CHAPTER 3

Effects of fibroblast growth factor overexpression and cellular localization on hematopoietic stem cell function

Joyce S. G. Yeoh, Ellen Weersing, Bert Dontje, Leonid Bystrykh, Ronald van Os, Gerald de Haan

Department of Cell Biology, Section Stem Cell Biology, University Medical Centre Groningen, The Netherlands

In preparation
Abstract
Exogenous addition of Fibroblast Growth Factor (FGF)-1 and FGF-2 maintains and expands long-term repopulating hematopoietic stem cells (HSCs) in vitro. These proteins are also highly expressed by Lin−Sca-1−c-Kit+ (LSK) cells. In this study we retrovirally transduced post 5-fluorouracil (5-FU) bone marrow (BM) cells with retroviral vectors which express wild-type (WT) FGF-1 and WT FGF-2 to examine their cell intrinsic role in hematopoietic cell expansion. In addition, we examine the role of nucleocytoplasmic trafficking of FGFs in hematopoietic cells by overexpressing two mutant isoforms of FGF-1 in which a serine residue at the phosphorylation site is exchanged for glutamic acid (S130E) or alanine (S130A). S130E mimics the phosphorylated state of FGF-1 and should hypothetically be constitutively exported to the cytoplasm whilst S130A should remain in the nucleus. A third mutant was created by inserting an artificial nuclear localization signal (NLS) upstream of FGF-2. Using fluorescence microscopy, we demonstrate that FGF-1, FGF-2 and S130E predominantly localizes to the cytoplasm whilst 2% of S130A and 11% of NLS/FGF-2 overexpressing cells shown nuclear localization of FGF-1 and FGF-2 respectively. We present evidence demonstrating that in an in vivo competitive repopulation assay, stem cells expressing S130A engrafted into secondary recipients with superior kinetics compared to WT cells, suggesting that nuclear localization of FGF-1 may improve hematopoietic stem cell functioning.
**Introduction**

A small population of HSCs plays a pivotal role in the lifelong maintenance of hematopoiesis. A critical property of HSCs is their ability to undergo self-renewal. The relative inability to expand HSCs ex vivo imposes substantial limitations on the current use of HSC transplantation. While studies have shown that some self renewal is clearly possible in vitro\(^1\)\(^-\)\(^3\), the magnitude of expansion obtained for human and murine HSCs is modest\(^4\)\(^-\)\(^7\).

In studies aimed to maintain HSCs in vitro, recent attention has been focused on the large family of FGFs which are involved in embryonic development and adult tissue homeostasis. To date, there are 22 members of the FGF family and only four distinct FGF receptors (FGFRs) (reviewed by Ornitz and Itoh 2001)\(^8\). There is a growing body of evidence demonstrating the role of FGFs in hematopoiesis in general and HSCs in particular. For example, previous in vitro studies showed impaired hematopoietic development in FGFR\(^-/-\) embryoid bodies (EB), such that the number of blast colonies, primitive erythroid and myeloid progenitors were greatly reduced\(^9\). FGFs, in particular FGF-2, have been shown to sustain the proliferation of hematopoietic progenitor cells, maintaining their primitive phenotype\(^10\)\(^;\)\(^11\). FGF-1 induces granulopoiesis\(^12\) and megakaryocytopoiesis\(^13\)\(^;\)\(^14\). Recently, our group showed that Fgfr-1, -3 and -4 are expressed by mouse primitive hematopoietic cell subsets and that FGF-1 was involved in the expansion of multi-lineage (lymphoid and myeloid), long-term (LT) repopulating HSCs \(^15\). Additionally, we recently reported that unfractionated bone marrow cells could be cultured in the combination of FGF-1 and FGF-2 for up to five weeks without loss of stem cell repopulation activity. We showed that this originated from FGF cultured HSCs\(^16\).

Several lines of evidence exist indicating that nuclear localization of FGFs may be required for the mitogenic effect in certain conditions, in different cells types. For example, radiolabeled exogenous FGF-1 localized to the nuclear fraction and was shown to stimulate DNA synthesis and cell proliferation in cells containing receptors for FGF-1\(^17\). In glioma cells and in primary cultures of human astrocytes, cell proliferation rate and nuclear association of FGF-2 was reported to change in parallel\(^18\). These observations support the notion that nuclear translocation of FGFs could be related to mitogenesis in different cells.
Studies have shown that the translocation of FGF-1 from the cytosol to the nucleus requires tyrosine kinase and phosphatidylinositol 3-kinase (PI3K) activity and that phosphorylation of FGF-1 occurs in the nucleus by protein kinase C (PKC) at the only functional phosphorylation site (Serine 130) \(^{19-22}\). In contrast to FGF-1, FGF-2 contains both autocrine and intracrine effects resulting from the existence of different isoforms. For example, human FGF-2 contains five different forms; a low molecular mass form (18kDa), which acts as an autocrine/paracrine factor and four high molecular mass forms (21-22, 22.5, 24 and 34kDa) which are intracrine effectors. These four high molecular mass forms are generated by differential initiation of translation sites from an upstream CUG codon. They contain at least two short N-terminal extensions in which the NLS is located\(^{23;24}\). Only N-terminally extended forms initiated at upstream CUG codons are translocated to the nucleus while the normal 18 kDa AUG-initiated form is confined to the cytoplasm\(^{25-28}\). When the intracrine FGF-2 forms were expressed in NIH 3T3 cells, high proliferation rates and growth in soft agar were observed\(^{29}\) and stimulated cell growth under low-serum conditions was evident\(^{30;31}\).

In previous studies, our goal was to maintain and expand HSCs in vitro by exogenously adding FGF-1 and FGF-2 to serum-free media\(^{15;16}\). In the current study we focused on retroviral overexpression of FGF-1 and FGF-2 on HSCs to promote expansion and maintenance of hematopoietic cells in both in vitro and in vivo studies. Parallel to this, we examined the effects of nuclear localized FGFs and their ability to provide long-term (LT) repopulation. To clarify the role of differential localization of FGFs in maintaining hematopoietic cells, we created two FGF-1 mutants. Firstly, a serine residue at the phosphorylation site (amino acid 130) was exchanged with glutamic acid to mimic phosphorylated FGF-1, which is expected to be constitutively transported to the cytosol (S130E)\(^{22}\). In the second mutant the same serine residue was exchanged for alanine, which is expected to remain in the nucleus (S130A)\(^{22}\). A third mutant for FGF-2 was also created whereby an upstream nuclear localization signal (NLS) was inserted into the FGF-2 coding sequence.

