose, or N-acetylglucosamine, are removed with the appropriate exoglycosidase, such as sialidases), sialidase A (which removes sialic acid-containing substitutions from core oligosaccharides), and the combination of the three glycosidases. Each glycosidase digestion reaction was carried out as recommended by the manufacturer. Briefly, the CMs were collected from the transduced cells, and 30 μl of each CM was mixed with 10 μl of 5× incubation buffer and 2.5 μl of denaturation solution. The samples were heated to 100°C for 5 min, followed by the addition of 2.5 μl of detergent solution. The PNGase, sialidase A, or endo-O-glycosidase (1 μl each), separately or all together, was added to the denatured CM protein samples. The samples were then incubated overnight at 37°C, and the FGF-2 proteins were identified by Western blots.

### FGF-2 Heparin Binding Affinity Assay

The relative heparin binding affinity of the wild-type FGF-2 protein and the cysteine-mutated variant was determined with an Affi-Gel heparin column (Bio-Rad Labs.), in which heparin was covalently crosslinked to agarose beads. Briefly, 1 ng each of wild-type and cysteine-mutated FGF-2, obtained from the CM of HT1080 cells transduced with pY-FGF and pY-BMPFGFC2SC3N vector, respectively, was adjusted to the same volume (729 μl) with phosphate-buffered saline (PBS), and each was applied to an Affi-Gel heparin column with a bed volume of 200 μl. Each column was washed with 2 ml of PBS and the bound FGF-2 was eluted four times with 100 μl of 10× PBS each. The amount of FGF-2 in each fraction was measured with ELISA as described above. Over 95% of the loaded FGF-2 protein was recovered from each column.

### Biological Activity of FGF-2 Assays

The in vitro biological activity of recombinant C2S/C3N-mutated FGF-2 was determined by measuring its ability to stimulate [$^3$H]thymidine incorporation in RSFs similar to a previously described assay [Kasperk et al., 1990]. Briefly, RSFs were plated at a density of 10,000 cells per well in 24-wells plates in serum-free DMEM for 24 h. Cell medium was changed to fresh serum-free DMEM for an additional 24 h. Various amounts of the CM wild-type FGF-2 protein and cysteine-mutated variant (produced by HT1080 cells) were added for an additional 24 h. [The amount of FGF-2 in each CM was pre-determined by ELISA.] [$^3$H]Thymidine (1.5 μCi/well) was added during the final 6 h of the incubation. The [$^3$H]thymidine incorporation into trichloroacetic acid-precipitable DNA was measured by liquid scintillation counting ($n = 6$ per each group).

The effects of wild-type and the cysteine-mutated FGF-2 on the Erk1/2 phosphorylation were determined with Western immunoblots using an antibody specific against phosphorylated Erk1/2 (pErk1/2) as described previously [Kapur et al., 2003]. Briefly, RSFs were treated with 1 ng/ml CM wild-type FGF-2 or C2S/C3N-FGF-2 mutant protein for 10 min and immediately lyzed in the radioimmunoprecipitation assay buffer. Twenty micrograms of cellular protein each was resolved on 10% SDS–PAGE and transferred to polyvinyl difluoride membrane for immunblot analysis. The membrane was probed with the anti-pErk1/2 and was visualized with the enhanced chemiluminescence system. The blots were then stripped and reblotted with anti-panErk1/2 (which recognized both Erk1 and Erk2) to determine the relative levels of total Erk1/2. The density of pErk1/2 bands was determined by laser densitometry, and the relative cellular pErk1/2 levels were normalized against the relative cellular total Erk1/2 levels.

The in vivo biological activity of wild-type FGF-2 and the C2S/C3N-FGF-2 mutant was determined in a subcutaneous skin implant rat model. Briefly, primary skin fibroblasts from inbred strain of Fisher 344 rats were transduced three times with each test MLV vector. The transduction efficiency was determined with the β-gal marker gene or FGF-2 expression and also by real-time PCR assay for FGF-2 mRNA levels. Greater than 75% of transduction efficiency has routinely been obtained. Four million each of transduced primary RSF expressing β-gal marker gene, wild-type FGF-2 gene, or C2S/C3N-FGF-2 mutant gene were incubated in a $1 \times 1$-cm Gelfoam disc (Pharmacia & Upjohn, Kalamazoo, MI) overnight as previously described [Gysin et al., 2002]. Each disc was implanted into a subcutaneous pocket at the back of a Fisher 344 rat. Fourteen days later, the serum level of FGF-2 was determined with ELISA and the weight of each implant was determined.

## RESULTS

### Effects of cys-70 (C2) and/or cys-88 (C3) Mutation on FGF-2 Secretion in HT1080 Cells

To evaluate whether C2 and/or C3 mutations would improve FGF-2 protein secretion, HT1080 fibrosarcoma cells, which do not express detectable amounts of FGF-2 protein, were transduced with MLV-based vectors expressing wild-type or the cysteine-mutated FGF-2 gene. Table II shows the amounts of FGF-2 protein in cell lysates and CMs of the transduced cells 48-h post-transduction. The

**TABLE II. Effects of cys-70 (C2) and cys-88 (C3) Mutations on FGF-2 Production and Secretion in HT1080 Cells (Mean ± SD, n = 2)[a]**

| Transduced cells | FGF-2 in CM | FGF-2 in lysate | Total FGF-2[b] | % Secretion[c] |
|---|---|---|---|---|
| pY-FGF | 16.2 ± 3.2[d] | 148.2 ± 39.5 | 164.4 ± 39.6 | 9.8 ± 3.2 |
| pY-FGFC2S | 47.4 ± 9.3 | 497.6 ± 22.3 | 545.0 ± 24.2** | 8.7 ± 1.8 |
| pY-FGFC3S | 99.4 ± 15.8 | 532.2 ± 69.4 | 631.6 ± 71.2* | 15.7 ± 3.2 |
| pY-FGFC3N | 120.6 ± 4.0 | 503.6 ± 111.6 | 624.2 ± 111.7* | 19.3 ± 4.3 |
| pY-FGFC2SC3S | 135.6 ± 9.0 | 843.2 ± 111.2 | 978.8 ± 111.6* | 13.9 ± 2.1 |
| pY-FGFC2SC3N | 129.4 ± 35.4 | 613.2 ± 2.6 | 742.6 ± 35.5** | 17.4 ± 4.8 |

[a]There is no detectable FGF-2 protein in CM or lysate in untreated or pY-β-gal-treated HT-1080 cells.
[b]Total FGF-2 is the sum of FGF-2 in CM and FGF-2 in lysate. *$P < 0.05$ and **$P < 0.01$, compared with the pY-FGF-treated cells.
[c]% FGF-2 secretion was calculated by dividing FGF-2 in CM by total FGF-2. None of the cysteine-mutated vector-treated cells was significantly different from the pY-FGF-treated cells.
[d]ng/$10^6$ cells per 48 h.