Using retroviral overexpression, long-term in vivo repopulation and principles of limiting dilution assays to quantitatively determine hematopoietic cell expansion for both WT FGF-1 and FGF-2 and their mutant forms, we demonstrate that in in vitro assay, endogenous FGFs confer increased mitogenic activity to BM cells. However, it does not provide enhanced long-term repopulation in primary and secondary in vivo
repopulating assays. Fluorescence microscopy images indicated that FGF-1, FGF-2 and S130E were preferentially located in the cytoplasm. In S130A and NLS/FGF-2 overexpressing cells, only 2% of FGF-1 and 11% of FGF-2 positive cells showed nuclear FGF activity, respectively. S130A mutants transplanted competitively engrafted into secondary recipients with improved efficiency compared to WT cells, indicating that targeting FGF-1 to the nucleus may provide improved stem cell quality.
Materials and Methods

Animals

Eight to twelve week old female B6.SJL-Ptprc<sup>a</sup>Pep<sup>b</sup>/BoyJ (CD45.1) mice, bred at our local animal facility, were used as a donor source of hematopoietic stem cells for in vitro cultures and transplantations. Female C57BL/6 (CD45.2) (B6) mice were used as recipients and were purchased from Harlan (Horst, The Netherlands). All animals were maintained under clean conventional conditions in the animal facilities of the Central Animal Facilities, University Medical Centre Groningen (The Netherlands). Mice were fed ad libitum with food pellets and acidified tap water (pH = 2.8). All animal procedures were approved by the local animal ethics committee of the University Medical Centre Groningen.

DNA constructs, expression vectors and retroviral vectors

The MIEV vector (kindly provided by Prof. C. Jordan, University of Rochester) contains an internal ribosomal entry site (IRES) sequence, ψ packaging signal and the reporter gene for enhanced green fluorescent protein (eGFP). The empty MIEV vector served as a control and was the backbone from which all other vectors were made.

FGF-1 was initially cloned into pCR4 vector following SalI end modification by using the following primers; 5’-CTACCACCGCTGCTTGC-3’ (forward), 5’-GTCGACCAAAATAGAGAACACTCAG-3’ (reverse) (fragment size 529bp). The FGF-1 coding sequence was cut with EcoRI/SalI and inserted into MunI/SalI site of the MIEV vector in between the pPGK promoter and IRES. We refer to this vector as WT FGF-1. A retroviral vector encoding for WT FGF-2 was created identically. The primers used were; 5’-CCCCAAGAGCTGCCACAG-3’ (forward) and 5’-TCAGTGACAGTGTCAAAAGTGAGTC-3’ (reverse) (fragment size 531bp).

Site directed mutagenesis was used to create two mutant isoforms of FGF-1. At the phosphorylation site, the serine at amino acid 130 was exchanged for either glutamic acid (S130E) or alanine (S130A). The initial FGF-1 cloned in pCR4 was used to create the two mutants by amplifying the whole fragment with the following primers; S130E, 5’-CAAGAAGAACGGGgagTGTAAGCGCGGTCC-3’ (forward) and 5’-GGACCGCGCTTACACTCCCGTGTTCTTTGTCC-3’ (reverse); S130A, 5’-CAAGAAGAACGGGgccTGTAAGCGCGGTCC-3’ (forward) and 5’-
Fibroblast growth factor overexpression

GGACCGCGCTTACAggcCCCGTTCTTCTTG-3’ (reverse). Lower case letters indicates the place of mismatch. Both mutants were subcloned from pCR4 vector at EcoRI/SalI site into MunI/SalI of MIEV vector.

Contrary to the human FGF-2 gene, in the mouse genome Fgf-2 does not contain an upstream NLS. The 5’ UTR NLS was artificially created based on homology to the human FGF-2 coding sequence using these primers; 5’-ACGGACTGGGAGGCTGGCAG-3’ (forward) and 5’-CGAGGTGATGCGCTGGCAG-3’ (reverse) (fragment size 157bp). Following successful sequencing results, an EcoRV and ATG site was inserted into the upstream sequence with the following primers; 5’-gatatcACGatggTACGGACTGGGAGGCTGGCAG-3’ (forward) and 5’-CGAGGTGATGCGCTGGCAG-3’ (reverse) (fragment size 140bp). Following cloning into pCR4 vector, the 5’UTR NLS was excised from the vector at EcoRV/NcoI site and ligated into the original FGF-2 pCR4 vector at SmaI/NcoI site. The ligated 5’UTR NLS/FGF-2 was removed from pCR4 vector with EcoRI/SalI and subcloned into MIEV at MunI/SalI site creating the NLS/FGF-2 retroviral vector.

Retroviral overexpression of FGFs in primary BM cells

Bone marrow cells were obtained from CD45.1 mice injected i.p. with 150mg/kg 5-Fluorouracil (5-FU, Pharmachemie Haarlem, The Netherlands), 4 days prior to BM isolation. Bone marrow cells were harvested by flushing the femoral content with StemSpan (Stem Cell Technologies, Vancouver, BC, Canada). Cells were cultured for 48 hours in StemSpan supplemented with 10% fetal calf serum (FCS; GibcoBRL, Invitrogen, CA), 300ng/ml Stem Cell Factor (SCF; Amgen, Thousand Oaks, CA, USA), 20ng/ml Interleukin-11 (IL-11; R&D Systems, Minneapolis, MN, USA), 1ng/ml Flt3 Ligand (Flt3L; Amgen, Thousand Oaks, CA, USA), penicillin and streptomycin (Invitrogen, Breda, The Netherlands).

Twenty-four hours prior to transfection, 3 x 10^5 ecotropic Phoenix packaging cells/well (ATCC-LGC Promochem, Middlesex, United Kingdom) were seeded onto 6-well plates. Using Fugene 6 (Roche, Basel, Switzerland), plasmid DNA (1µg) was transfected on to pre-seeded ecotropic phoenix packaging cells. Virus-containing supernatants from transfected ecotropic Phoenix packaging cells were harvested 24 hours after transfection and seeded onto retronecctin (Takara, Kyoto, Japan) coated 6-well plates. Plates containing viral supernatant were centrifuged for 1 hour at
2200rpm at room temperature and then incubated at 37°C with 5% CO₂ for 4 hours. The viral supernatant was then removed from the well prior to the incubation of 7.5 x 10^5 cultured BM cells with 4µg polybrene (Sigma, St Louis, MO, USA). The whole procedure was repeated 48 hours after the initial transfection and 2µg of polybrene was added. Four days after transduction, the transduction efficiency was determined by flow cytometry (FACS Calibur, Becton Dickinson, Palo Alto, CA).

**In vitro proliferation of BM cells**

Four days following transduction, Green Fluorescent Protein (GFP⁺) cells were sorted by a MoFlow cell sorter (DakoCytomation, Fort Collins, CO). 6 x 10^5 to 1 x 10^6 GFP⁺ cells were placed in 6-well plate in StemSpan supplemented with 10% fetal calf serum, 300ng/ml SCF, 20ng/ml IL-11, 1ng/ml Flt3L, penicillin and streptomycin. On a weekly basis, cells were counted, cultures were depopulated and passaged.

**In vitro hematopoietic cell assays**

Cobblestone Area Forming Cell Assays (CAFC) were performed as previously described to assess the number of hematopoietic progenitor cells (CAFC day 7) or more primitive stem cells (CAFC day 28-35) among the transduced BM cells. Colony Forming Unit Granulocyte-Macrophage (CFU-GM) was determined using standard methylcellulose cultures (0.8% methylcellulose, 30% FCS in α-MEM). Transduced BM cells were added to methylcellulose cultures supplemented with 100ng/ml SCF and 10ng/ml recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF; Behringwerke, Marburg, Germany). Cultures were cultured at 37°C with 5% CO₂ and scored after 6-7 days.

**In vitro proliferation of BM cells co-cultured on a stromal layer**

Following retroviral transduction, 5,000 or 10,000 GFP⁺ cells were sorted and co-cultured with Fetal Bone Marrow Derived (FBMD)-1 stromal layer in a culture flask. Cells were cultured in Iscoves modified DMEM (IMDM; GibcoBRL, Paisley, Scotland) supplemented with 20% horse serum (GibcoBRL, Paisley, Scotland), pencilllin/streptomycin (Gibco, Paisley, Scotland) with β-mercaptoethanol (Merck Schuchardt, Hohenbrunn, Germany) and hydrocortisone (Sigma, St Louis, MO). Nine
and 14 days after initiation of culture, all non-adherent cells were removed, counted and placed in CFU-GM and CAFC assays.

**Sorting of hematopoietic cell populations**

Hematopoietic cells were stained and sorted for populations of cells that differentially expressed Lin⁻, Sca-1 and c-Kit using the MoFlow cell sorter as previously described. BM cells were stained with biotinylated lineage-specific antibodies Mouse Lineage Panel, containing anti-CD45R, anti-CD11b, anti-TER119, anti-Gr-1 and anti-CD3e (BD Pharmingen, San Diego, CA), FITC-anti-Sca-1 and APC-anti-c-kit (BD Pharmingen, San Diego, CA). Biotinylated antibodies were visualized with streptavidin-PE (Pharmingen, San Diego, CA). Four different populations were sorted, Lin⁻Sca-1⁻c-Kit⁻ (L⁻S⁻K⁻), Lin⁻Sca-1⁺c-Kit⁻ (L⁻S⁺K⁻), Lin⁻Sca-1⁻c-Kit⁺ (L⁻S⁻K⁺) and Lin⁻Sca-1⁺c-Kit⁺ (L⁻S⁺K⁺).

**Reverse Transcriptase (RT-PCR) and Quantitative PCR (Q-PCR)**

Total RNA was prepared from approximately 1 x 10⁶ to 3 x 10⁶ sorted GFP⁺ cells using RNeasy Mini kit (Qiagen). M-MLV reverse transcriptase (Invitrogen, Breda, The Netherlands) was used to synthesize cDNA. Expression of FGF-1 and FGF-2 transcripts was assessed by RT-PCR with the following primers; FGF-1, 5’-CGGCTCGACAGACACAAATGAGG-3’ (forward) and 5’-GTCGACCACCAAATGAGGACCTCAG-3’ (reverse) (fragment size 238bp); FGF-2, 5’–CCCCAAGAGCTGCCACAG-3’ (forward) and 5’-TCAGTGACAGTGTCAAAGATGAGTC-3’ (reverse) (fragment size 531bp). The cDNA products were quantified using SYBR Green (Bio-rad) in a 96-well microtiter plates in an iCycler thermal cycler (Bio-rad, Hercules, CA, USA). Primers used for quantifying Fgf-1 and Fgf-2 expression were as follows; Fgf-1, 5’-CGGCTCGACAGACACAAATGAGG-3’ (forward), 5’-CCATAGTGAGTCCGAGGACC-3’ (reverse) (fragment size 155bp) and Fgf-2, 5’-CGACCCCACACGTCAAAACTACACACT-3’ (forward), 5’-GAAGCCGAGCCGCTCCATC-3’ (reverse) (fragment size 113bp).