amounts of FGF-2 in CMs of the wild-type FGF-transduced cells (pY-FGF) represented ~10% of the total FGF-2 protein produced, a finding consistent with the premise that the secretion of unmodified FGF-2 in mammalian cells is inefficient. Mutation of C2 or C3 to serine or asparagine alone each significantly increased the amounts of FGF-2 in cell lysates and CMs by two- to threefold. However, the FGF-2 secretion was enhanced by 40%–100% in cells transduced with vectors containing the C3 mutation (C3S and C3N), but not the C2S mutation. Double mutations of C2 and C3 increased the total amounts of FGF-2 in pY-FGF-transduced cells but did not further enhance FGF-2 secretion when compared to the C3 single mutation. Western immunoblot analysis (Fig. 2) shows that: (a) each group of transduced cells produced a major immunoreactive band of 21 kD, that co-migrated with the mature FGF-2 protein standard, in cell lysates and CMs, and (b) the amounts of FGF-2 protein in lysates and CMs were significantly higher in the cysteine-mutated FGF-2 vector-transduced cells with than those in wild-type pY-FGF vector-transduced cells. Cells transduced with the pY-LMPHA vector (negative control) produced no detectable FGF-2 protein in lysates or CMs.

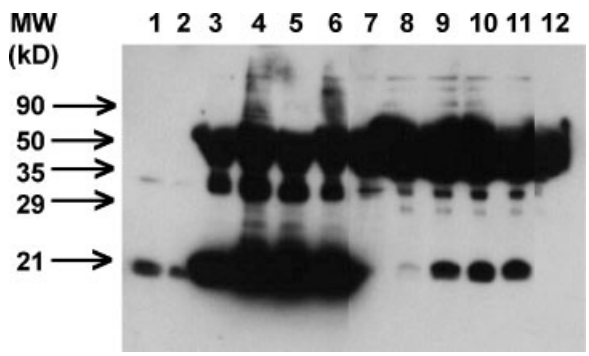


**Fig. 2.** Western immunoblotting analysis of FGF-2 immunoreactive proteins in the cell lysates and conditioned medium (CM) of HT1080 cells 24 h after transduction with MLV-based vector expressing wild-type or C2- and/or C3-mutated FGF-2 genes. **Lane 1**: 10 ng of recombinant human FGF-2 standard; **Lane 2**: 5 ng of recombinant human FGF-2 standard; **Lane 3**: 10 μl of pY-FGF-treated cell lysate; **Lane 4**: 10 μl of pY-FGFC2S-treated cell lysate; **Lane 5**: 10 μl of pY-FGFC2SC3S-treated cell lysate; **Lane 6**: 10 μl of pY-FGFC2SC3N-treated cell lysate; **Lane 7**: 10 μl of the control vector pY-LMPHA-treated cell lysate; **Lane 8**: 10 μl of pY-FGF-treated cell CM; **Lane 9**: 10 μl of pY-FGFC2S-treated cell CM; **Lane 10**: 10 μl of pY-FGFC2SC3S-treated cell CM; **Lane 11**: 10 μl of pY-FGFC2SC3N-treated cell CM; and **Lane 12**: 10 μl of the control vector pY-LMPHA-treated cell CM.

### Effects of the Addition of BMP2/4 Secretion Signal Sequence and C2 and/or C3 Mutation on FGF-2 Secretion in HT1080 Cells

Table III shows that addition of the BMP2/4 hybrid secretion signal sequence alone (pY-BMPFGF), surprisingly, resulted in a sevenfold reduction in total amounts of FGF-2 produced compared to pY-FGF-transduced cells. However, the relative amount of FGF-2 protein in the CMs of pY-BMPFGF-treated cells accounted for 54.2% of total FGF-2 proteins produced, which was >fivefold more than that in CMs of pY-FGF-treated cells (9.8%), indicating that the BMP2/4 signal sequence enhanced the secretion of FGF-2 in HT1080 cells. The single C2S mutation along with the BMP2/4 secretion signal sequence (pY-BMPFGFC2S) did not increase total amount or secretion of the FGF-2 protein compared to the pY-BMPFGF-transduced cells. Conversely, double C2/C3 mutations markedly increased total amounts of FGF-2 protein in CMs and lysates compared to the pY-BMPFGF group. The enhancement in total FGF-2 produced was significantly ($P < 0.01$) larger in C3N mutated group compared to that in C3S mutated group: C3S mutation yielded a ~threefold increase, while C3N mutation produced a sevenfold enhancement. The increase in the amounts of FGF-2 in CMs were also higher in cells transduced with vectors containing the C3N mutation (~60-fold) than in cells transduced with C3S mutated vectors (~30-fold). With respect to FGF-2 secretion, cells treated with C2/C3 double mutations vector showed an increase in FGF-2 secretion by ~fourfold compared with pY-BMPFGF-treated cells.

Figure 3 shows a representative Western immunoblot of FGF-2 immunoreactive protein bands in lysates and CMs of HT1080 cells transduced with the test vectors. Lysate of cells transduced with the C2S vector and/or the C3S vector (but not the non-cysteine-mutated vectors) showed an additional immunoreactive band of ~26 kD, in addition to the 21-kD immunoreactive protein band that co-migrated with the FGF-2 protein standard. Lysates of cells transduced with the C2SC3N vector showed yet another immunoreactive band of ~29 kD. Because (a) CMs of cells transduced with vectors that did not have the C3 mutation contained relatively low levels of FGF-2 protein (Table III) and (b) a small amount of CM (10 μl)

**TABLE III. Effects of the Addition of BMP2/4 Secretion Signal Sequence and C2/C3 Mutations on FGF-2 Production and Secretion in HT1080 Cells (Mean ± SD, n = 2)**

| Transduced cells | FGF-2 in CM | FGF-2 in lysate | Total FGF-2[a] | % Secretion[b] |
|---|---|---|---|---|
| pY-FGF | 16.2 ± 3.2[c] | 148.2 ± 39.5 | 164.4 ± 39.6 | 9.8 ± 3.2 |
| pY-BMPFGF | 13.0 ± 1.0 | 11.0 ± 0.3 | 24.0 ± 1.0* | 54.2 ± 4.4** |
| pY-BMPFGFC2S | 15.6 ± 0.4 | 16.2 ± 3.1 | 31.8 ± 3.1* | 49.1 ± 9.5* |
| pY-BMPFGFC2SC3S | 318.8 ± 5.1 | 153.2 ± 0.7 | 472.0 ± 5.1** | 67.5 ± 1.1*** |
| pY-BMPFGFC2SC3N | 916.2 ± 203.1 | 155.4 ± 31.8 | 1071.6 ± 205.6* | 85.5 ± 25.8* |