FGF expression levels were compared with expression of housekeeping genes Gapdh and Actin using relative quantification ΔΔCT technique. This value was then corrected for the initial number of cells used.
Western blotting
Cell lysates were prepared using the acid-acetone precipitation method. 3 x 10^6 transduced cells were pelleted and washed with 0.1M hydrochloric acid (MERCK KGaA, Darmstadt, Germany), followed by a 60 minute incubation at -20°C. Samples were centrifuged for 30 minutes at maximum speed at 4°C, washed in 70% ethanol and resuspended in chilled (-20°C) acetone. All acetone was removed and the pellets were left to dry at 37°C for 1-5 minutes. The pellets were redissolved in sample buffer at 60°C. Lysates were then boiled for 5 minutes. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Following overnight blocking in 5% skim milk, membranes were probed overnight with 1:1000 dilution of rabbit anti-FGF-1 (Sigma, Saint Louis, MO, USA) and rabbit anti-FGF-2 (Santa Cruz Biotechnology, Santa Cruz, California, USA). Membranes were washed and incubated with anti-rabbit IgG Horseradish peroxidase secondary antibody (Amersham Biosciences, Buckinghamshire, UK). ECL western blotting detection reagents (Amersham, Biosciences, Buckinghamshire, UK) were used to develop the membranes. Equal loading of membranes were verified with commassie staining of SDS-PAGE gels.

Immunocytochemistry
Transduced GFP^+ cells were spotted on to cytospin preparations following one week of culturing in culture conditions described above. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100/PBS. FGF-1 expression was detected using an anti-FGF-1 (C-19) goat polyclonal IgG antibody (1:20 dilution) (Santa Cruz Biotechnology, Santa Cruz, California, USA) followed by a Donkey Anti-Goat IgG-Cy3 (1:1500 dilution) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) secondary antibody. FGF-2 expression was detected using an anti-FGF-2 (147) rabbit polyclonal IgG antibody (1:20 dilution) (Santa Cruz Biotechnology, Santa Cruz, California, USA) followed by a Goat Anti-Rabbit IgG-Cy3 (1:1500 dilution) (Santa Cruz Biotechnology, Santa Cruz, California, USA) secondary antibody. DAPI (0.2µg/ml) (Sigma, St Louis, Missouri, USA) was used to stain the nuclei. Images were taken with Leica DM6000B (Wetzlar, Germany, www.leica-microsystems.com).
**Primary transplantations of transduced cells**

Following transduction, 3 x 10^6 CD45.1 BM cells were transplanted into lethally irradiated (9 Gy, IBL 637 Csi-source 0.7026Gy/min, CIS Biointernational, Gif-sur-Yvette, France) CD45.2 recipients. GFP^+ cells were not selected, thus transplants consisted of a mixture of both transduced and non-transduced cells. Each transplant group consisted of 10 recipients, and two independent experiments were performed. Following transplantation, blood samples (60μl) were taken monthly to determine donor chimerism. Levels of chimerism were determined by detecting the presence of CD45.1^+ and GFP^+ cells in transplanted mice. To detect CD45.1^+ cells, cells were stained with anti-CD45.1 (PE) antibody (BD Pharmingen, San Diego, CA) for 30 minutes and analyzed on a flow cytometer (FACS Calibur; Becton Dickinson Biosciences, San Jose, CA).

**Secondary transplantations of transduced cells**

Four months after primary transplantation, five recipients from each group were randomly selected, sacrificed and BM cells were isolated from two hind legs. Unfractionated BM cells were transplanted into lethally irradiated (9Gy) CD45.2 secondary recipients in three limiting dilutions (1:1, 1:2 and 1:4) with a fixed number of 4 x 10^5 CD45.2 competitor cells. Blood analysis was carried out each month to determine original donor CD45.1^+GFP^+ chimerism levels. Each transplanted group consisted of 5 recipients and two independent experiments were performed.

**Transplantation of cultured transduced cells**

Ten days after cells were plated onto a FBMD-1 stromal layer, all cells (including stromal cells) were removed and CD45.1^+GFP^+ cells were selected by FACS. Lethally irradiated (9Gy) B6 recipients were transplanted with CD45.1^+GFP^+ cells in competition with freshly isolated B6 BM cells. Recipients received; (i) 5 x 10^5 control cells + 5 x 10^5 competitor cells, (ii) 2.8 x 10^5 WT FGF-1 cells + 8.5 x 10^5 competitor cells, (iii) 1.5 x 10^5 S130E cells + 4.4 x 10^5 competitor cells and (iv) 2.5 x 10^5 S130A cells + 7.5 x 10^5 competitor cells. On a monthly basis, blood was analyzed for CD45.1^+GFP^+ chimerism.
Statistical Analysis

P-values were calculated using the Mann-Whitney test or the student’s t-test (assuming unequal variances of the two variables) to test the statistical significance ($p < 0.05$) of different groups. Quantification of CAFC at day 7, 28 and 35 was performed by using maximum likelihood ratio method. The Poisson-based limiting dilution analysis calculation was used with a 95% confidence interval (CI) to determine significant differences at $p < 0.05$. 
Results

Relative expression of FGF-1 and FGF-2 in HSCs

To quantify the normal expression levels of FGF-1 and FGF-2 in hematopoietic stem cells, freshly isolated BM cells were stained with lineage (Lin) specific markers and hematopoietic stem cell markers, Sca-1 and c-Kit. As shown in Figure 3.1A, 5% Lin' cells were separated into four populations; Sca-1'c-Kit', Sca-1'c-Kit', Sca-1'c-Kit' and Sca-1'c-Kit'. The levels of expression of FGF-1 and FGF-2 relative to Sca-1'c-Kit' is shown in Figure 3.1B and 3.1C. Both FGFs are expressed in all four populations. Interestingly, the highest expression of FGF-1 and FGF-2 was in the Lin'Sca-1'c-Kit' population, a population which is highly enriched for primitive hematopoietic stem cells. This further confirmed our hypothesis that FGFs may play an important role in HSCs regulation.

Figure 3.1: Relative expression of FGF-1 and FGF-2 in Lin' Sca-1 and c-Kit populations. (A): FACs plot of Lineage (Lin'), Sca-1 and c-Kit gates from B6 BM cells. Lineage' cells were sorted into four populations, Sca-1'c-Kit', Sca-1'c-Kit', Sca-1'c-Kit' and Sca-1'c-Kit'. (B): Relative expression of FGF-1 by Q-PCR in the four populations of Sca-1 and c-Kit relative to Sca-1'c-Kit' BM cells. (C): Relative expression of FGF-2 by Q-PCR in Sca-1 and c-Kit populations relative to Sca-1'c-Kit' BM cells.
Expression of FGF-1 and FGF-2 in BM cells

A schematic representation of the control, WT FGF-1 and WT FGF-2 retroviral vectors used to transduced 5-FU treated BM cells is shown in Figure 3.2A. The control vector expressing only eGFP was used as a control in all assays. Bone marrow cells harvested from 5-FU treated CD45.1 donor mice were transduced with control, WT FGF-1 and WT FGF-2 retroviral vectors. Transduction efficiencies of 30-40% were always achieved (data not shown). Following retroviral transduction, GFP⁺ cells were selected and cultured for a total of 33 days in StemSpan supplemented with 10%FCS, IL-11, SCF and Flt3L. On a weekly basis, cells were counted for growth kinetic analysis. Cell growth in control, FGF-1 and FGF-2 overexpressing cells was comparable (Figure 3.2B). FGF-1 overexpressing cells maintained slightly higher but not statistically significant cell numbers throughout the 33 days of culturing, peaking at day 26 (Figure 3.2B). At day 26, a 2.6-fold and 16-fold increase (although not statistically significant $p > 0.05$) in FGF-1 overexpressing BM cells was observed when compared to FGF-2 and control cells respectively (Figure 3.2B). Twelve days into the culture period the expression level of FGF-1 and FGF-2 in BM transduced cells was assessed by RT-PCR (Figure 3.2C). The expression of FGF-1 was only detectable in FGF-1 overexpressing BM cells and FGF-2 expression was detected only in FGF-2 overexpressing BM cells (Figure 3.2C). Although both FGF-1 and FGF-2 expression were detected by Q-PCR (Figure 3.1B), their expression in LSK cells by RT-PCR were below detection levels, possibly due to the use of different primers. Protein levels in FGF-1 and FGF-2 overexpressing BM cells correlated with RT-PCR results (Figure 3.2D). The relative expression of both FGFs in transduced BM cells was also examined by Q-PCR. As expected, high expression levels of FGF-1 and FGF-2 were detected in FGF-1 and FGF-2 overexpressing BM cells respectively (Figure 3.2E). It should be pointed out that expression of FGF-1 and FGF-2 in control cells was extremely low with $C_T$ value of 33 whilst both FGF-1 and FGF-2 displayed $C_T$ values of ~17.
Figure 3.2: Overexpression of WT FGF-1 and WT FGF-2 in 5-FU bone marrow cells. (A): Schematic representation of control, WT FGF-1 and WT FGF-2 vectors used to retrovirally transduce 5-FU BM cells. (B): Cumulative cell growth curve of 5-FU BM cells retrovirally transduced with control, WT FGF-1 and WT FGF-2. Following retroviral transduction, GFP+ cells were selected and cultured for 33 days in StemSpan containing 10%FCS, IL-11, SCF and Flt3L. On a weekly basis cells were counted and passaged. (C): RT-PCR analysis for FGF-1 and FGF-2 expression in cells transduced with control vector, WT FGF-1 and WT FGF-2 after 12 days of culturing. As a further control RNA from non-transduced LSK cells was included. (D): FGF-1 and FGF-2 protein expression in control, WT FGF-1 and WT FGF-2 transduced cells after 12 days of culturing. (E): Using Q-PCR, the relative expression of FGF-1 and FGF-2 in WT FGF-1 and WT FGF-2 transduced cells relative to control transduced cells, was determined five days after initiation of culture respectively. C<sub>T</sub> value of 33 was recorded for control cells, whilst both FGF-1 and FGF-2 contained C<sub>T</sub> values of ~17.
**Subcellular localization of FGF-1 and FGF-2**

A schematic representation of S130E, S130A and NLS/FGF-2 retroviral vectors is shown in Figure 3.3A. Phosphorylation of FGF-1 by protein kinase C occurs at serine 130\(^{22}\). Phosphorylation at this site should initiate the translocation of FGF-1 to the cytosol from the nucleus. Mutating the serine in the phosphorylation site to a negatively charged glutamic acid (E) (S130E) should mimic the constitutively phosphorylated state of FGF-1. Substituting serine to the uncharged alanine (A) (S130A) should mimic the unphosphorylated state of FGF-1 and causing the growth factor to remain in the nucleus\(^{21}\). Similar successful strategies had previously been used with other proteins\(^{38-40}\). The artificial addition of NLS upstream of FGF-2 coding sequence is expected to translocate FGF-2 from the cytosol into the nucleus.

The subcellular localization of FGF-1 and FGF-2 in transduced BM cells was examined using fluorescence microscopy. We were not able to detect GFP and FGF proteins in all spotted cells. Localization patterns of FGF-1 and FGF-2 were therefore only quantified in GFP\(^+\) and FGF double positive cells. FGF-1 and FGF-2 were not detected in control cells (Figure 3.3B and 3.3F respectively), correlating with RT-PCR and western blot data in Figure 3.2C-E. Fluorescent images of BM cells overexpressing WT FGF-1 and WT FGF-2 indicated that both FGF-1 and FGF-2 were localized in the cytoplasm in all cells in which FGF expression could be detected (Figure 3.3C and 3.3G respectively).

The S130E mutant mimics phosphorylated FGF-1. Thus, we hypothesize that it should be constitutively transported to the cytosol. Overlay images of FGF-1 positive cells indicate that the protein is indeed predominantly localized in the cytoplasm (Figure 3.3D). The S130A mutant lacks a phosphorylation site. Therefore, FGF-1 was expected to be localized in the nucleus. In FGF-1 positive cells (n = 100), 2% of cells showed nuclear staining (Figure 3.3Ei) and the remaining 98% showed cytoplasmic staining (Figure 3.3Eii). Similar to S130A mutant, in NLS/FGF-2 overexpressing cells, FGF-2 was expected to be present in the nucleus of cells. In 11% (n = 100) of FGF-2 positive cells, FGF-2 was indeed localized in the nucleus (Figure 3.3H). In remaining cells, FGF-2 was found in the cytoplasm (results not shown). Although only a small percentage of FGF-1 and FGF-2 were localized to the nucleus, the localization pattern was distinct when compared to WT FGF-1, WT FGF-2 and S130E, indicating that nuclear localization did occur.
Our findings indicate that FGF-1 and FGF-2 are localized in the cytoplasm of BM cells overexpressing WT FGFs. The localization distribution of FGF-1 and FGF-2 in S130A and NLS/FGF-2 mutants respectively, suggests that the transport of FGF-1 and FGF-2 to and from the nucleus may be a dynamic process. FGFs enter the cytoplasm following FGFR binding. Their NLS will transport FGFs into the nucleus and upon phosphorylation, FGFs exit the nucleus into the cytoplasm. Cellular stress will transport FGFs out of the cell.41