[a]Total FGF-2 is the sum of FGF-2 in CM and FGF-2 in lysate. *$P < 0.05$ and **$P < 0.01$, compared with the pY-FGF-treated cells.
[b]% FGF-2 secretion was calculated by dividing FGF-2 in CM by total FGF-2. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the pY-FGF-treated cells.
[c]ng/$10^6$ cells per 48 h.

was analyzed by Western immunoblots, only CMs of cells transduced with vectors containing the C3 mutation showed detectable FGF-2 immunoreactive bands. Since serine and asparagine are potential sites of O- and N-glycosylation, respectively, the larger immunoreactive bands could be glycosylated forms of FGF-2: the 26-kD band may be O-glycosylated and the 29-kD band may be both O- and N-glycosylated.

### Effects of the Addition of BMP2/4 Signal Sequence and C2 and C3 Double Mutations on FGF-2 Secretion in RSFs

We next determined whether the modifications would similarly affect the secretion and/or the total amounts of FGF-2 protein produced in normal, untransformed cells. Accordingly, primary RSFs were transduced with the pY-β-galactosidase (pY-β-gal) (control vector), pY-FGF, or pY-BMP-FGF-C2SC3N. Staining for β-gal expression with x-gal in pY-β-gal-transduced cells indicated that the transduction efficiency was >90% (data not shown). Table IV summarizes the amounts of FGF-2 protein in CMs and in lysates of RSFs 48-h post-transduction. Both CMs and lysates of cells transduced with pY-β-gal showed very low (almost undetectable) levels of FGF-2 protein. Transduction of RSFs with the pY-FGF vector increased the FGF-2 protein level in the cell lysate, but the amount of FGF-2 protein in the CM was still very low (accounting for ~1% of the total FGF-2 produced). Similar to HT1080 cells, addition of the BMP2/4 secretion signal suppressed FGF-2 biosynthesis in RSFs, as the total amounts of FGF-2 protein synthesized by RSFs transduced with the pY-BMPFGFC2SC3N vector were reduced by ~60% compared to pY-FGF-treated cells. Real-time PCR analysis of genomic DNA of the transduced cells indicated that the relative copies of the transgene incorporated into the host genomic DNA was not significantly different among cells transduced with the pY-BMPFGF vectors and pY-FGF vector (data not shown). The large majority of FGF-2 protein (~90%) in the pY-BMPFGFC2SC3N-transduced cells was in CMs, suggesting that addition of the BMP2/4 signal sequence increased secretion of the FGF-2 chimeric protein in RSFs. Despite the ~60% reduction in the total amounts of FGF-2 produced, the actual amounts of FGF-2 secreted into CMs of the pY-BMPFGFC2SC3N-transduced cells was 37-fold higher than that in CMs of the pY-FGF-transduced cells.

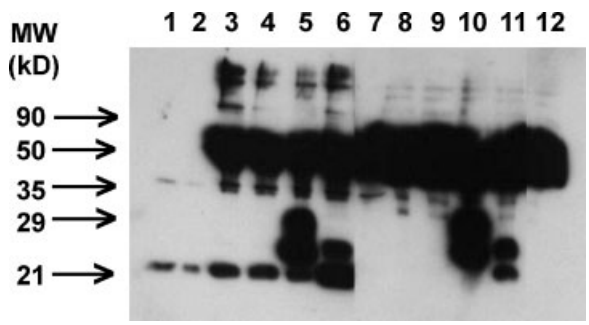


**Fig. 3.** Western immunoblotting analysis of FGF-2 immunoreactive proteins in the cell lysates and CM of HT1080 cells 24 h after transduction with MLV-based vector expressing BMP2/4-FGF-2 hybrid genes. **Lane 1**: 10 ng of recombinant human FGF-2 standard; **Lane 2**: 5 ng of recombinant human FGF-2 standard; **Lane 3**: 10 μl of pY-BMPFGF-treated cell lysate; **Lane 4**: 10 μl of pY-BMPFGFC2S-treated cell lysate; **Lane 5**: 10 μl of pY-BMPFGFC2SC3N-treated cell lysate; **Lane 6**: 10 μl of pY-BMPFGFC2SC3S-treated cell lysate; **Lane 7**: 10 μl of the control vector pY-LMPHA-treated cell lysate; **Lane 8**: 10 μl of pY-BMPFGF-treated cell CM; **Lane 9**: 10 μl of pY-BMPFGFC2S-treated cell CM; **Lane 10**: 10 μl of pY-BMPFGFC2SC3N-treated cell CM; **Lane 11**: 10 μl of pY-BMPFGFC2SC3S-treated cell CM; and **Lane 12**: 10 μl of the control vector pY-LMPHA-treated cell CM.

**TABLE IV. Effects of the Test Modification of FGF-2 Gene on FGF-2 Production and Secretion in Primary Rat Skin Fibroblasts**

| Transduced cells | FGF-2 in CM | FGF-2 in lysate | Total FGF-2[a] | % Secretion[b] |
|---|---|---|---|---|
| pY-β-gal | $0.003 \pm 0.000$[c,d] | $0.521 \pm 0.018$ | $0.52 \pm 0.02$ | $0.48 \pm 0.09$ |
| pY-FGF | $0.881 \pm 0.013$ | $91.7 \pm 0.000$ | $92.6 \pm 0.0$ | $0.95 \pm 0.10$ |
| pY-BMPFGFC2SC3N | $33.04 \pm 0.421$ | $4.83 \pm 0.530$ | $37.9 \pm 0.6$* | $87.3 \pm 1.7$** |

[a]Total FGF-2 is the sum of FGF-2 in CM and that in lysate. *$P < 0.05$ compared with the pY-FGF-treated cells.
[b]% FGF-2 secretion was calculated by dividing FGF-2 in CM by total FGF-2. **$P < 0.001$, compared with the pY-FGF-treated cells.
[c]ng/$10^6$ cells per 48 h.
[d]Most of the values were below the detection limit.

Figure 4 shows a representative Western immunoblot of FGF-2 proteins in CMs of three preparations of pY-BMPFGFC2SC3N-transduced RSFs. In contrast to HT1080 cells which primarily produced the 26- and 29-kD immunoreactive protein bands, CMs of pY-BMPFGFC2SC3N-transduced RSF contained a major 27-kD immunoreactive protein band, in addition to the 21-kD mature FGF-2 band. This 27-kD band was not unique to primary RSFs, since the transduced primary rat marrow stromal cells also yielded this major 27-kD band (data not shown). Thus, the glycosylation of cysteine-mutated FGF-2 proteins in primary untransformed cells is probably different from that in transformed cells.