Growth kinetics of S130 and NLS/FGF2 mutants

Overexpression of both S130 mutants showed similar cell growth kinetics when compared to the control vector in BM cells (Figure 3.4A). However, WT FGF-1 appears to have a slight increase in cell numbers suggesting that the change in phosphorylation status of FGF-1 may not affect the mitogenic effect of BM cells. It should be noted that growth kinetics of WT FGF-1 and control BM cells are the same data as shown in Figure 3.2B.

It is often thought that nuclear localization of FGFs would increase its mitogenic effect17;18;29. Bone marrow cells overexpressing NLS/FGF-2 displayed an increase in cell growth during the culture period compared to WT FGF-2 and control BM cells. Thirty-three days after initiation of culture there was a 7-fold increase in NLS/FGF-2 overexpressing cells when compared to WT FGF-2 overexpressing BM cells respectively (Figure 3.4B). This suggests that NLS/FGF-2 increases the mitogenic activity of BM cells. It should be noted that growth kinetics of WT FGF-2 and control BM cells are the same data as shown in Figure 3.2B.
Figure 3.3: Overexpression and subcellular localization of S130 mutants and NLS/FGF2 in 5-FU bone marrow cells. (A): Schematic representation of S130 and NLS/FGF-2 retroviral vectors. At the phosphorylation site of FGF-1 serine 130 is replaced with glutamic acid (E) (S130E) or alanine (A) (S130A). Subcellular localization of FGF-1 and FGF-2 in transduced BM cells. Fluorescent microscope images of BM cells transduced with (B): control vector stained with anti-FGF-1, (C): WT FGF-1, (D): S130E, (Ei): S130A with cytoplasmic localization, (Eii): S130A with nuclear localization, (F): control vector stained with anti-FGF-2, (G): WT FGF-2 and (H): NLS/FGF-2. Localization pattern was quantified in 100 GFP+ cells expressing FGF-1 or FGF-2.
Figure 3.4: Cell growth of 5-FU BM cells overexpressing S130 mutants and NLS/FGF-2. (A): Cumulative cell growth of 5-FU BM cells transduced with control, WT FGF-1, S130E and S130A vectors. Following transduction GFP+ cells were selected and cultured for a total of 33 days in StemSpan supplemented with 10% FCS, IL-11, SCF and Flt3L. Growth curves for control and WT FGF-1 cells are the same as those in Figure 3.1 and were used for comparison purposes. (B): Cumulative cell growth of 5-FU BM cells transduced with control, WT FGF-2 and NLS/FGF-2. Growth curves for control and WT FGF-2 are the same as those in Figure 3.1 and were used for comparison purposes.

Co-culturing of transduced cells on a stromal layer

Previous studies in our group demonstrated that we could culture purified LSK cells in the presence of unfractionated BM16. Based upon these results we sorted 5,000 or 10,000 GFP+ cells transduced with control, WT FGF-1, S130E and S130A onto FBMD-1 stromal layer (Figure 3.5A). Cultures were maintained for a maximum of 14 days. At each data point (day 9 and day 14), non-adherent cells were removed, analyzed and discarded. Analysis of growth kinetics revealed that control and WT
Fibroblast growth factor overexpression

FGF-1 transduced BM cells displayed a slight increase (~ 1.3 fold) in cell numbers compared to both S130 mutants (Figure 3.5B). The co-culturing of transduced BM cells on a stromal layer results in the widespread presence of cobblestones, as can easily be seen from images of co-cultures, taken eleven days after initiation (Figure 3.5C-F). More cobblestones were observed in co-cultures with WT FGF-1 and S130A at day 11 (Figure 3.5D and 3.5F). S130E co-cultures contained more non-adherent cells than the other co-cultures (Figure 3.5E).

**Figure 3.5:** Co-culturing of control, WT FGF-1, S130E and S130A cells with FBMD-1 stromal cells. (A): Schematic representation of co-culturing procedure. BM cells were retrovirally transduced with control, WT FGF-1, S130E and S130A vectors. Following transduction, 5,000 or 10,000 GFP<sup>+</sup> cells were selected and co-culture in a culture flask containing pre-seeded FBMD-1 stromal cells. Cells were cultured in IMDM supplemented with 20% horse serum, hydrocortisone and penicillin/streptomycin containing β-mercaptoethanol. (B): Cumulative cell growth of co-cultured cells. Images of co-cultured cells 11 days after initiation of culture transduced with (C): control, (D): WT FGF-1, (E): S130E and (F): S130A.
Co-cultured cell do not provide long-term repopulation

Nine and 14 days after initiation of co-cultures, non-adherent cells were placed in CFU-GM assay. The number of CFU/GM per $10^5$ cells is shown in Figure 3.6A. CFU-GMs were not detected for control cells and no significant differences were observed between WT FGF-1 and S130 mutants after nine days of culturing (Figure 3.6A). After 14 days of culturing, there was an increase in CFU-GM for S130A transduced cells over the control, WT FGF-1 and S130E transduced cells (Figure 3.6A). In addition, 14 days after initiation of culture, non-adherent cells were placed in a CAFC assay. S130A cells contained higher CAFC day 7 activity, correlating with CFU-GM data (Figure 3.6B). S130E transduced cells were observed to have extremely high CAFC day 28 activity ($30/10^5$, 17-61; 95% confidence interval) whilst CAFC day 28 activity was not detected for the other groups (Figure 3.6B).

Ten days after co-culturing of GFP$^+$ cells with the stromal layer, the whole culture was harvested and CD45.1$^+$GFP$^+$ cells were sorted. CD45.1$^+$GFP$^+$ cells were transplanted in competition with freshly isolated B6 BM cells. Disappointingly, CD45.1$^+$GFP$^+$ cells co-cultured cells failed to provide engraftment 18 weeks after transplant (Figure 3.6C). It should be noted that the calculated chimerism levels have not been corrected for the different number of CD45.1$^+$GFP$^+$ cell transplanted. In conclusion, these results demonstrate that co-culturing of retrovirally transduced BM cells is ineffective.

Primary transplantation of WT FGF-1 and S130 mutants

Bone marrow cells from CD45.1 donor mice were isolated four days after 5-FU administration and retrovirally transduced with control vector and vectors encoding WT FGF-1, S130E and S130A. Following transduction, cells were assayed for CAFC content and transplanted into primary recipients (Figure 3.7A). Transduction efficiencies of $\sim 50\%$ were achieved (data not shown). Unsorted transduced BM cells were placed in a 4-fold limiting dilution in in vitro CAFC assays. No significant differences in CAFC day 7 activity were observed between the different cell sources (Figure 3.7B). Low CAFC day 28 activity was observed for all cells, however control cells contained higher CAFC day 28 activity compared to WT FGF-1, S130E and S130A (Figure 3.7B). Following transduction, $3 \times 10^6$ non-fractionated BM cells were transplanted into primary lethally irradiated recipients. Donor chimerism analysis of CD45.1$^+$GFP$^+$ cells showed no differences in engraftment between control, S130E
and S130A overexpressing cells (Figure 3.7C). Engraftment of FGF-1 overexpressing cells throughout the transplant period was lower compared to control and S130 cells. A ~2.5-fold decrease in engraftment was observed, 24 weeks after transplant (Figure 3.7C). Donor chimerism levels of S130E and S130A transplanted animals were significantly higher than control and WT FGF-1 chimerism levels ($p < 0.05$) only at 20 weeks post transplant (Figure 3.7C).

**Figure 3.6:** In vitro assays and in vivo repopulating assay of co-cultured cells. (A): CFU-GM analysis of control, WT FGF-1, S130E and S130A overexpressing cells, 9 and 14 days after initiation of culture. (B): Day 7 and day 35 CAFC activity of co-cultured cells 14 days after initiation of culture. (C): Average CD45.1$^*$GFP$^*$ donor chimerism levels 18 weeks post transplant from recipients transplanted with co-cultured control, WT FGF-1, S130E and S130A cells 10 days after initiation of culture.
Figure 3.7: Transplantation of WT FGF-1, S130E and S130A overexpressing cells into primary and secondary recipients. (A): Schematic representation of transplantation strategy. 5-FU treated BM cells were retrovirally transduced with control, WT FGF-1, WT FGF-2, S130E, S130A and NLS/FGF-2 vectors. Following transduction, 3 x 10^6 unsorted BM cells were transplanted into lethally irradiated (9Gy) primary B6 recipients. Parallel to this, transduced cells were analyzed for CAFC. Twenty weeks after transplant, BM cells were harvested from primary recipients and transplanted in limiting dilutions (1:1, 1:2 and 1:4) with 4 x 10^5 competitors into lethally irradiated secondary recipients. (B): Day 7 and day 28 CAFC activity of control, WT FGF-1, S130E and S130A overexpressing cells. (C): Average CD45.1+GFP+ donor chimerism levels of primary recipients (n=10 per group) transplanted with control, WT FGF-1, S130E and S130A overexpressing cells. Chimerism in blood was analyzed on a monthly basis. (D): LSK analysis of BM harvested from primary recipients 20 weeks after transplant.
Secondary transplantation of WT FGF-1 and S130 mutants

Twenty weeks after transplant, BM was harvested from primary recipient mice and analyzed for Lin−Sca-1−c-Kit+ (LSK) expression. In a normal C57BL/6 mouse, BM cells contained 0.22 ± 0.05% LSK cells (Figure 3.7D). The percentages of LSK cells in control, WT FGF-1 and S130A transplanted mice were comparable to that of B6 BM (Figure 3.7D). A small increase (1.45-fold) in the percentage of LSK cells was observed in mice transplanted with S130E overexpressing BM cells (Figure 3.7D).

Secondary recipients were transplanted with 2 x 10^5 unfractionated donor BM cells in competition with a fixed dose of 4 x 10^5 freshly isolated B6 CD45.2 BM cells (Figure 3.7A). Recipients were analyzed for the presence of CD45.1+GFP+ cells on a monthly basis. As shown in Figure 3.8A, 24 weeks after transplant mice transplanted with control, WT FGF-1 and S130E retrovirally transduced cells displayed CD45.1+GFP+ chimerism levels of ~8-10%. In contrast, S130A transduced cells displayed high donor derived contribution in the peripheral blood. Chimerism levels of ~40% were reached (Figure 3.8A). Individual donor chimerism levels of S130A secondary recipients demonstrate that the effect brought about by S130A was delayed, with chimerism levels only beginning to increase 12 weeks after transplant (Figure 3.8B). FACS plots (inset) illustrate the increase in GFP+ cells from 4 weeks to 24 weeks after transplant (Figure 3.8B). Comparison of S130A transduced cells (GFP+) over non-transduced cells (GFP−), revealed a significant (p < 0.05) 10-fold increase in GFP+ cells compared to control, WT FGF-1 and S130E transduced cells, 24 weeks after transplant (Figure 3.8C). These results suggest that transport of FGF-1 into the nucleus may have an intracrine function maintaining stem cell quality.
Figure 3.8: S130A increases donor chimerism levels in secondary recipients transplanted with $2 \times 10^5$ S130A overexpressing cells and $4 \times 10^5$ competitor cells. (A): Average donor chimerism (CD45.1^+GFP^+) levels 24 weeks post transplant of secondary transplant recipients. (n=5 per group). (B): Individual donor chimerism of secondary recipients transplanted with S130A transduced cells. FACS plots highlight the increase in GFP^+ cells during the transplant period. (C): Ratio of transduced GFP^+ cells and non-transduced GFP^− cells.