### Glycosylation of Cysteine-Mutated FGF-2

To confirm that the 26- and 29-kD immunoreactive bands in HT-1080 cells and the 27-kD band in RSFs were glycosylated species of FGF-2, the CM of pY-BMPFGFC2SC3N-treated HT1080 cells and also that of pY-BMPFGFC2SC3N-treated RSF was each treated with PNGase F, endo-O-glycosidase, sialidase A, or all three glycosidases. With the HT1080 CM (Fig. 5A), combination treatment with all three glycosidases converted the 26- and 29-kD bands to the 21-kD FGF-2 band, confirming that both bands were glycosylated species of FGF-2. Sialidase A alone converted both the 26- and 29-kD bands to a single band of an apparent size between 21 and 26 kD. The PNGase F treatment alone converted the 29 kD to a major band of slightly greater than 26 kD in addition to the 21 kD non-glycosylated FGF-2 band. The endo-O-glycosidase treatment (without sialidase A) had no significant effects on the 29- or 26-kD band, suggesting that the O-linked oligosaccharide core contained sialic acid substitutions. With respect to the 27-kD band in RSF CM (Fig. 5B), the combination treatment converted the 27-kD band to the 21-kD non-glycosylated FGF-2 band. However, the PNGase F treatment, but not endo-O-glycosidase or sialidase A treatments, converted the 27-kD band to the 21-kD non-glycosylated

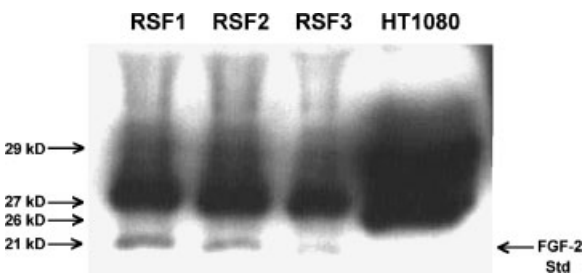


**Fig. 4.** Western blot of FGF-2 proteins secreted by pY-BMPFGFC2SC3N-transduced primary rat skin fibroblasts (RSF). The CMs (50 μl each) of three separate preparations of RSF transduced with the pY-BMPFGFC2SC3N expression vector (RSF1, RSF2, and RSF3, respectively) were analyzed by Western analysis as described in Materials and Methods.

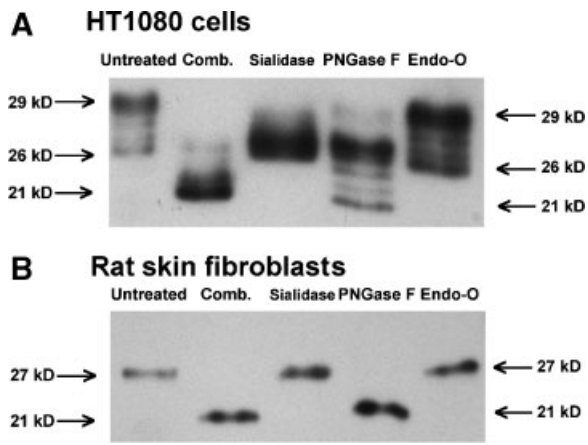


**Fig. 5.** Treatment of the secreted FGF-2 protein bands by glycosidases. The CMs of pY-BMPFGFC2SC3N-transduced HT1080 cells (**A**) and pY-BMPFGFC2SC3N-transduced RSFs (**B**) were each treated with sialidase A, PNGase F, endo-O-glycosidase, or the combination of the three glycosidases as described in Materials and Methods. Each treated CM sample was then subjected to Western analysis as described in Materials and Methods.

FGF-2, indicating that the 27-kD band is primarily of N-linked glycosylation.

### Effects of Glycosylation on the Secretion of the FGF-2 Chimeric Protein by Transduced Cells

To determine if glycosylation is essential for promoting secretion of the FGF-2 chimeric protein, we evaluated the effect of mutation of C2 and/or C3 to non-glycosylatable alanine on FGF-2 secretion by measuring the amounts of the FGF-2 protein in CMs in transduced HT1080 cells and RSFs. Table V shows that the C2A mutation alone increased the amounts of FGF-2 protein by threefold and ~20-fold in HT1080 cells and RSFs, respectively, compared with pY-BMPFGF-transduced control cells. In contrast, the C3A mutation alone increased the CM FGF-2 level by 15-fold and ~100-fold, respectively, in HT1080 cells and RSFs. Double C2A/C3A mutations did not further increase the secretion compared to C3A mutation alone. However, by allowing N-glycosylation at C3 with the C2A/C3N mutation, the amounts of FGF-2 protein level in CMs of transduced HT1080 cells and RSFs were increased by 170- and 500-fold, respectively, which was ~twofold greater than the C2S/C3N double mutants in both cell types. These findings suggest that N-glycosylation at the C3 site (but not O-glycosylation at the C2 site) is essential for promoting secretion. It is possible that glycosylation at the C2 site might even have a negative impact on the secretion and/or stability of the FGF-2 chimeric protein.

To assess the possibility that glycosylation at the C2 site might negatively affect the stability and/or secretion of the FGF-2 chimeric protein, we compared the relative FGF-2 amounts in CMs and cell lysates of pY-BMPFGFC2SC3N- and pY-BMPFGFC2AC3N-transduced RSFs (Table VI). C2A mutation rather than C2S mutation increased the levels of FGF-2 protein in cell lysates and CMs. On the other hand, the relative percentage of FGF-2 secretion was not significantly different between the two mutations. These results indicate that the C2A mutation did not enhance secretion compared to the C2S mutation, and suggest that the increase in FGF-2 levels in CMs (and cell lysates) could be due to an increased stability of the C2A mutant compared to the C2S mutant.

### Biological Activities of the Glycosylated FGF-2 Chimeric Protein

The pY-BMPFGFC2SC3N-transduced RSFs displayed an altered morphology compared to pY-β-gal-transduced RSF in that these cells were much smaller than the pY-β-gal-transduced control cells (Fig. 6). They were also changed to spindle-like shape from the elongated shape that is typical of flibroblasts (Fig. 6C). This morphology was similar to that of primary RSFs after treatment with recombinant FGF-2 protein for 1 week (Fig. 6D). No such morphological change was seen in pY-β-gal-transduced RSFs (Fig. 6B) or in pY-FGF-transduced RSFs (data not shown). Similar morphological change was seen with pY-BMPFGFC2SC3N-transduced primary rat marrow stromal cells (data not shown).

All members of the FGF family have a high affinity for heparin and for cell surface heparin

**TABLE V. Comparison of the C2 and C3 Mutations on the Secretion of Recombinant FGF-2 Chimeric Protein in HT1080 Cells and Normal RSF***

| Transduced cells | FGF-2 in CM of HT1080 cells (ng/$10^6$ cells/96 h) | FGF-2 in CM of RSF (ng/$10^6$ cells/96 h) |
|---|---|---|
| pY-β-gal | 0.096 ± 0.025 | 0.003[a] |
| pY-FGF | 0.350 ± 0.040 | 0.013 ± 0.004 |
| pY-BMPFGF | 0.343 ± 0.085 | 0.035 ± 0.010 |
| pY-BMPFGFC2A | 1.021 ± 0.163 | 0.759 ± 0.012 |
| pY-BMPFGFC3A | 5.174 ± 0.121 | 3.642 ± 0.404 |
| pY-BMPFGFC2AC3A | 8.764 ± 0.438 | 2.589 ± 0.186 |
| pY-BMPFGFC2AC3N | 59.825 ± 3.715 | 18.674 ± 2.043 |
| pY-BMPFGFC2SC3S | 9.315 ± 0.622 | 3.255 ± 0.068 |
| pY-BMPFGFC2SC3N | 26.988 ± 0.723 | 8.481 ± 0.508 |

*The FGF-2 levels in these transduced cells were significantly lower than those in Table II and III. This might be due to a lower viral vector concentration that was used in the transduction, since the cells were transduced only once with 100 μl viral stock, rather than the standard protocol of a total of three rounds of transduction with 200 μl viral stock each.
[a]The result was obtained from a single sample without repeats.

**TABLE VI. Comparison of the C2A and the C2S Mutation on the Secretion of Recombinant FGF-2 Chimeric Protein in Normal RSF[a]**

| Transduced cells | FGF-2 in CM | FGF-2 in lysate | Total FGF-2[a] | % Secretion[b] |
|---|---|---|---|---|
| pY-FGF | $0.24 \pm 0.01$[c] | $8.51 \pm 3.83$ | $8.75 \pm 3.83$ | 2.74 |
| pY-BMPFGFC2SC3N | $20.61 \pm 0.00$ | $5.53 \pm 1.65$ | $26.14 \pm 1.65$ | 78.84 |
| pY-BMPFGFC2AC3N | $64.05 \pm 6.38$** | $12.41 \pm 0.87$* | $76.46 \pm 6.44$** | 83.77 |

[a]Total FGF-2 is the sum of FGF-2 in CM and FGF-2 in lysate. *$P < 0.05$ and **$P < 0.01$, compared with the pY-BMPFGFC2SC3N-treated cells.
[b]% FGF-2 secretion was calculated by dividing FGF-2 in CM by total FGF-2.
[c]ng/$10^6$ cells per 72 h.

sulfate proteoglycans, which participate in formation of stable and active FGF-receptor complexes [Wiedlocha and Sorensen, 2004]. To assess if glycosylation of the modified FGF-2 would affect its heparin-binding ability, we compared the elution profile of the C2S/C3N FGF-2 mutant protein in a small heparin-agarose affinity column with that of wild-type FGF-2 protein. Figure 7A indicates that the glycosylated C2S/C3N FGF-2 mutant was able to bind heparin but it also shows that its heparin affinity was slightly lower than that of the wild-type FGF-2 molecule, since the C2S/C3N FGF-2 protein can be eluted out slightly earlier than wild-type FGF-2. Thus, the glycosylation appeared to slightly affect the heparin binding affinity of the FGF-2 protein. To test if the apparent decrease in heparin binding affinity would alter its biological activity and/or the signaling mechanism, we compared the relative ability of the C2S/C3N FGF-2 mutant to stimulate the cell proliferation (Fig. 7B) and stimulate Erk1/2 phosphorylation (Fig. 7C) of RSFs with those of wild-type FGF-2 protein. There did not appear to be a significant difference in the ability to stimulate RSF cell proliferation and Erk1/2 phosphorylation between the C2S/C3N FGF-2 mutant and the wild-type FGF-2 protein. Thus, the modification (glycosylation) did not appear to significantly affect the biological activity of the FGF-2 protein.

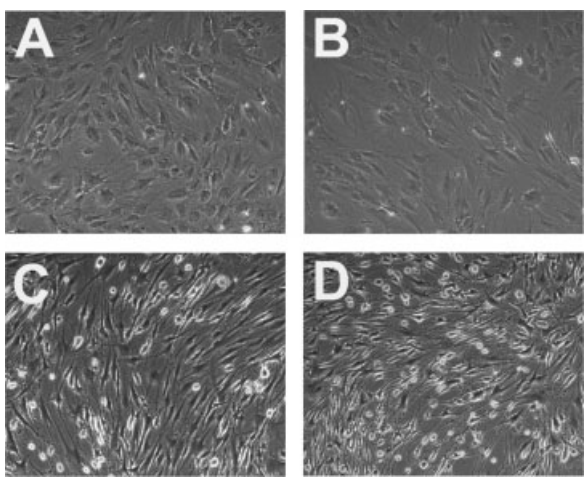


**Fig. 6.** Effects of the pY-BMPFGFC2SC3N transduction on the morphology of transduced RSFs. Primary RSF ($1 \times 10^5$ cells per well in six-well plates) were transduced with either pY-β-gal control vector (**left**) or the pY-BMPFGFC2SC3N. One week after the transduction, the cells were stained with Fast Red. **A**: Untransduced RSF; (**B**) pY-β-gal-transduced RSF; (**C**) the pY-BMPFGFC2SC3N-transduced RSF; and (**D**) untransduced RSF treated with 200 ng/ml recombinant FGF-2 for the same 7-day duration. Magnification = 200× in each panel.

### Biological Effects of Subcutaneous Implantation of pY-BMPFGFC2SC3N-Transduced Rat Skin Fibroblasts in Syngenic Rats

To initiate an evaluation of the utility of the pY-BMPFGFC2SC3N vector in gene transfer protocols, we investigated whether subcutaneous implantation of RSF transduced with the pY-BMPFGFC2SC3N vector in the dorsal back of syngenic rats would increase serum FGF-2 levels and enhance the growth of the implant 14 days after the implantation. Figure 8A shows that the serum FGF-2 level in rats with implants containing the control pY-β-gal-transduced RSF or pY-FGF-transduced RSF were very low (i.e., <10 pg/ml). By contrast, every rat that had the implant containing the pY-BMPFGFC2SC3N-transduced RSF showed very high serum FGF-2 levels (~150 pg/ml). The weight of implants was measured at 14 days post-implantation (Fig. 8B). It was significantly greater (two- to threefold) in the pY-BMPFGFC2SC3N group than that in the pY-β-gal or in the pY-FGF control groups. Gross anatomical examination of the implants showed that the pY-BMPFGFC2SC3N implants, but not the pY-β-gal or pY-FGF implants, were reddish in color (Fig. 8C) and rich in blood, suggesting that extensive vascularization had occurred.

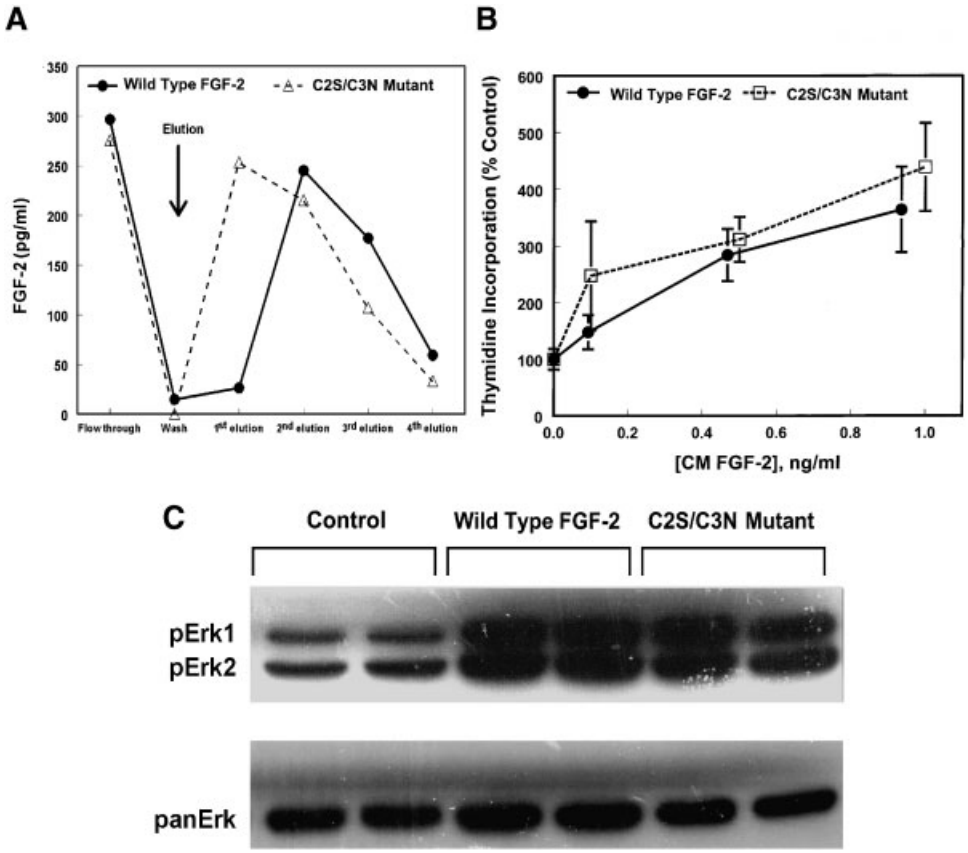


**Fig. 7.** Effects of C2S/C3N mutations and glycosylation on the heparin binding affinity (**A**), and its ability to stimulate cell proliferation, (**B**) and Erk1/2 activation (**C**) in RSFs. HT1080 cells were transduced with pY-FGF or pY-BMPFGFC2SC3N. CM of cells transduced with pY-FGF and pY-BMPFGFC2SC3N contained wild-type and cysteine-mutated, glycosylated FGF-2, respectively. The concentration of FGF-2 in each CM was measured with ELISA. The CM of pY-BMPFGFC2SC3N-transduced cells was diluted with PBS so that the concentration of FGF-2 protein in the two CMs was equivalent. In A, 1 ng of FGF-2 protein from each CM was applied to a small Affi-Gel heparin column (bed volume of 200 μl) and eluted with 10× PBS as described in Materials and Methods. The recovery of FGF-2 in either column was similar and >95%. In B, the indicated amounts of CM wild-type or cysteine-mutated FGF-2 were added to monolayer cultures of quiescent RSFs and [$^3$H]thymidine incorporation, as an index of cell proliferation, was measured as described in Materials and Methods. Each data point contains six replicates and the results are shown as mean ± SD. In C, monolyaer cultures of RSFs were treated with 1 ng/ml of either CM wild-type FGF-2 or CM C2S/C3N FGF-2 mutant for 10 min. The relative phosphorylated Erk1/2 (pErk1 or pErk2) levels were identified on Western immunoblot as described in Materials and Methods. The blots were then stripped and reblotted with the anti-panErk antibodies to show relative levels of total Erk1/2.

## DISCUSSION

A significant problem that hindered the progress of the FGF-2 gene therapy has been the inconsistent and inefficient secretion of FGF-2 protein in mammalian cells [Mignatti et al., 1992; Florkiewicz et al., 1995; Dahl et al., 2000]. It has been difficult to reproducibly and consistently achieve therapeutic levels of FGF-2 through gene transfer approaches. This problem is further exaggerated by the possibility that recombinant FGF-2 proteins can be inactivated by intra- and/or intermolecular disulfide formation [Iwane et al., 1987]. In this study, we have shown that these problems can be ameliorated by adding to the FGF-2 transgene a potent secretion signal sequence, such as the BMP2/4 hybrid signal sequence, and also by mutation of cys-70 (C2) and cys-88 (C3) to a serine or an asparagine. These modifications together have led to an overall ~60-fold and ~40-fold increase in the actual amounts of FGF-2 protein secreted into CMs of transduced HT1080 cells and primary RSFs, respectively, compared to the unmodified FGF-2 gene. More importantly, the ex vivo application of the modified FGF-2 MLV-based vector, but not the unmodified, wild-type FGF-2 vector, in a subcutaneous transplant rat model resulted in a ~20-fold increase in serum FGF-2, a

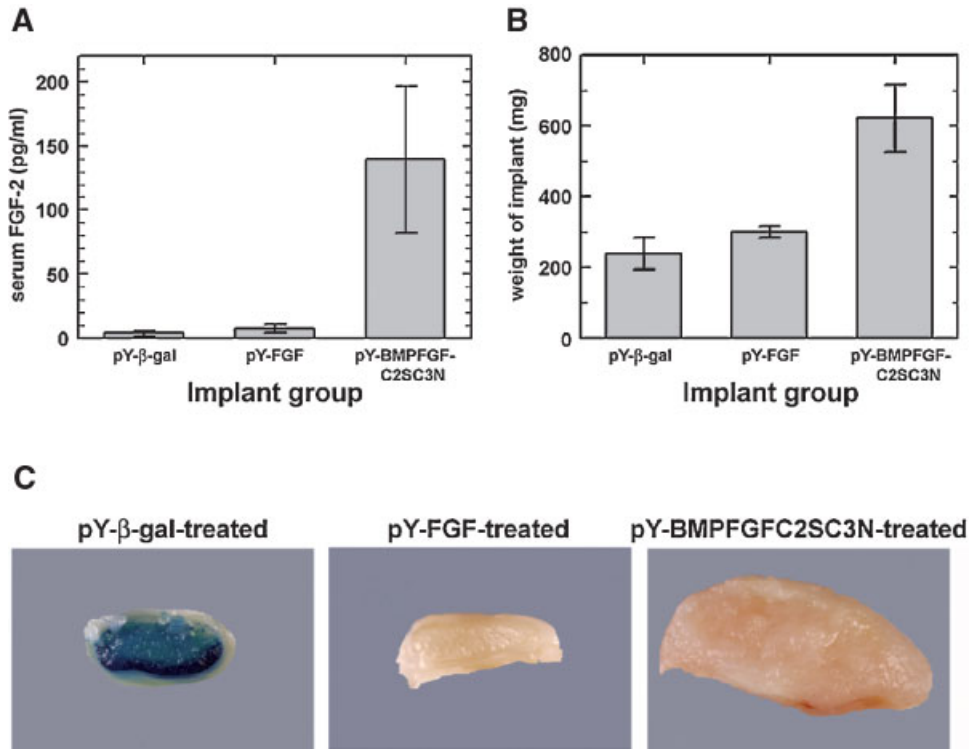


**Fig. 8.** Effects of ex vivo administration of pY-BMPFGFC2SC3N-transduced RSFs on the serum FGF-2 level (**A**), the growth of implants (**B**), and the extent of vascularization of the implants (**C**) in a subcutaneous dorsal back implant rat model. Gel-foam squares (1 $cm^2$) were impregnated with 4 million of primary RSFs transduced with pY-β-gal, pY-FGF control vector, or pY-BMPFGFC2SC3N vector overnight and implanted subcutaneously into the dorsal back of each syngenic rat. Fourteen days after the implantation, animals were sacrificed and serum FGF-2 was determined with an ELISA assay (A). The implants were dissected and weighed (B) and are shown in C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

~threefold increase in the weight, and the apparent vascularization of the implants. These findings led us to conclude that the secretion and also, presumably, the stability problem can be overcome by these modifications of the FGF-2 transgene (i.e., addition of the BMP2/4 secretion signal sequence and the C2/C3 mutations), and that our modified FGF-2 expression vector may be useful in a gene transfer approach to yield consistent therapeutic levels of FGF-2.

The reason for C2/C3 mutations to increase the total amounts of FGF-2 proteins in the transduced cells has not been determined. Because the C2/C3-mutated FGF-2 expression vectors have the same viral backbone as the wild-type FGF-2 expression vector, and because the viral titers and transduction efficiency (determined by real-time PCR analysis of FGF-2 mRNA transcript levels) of the C2/C3-mutated FGF-2 and wild-type FGF-2 expression vectors were very similar, we do not believe that the increased amounts of FGF-2 proteins in cells transduced with the C2/C3-mutated vectors were caused by an increase in gene expression or protein biosynthesis. We tentatively conclude that the increase in total FGF-2 proteins in cells transduced with the C2/C3-mutated FGF-2 vector was due to enhanced stability (and/or decreased degradation) of the FGF-2 chimeric protein. Although we do not yet have definitive evidence that the modified FGF-2 protein was indeed more stable than the wild-type FGF-2, our conclusion is consistent with findings of previous studies, which also suggested that C2/C3 mutations increased stability of the FGF-2 protein [Seno et al., 1988; Sasada et al., 1991].

The underlying mechanism for the presumed increased stability of C2/C3 mutated FGF-2 variant is unclear. A previous study [Seno et al., 1988] has concluded that the enhanced stability of the cysteine-mutated FGF-2 was due largely to the fact that the C2/C3 mutation prevented oxidation of and/or formation of intra- or intermolecular disulfides with these two key cysteines that have been shown to

decrease FGF-2 stability [Iwane et al., 1987]. While our findings are compatible with this interpretation, our results suggest that glycosylation in response to cysteine to serine or cysteine to asparagine mutations may also play an important role in increasing the total amounts of FGF-2 protein in CMs of transduced cells. Our conclusion was based on two observations: first, the increase in total amounts of FGF-2 protein in CMs of HT1080 cells transduced with C2/C3-mutated vectors without the BMP2/4 signal sequence (which produced only the non-glycosylated FGF-2 (Fig. 2)) was only ~sevenfold (Table II). In contrast, the increase in total FGF-2 protein in CMs of cells transduced with C2/C3-mutated vectors containing the BMP2/4 signal sequence (which produced primarily glycosylated FGF-2 (Fig. 3)) was ~60-fold. Moreover, the fact that glycosylation could occur at the C2/C3 sites when they were mutated to a serine or an asparagine strongly suggests that these two cysteine residues are exposed and, thus, it is entirely consistent with the possibility that these two cysteine residues could be involved in intermolecular disulfide formation as previously suggested by Iwane et al. [1987]. Secondly, the findings that the C2A/C3A mutations, that do not permit glycosylation, did not enhance the total amounts of CM FGF-2 chimeric protein (Table V) is consistent with our contention that glycosylation may have a role in enhancing the stability of the chimeric FGF-2 protein produced by the transduced cells. In this regard, there is abundance of evidence supporting a role of glycosylation in protein stability (e.g., Wormald and Dwek, 1999).

Five observations concerning glycosylation of the cysteine-mutated FGF-2 chimeric proteins are noteworthy: First, the chimeric FGF-2 protein produced by cells transduced with the cysteine-mutated FGF-2 vectors were glycosylated only if BMP2/4 secretion signal sequence was included in the construct. The BMP2/4 secretion signal sequence is a member of the large family of cleavable classical signal sequences [Hammonds et al., 1990] that direct the secretory proteins to the ER and golgi for processing (including glycosylation) and secretion [Brodsky, 1998]. Thus, glycosylation of the modified FGF-2 most likely took place within the ER/golgi, where glycosidases and glycosyltransferases are located [Verbert and Cacan, 1999]. Therefore, the fact that glycosylation occurs only when the BMP2/4 signal sequence is included supports the assumption that the BMP2/4 signal sequence redirected FGF-2 chimera to ER/golgi for secretion. Consistent with this conclusion are previous findings that addition of the growth hormone secretion signal (a member of the cleavable classical signal sequences) to the FGF-2 transgene also resulted in secretion of glycosylated FGF-2 [Blam et al., 1988].

Secondly, glycosylation of C2S/C3N-mutated FGF-2 chimera in primary untransformed RSFs (and rat marrow stromal cells) was different from that in transformed HT1080 cells. The C2S/C3N-mutated FGF-2 secreted by transduced HT1080 cells was both O- and N-linked glycosylated, whereas the chimeric protein secreted by transduced primary RSFs (or rat marrow stromal cells) was primarily N-linked glycosylated. The reason for N-linked glycosylation being preferred over O-linked glycosylation in primary untransformed cells is unclear at this time. Thirdly, the presumed enhancement in FGF-2 stability appeared to be dependent on the type of glycosylation at the C3 site: the C3S mutation (i.e., O-linked glycosylation) yielded a ~threefold enhancement, but the C3N mutation (i.e., N-linked glycosylation) produced a ~sixfold increase in the amounts of chimeric FGF-2 in CMs of transduced cells. However, it is unclear why N-linked glycosylation of C3 would appear to have a bigger enhancing effect on the stability of the FGF-2 chimeric protein than the O-linked glycosylation of C3. It is further intriguing that there is circumstantial evidence that O-glycosylation at the C2 site might even have a negative effect on the stability of the FGF-2 chimeric protein (Table VI). Fourthly, this study offers the circumstantial evidence that C3 mutations, but not C2 mutations, seem to have a small but significant enhancing effect (>twofold) on secretion of the chimeric FGF-2 protein with or without the BMP2/4 secretion sequence. It may be speculated that the C2/C3 mutation, particularly the C3 mutation, could prevent intermolecular disulfide formation of FGF-2 with other cellular proteins. It has been suggested that blocking of the intermolecular disulfide formation could lead to a small increase in FGF-2 secretion through its own energy- dependent, non-ER/golgi pathway [Mignatti et al., 1992; Florkiewicz et al., 1995; Dahl et al., 2000]. Future work is needed to

confirm this speculation. Finally, the cysteine mutations and/or glycosylation did not appear to significantly affect the ability of chimeric FGF-2 protein to stimulate cell proliferation, to activate the mitogenic Erk1/2 signaling, and to bind heparin, although the heparin binding affinity of the modified FGF-2 protein appeared to be slightly lower than that of the wild-type FGF-2 (Fig. 7).

One of the surprising observations of this study was that the addition of the BMP2/4 secretion sequence to the FGF-2 gene led to a drastic reduction in the total amounts of FGF-2 proteins produced by transduced HT1080 cells (sevenfold decrease) or transduced RSFs (60% decrease). This reduction in total FGF-2 protein levels produced was not due to reduced transduction efficiency or reduced numbers of gene copies inserted in the transduced cells. It was most probably the result of the suppression of biosynthesis of FGF-2 chimeric proteins. The possibility of BMP2/4 secretion signal to suppress FGF-2 biosynthesis is puzzling. An inspection of the amino acid sequence of the BMP2/4 secretion signal (Fig. 1) did not reveal obvious mechanistic reasons for the suppression. The replacement of the secretion signal of BMP4 with that of BMP2/4 hybrid signal sequence did not lead to a significant inhibition of the biosynthesis of BMP2/4 chimeric protein in the test cell types, including HT1080, RSFs, and rat marrow stromal cells [Peng et al., 2001]. Thus, the suppression might be specific for FGF-2.

Addition of a classical secretion signal, such as that of BMP2/4, would direct the biosynthesis and secretion of the FGF-2 protein to rough ER and golgi. There is evidence that the process of protein secretion is intimately linked to the rate and potential of proper folding and assembly and that proper folding during translation in ER plays an important role in the regulation of mRNA translation [Kaufman, 2004]. Thus, we cannot rule out the possibility that the relatively large BMP2/4 secretion signal may affect the folding of the nascent FGF-2 protein in such a way that the biosynthesis of the FGF-2 protein is suppressed. If this hypothesis is correct, addition of other classical secretion signals to the FGF-2 gene should also yield similar suppression of the biosynthesis of FGF-2. Unfortunately, information about whether the addition of a classical secretion signal, such as FGF-4 [Sohn et al., 2001], IL-2 [Sasada et al., 1991], or growth hormone [Blam et al., 1988], would also affect the biosynthesis of FGF-2 chimeric proteins was not available in previous studies. Therefore, it is not clear if the suppressive effect was unique to the BMP2/4 secretion signal. However, we should emphasize that the addition of this same BMP2/4 secretion signal did not seem to affect the cleavage of the BMP2/4 signal peptide and the FGF-2 pre-peptide, as the molecular size of the secreted, mature FGF-2 protein was very similar, if not identical, to the 21-kD mature FGF-2.

Finally, three lines of evidence indicate that the FGF-2 chimeric protein produced by cells transduced with the modified FGF-2 expression vector is biologically active: (1) the in vitro mitogenic assay indicated that the chimeric FGF-2 protein in the CMs of the transduced cells stimulated the proliferation of quiescent RSFs and activated Erk1/2 signaling to an extent similar to those induced by recombinant FGF-2 protein (Fig. 7B,C). (2) The observation that the morphology of the cells transduced with the modified FGF-2 expression, but not that of cells transduced with control vectors, was altered to one that is consistent with highly proliferative activity supports our premise that the FGF-2 protein produced by the transduced cells is biologically active (Fig. 6). (3) The findings that ex vivo administration of cells transduced with the modified FGF-2 vector promoted the growth and vascularization of the implants provide strong evidence that the modified FGF-2 protein is biologically active in vivo (Fig. 8).

In summary, this study demonstrated that addition of a potent classical secretion signal, that is, that of BMP2/4, to the human FGF-2 transgene along with the mutation of cys-70 and cys-88 to glycosylable residues, that is, serine and asparagine, markedly enhanced the amounts of functionally active FGF-2 secreted by mammalian cells. On the basis of these findings, we believe that these modifications together might alleviate the problem of ineffective and inconsistent secretion of FGF-2 proteins by mammalian cells through gene transfer approaches. This modified FGF-2 expression vector should be useful in FGF-2-based gene therapy of various disorders, including wound healing and tissue repairs. Although we used the BMP2/4 secretion signal to direct FGF-2 secretion, it is possible that any potent member of the classical secretion signal

family may be used. Finally, it is conceivable that the application of this modified FGF-2 expression vector in gene transfer approaches may also be further improved with the use of tissue-specific promoters and/or regulatory promoters to regulate expression of this potent growth factor in tissue-specific manners.

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