Primary transplantation of WT FGF-2 and NLS/FGF-2

Similar to WT FGF-1 and S130 mutants, 5-FU treated BM cells were retrovirally transduced with control, WT FGF-2 and NLS/FGF-2. Transduction efficiencies of
~50% were achieved (data not shown). Following transduction, unfractionated cells were assayed for CAFC content in 3-fold limiting dilutions. CAFC day 7 activity for control transduced cells was 3- and 2-fold higher than WT FGF-2 and NLS/FGF-2 transduced cells respectively (Figure 3.9A), indicating that control cells contain more progenitors. Low CAFC day 35 activity was observed for all three groups (Figure 3.9A).

Lethally irradiated mice were transplanted with 3 x 10⁶ unsorted transduced BM cells. Monthly donor chimerism analysis (CD45.1⁺GFP⁺) showed that WT FGF-2 and NLS/FGF-2 transduced cells had comparable chimerism levels (~16% ± 5, 20 weeks after transplant) whilst control transduced cells maintain significantly higher chimerism levels (29% ± 6, 20 weeks after transplant) during the 20 week transplantation period (p < 0.05) (Figure 3.9B).

**Secondary transplantation of WT FGF-2 and NLS/FGF-2**

Unfractionated bone marrow from primary recipient mice 20 weeks post-transplant were harvested and analyzed for the percentage of LSK cells. Similar to normal B6 BM cells (0.22 ± 0.05%), WT FGF-2 and NLS/FGF-2 BM cells contained 0.15 ± 0.02% and 0.27 ± 0.05% LSK cells, respectively (Figure 3.9C). Control transduced cells contained 1.7-fold increase in LSK cells compared to B6 BM cells (Figure 3.9C).

As outlined above, secondary recipients were transplanted with harvested donor BM and transplanted in limiting dilutions in competition with 4 x 10⁵ freshly isolated B6 BM cells. Analysis of CD45.1⁺GFP⁺ chimerism levels 24 weeks after transplant indicated that WT FGF-2 and NLS/FGF-2 provided little to no contribution in the peripheral blood (Figure 3.9D). As expected, because of the higher levels of donor chimerism levels in the primary transplant, slightly higher CD45.1⁺GFP⁺ engraftment levels were also detected in secondary recipients of control transduced cells (~4 ± 8%) (Figure 3.9D). Comparison of chimerism levels from the control with that of the previous control group (Figure 3.8A) indicated that chimerism levels from both groups were similar. This confirms that in this experiment the control cells do not have an increase in engraftment, but that WT FGF-2 and NLS/FGF-2 transduced donor cells have poorer stem cell quality and thus poorer engraftment ability.
Figure 3.9: Transplantation of WT FGF-2 and NLS/FGF-2 cells into primary and secondary recipients. (A): Day 7 and day 35 CAFC activity of control, WT FGF-2 and NLS/FGF-2 overexpressing cells. (B): Average CD45.1+GFP+ donor chimerism levels of primary recipients (n=10 per group) transplanted with control, WT FGF-2 and NLS/FGF-2 overexpressing cells. Chimerism in blood was analyzed on a monthly basis. (C): LSK analysis of BM harvested from primary recipients 20 weeks after transplant. (D): Average donor chimerism (CD45.1+GFP+) levels 24 weeks post-transplant of secondary transplant recipients transplanted (n=5 per group).
Discussion
In this study we undertook a detailed analysis of the ability of enforced overexpression of FGF-1 and FGF-2 to maintain and expand hematopoietic cells in vitro and in vivo. We demonstrate that overexpression of FGF-1 slightly increases the mitogenic effect of 5-FU BM cells and that both FGF-1 and FGF-2 are almost exclusively expressed in Lin⁻Sca-1⁺c-Kit⁺ cells, a primitive hematopoietic cell subset. Finally, we show that secondary recipients transplanted with S130A mutant FGF-1 showed a delayed increase in donor chimerism levels and a 10-fold increase in GFP⁺ cells, implying that nuclear localized FGF-1 may play a role in maintaining stem cell quality.
Unfortunately, in in vivo repopulation assays, low donor chimerism levels (~10% to 15%) were detected in primary recipients transplanted with WT FGF-1 and WT FGF-2 and little to no reconstitution was observed in secondary recipients. This was unexpected as previous studies had demonstrated that the exogenous addition of FGFs could maintain whole BM cultures for up to five weeks and generate large numbers of cells with lymphoid and myeloid long-term repopulating capacity. Additionally, the use of FGF-1 expanded BM cells as a source of stem cells for retroviral gene delivery generated 15.5-fold increase in the number of bone marrow-derived competitive repopulating units per mouse and provided radioprotection and long-term BM reconstitution with average myeloid and lymphoid chimerisms of 70% and 50% respectively.
It is well known that FGFs are exogenous growth factors that activate the cell surface receptors, thereby inducing activation of intracellular second messengers. In addition, the receptor-bound growth factor is endocytosed and translocated across the vesicular membrane to reach the cytosol. Several lines of evidence exist indicate that after binding to receptors, FGF-1 and FGF-2 enter the nucleus and that this is required for mitogenic response in certain cell types. This led us to assume that both WT FGF-1 and WT FGF-2 may not be entering the nucleus in order to exert its full mitogenic activity. As a result, two S130 mutants and one NLS/FGF-2 were created to further examine the effect of nuclear localized FGFs on BM cells.
Immunocytochemistry analysis of S130A and NLS/FGF-2 overexpressing cells indicated that FGF-1 and FGF-2 were not exclusively localized in the nucleus in all FGF positive cells. Considering that in only 2% of FGF-1 positive cells the protein...
was localized in the nucleus, the delayed but marked increase in CD45.1^-GFP^+ chimerism levels and the significant 10-fold increase in GFP^+ cells of S130A transduced cells in secondary recipients was exceptional. This suggests that the localization of FGF-1 in the nucleus may play a role in maintaining stem cell quality.

The translocation of FGFs appears to be a dynamic process. For example, FGF-2 has been shown to enter the nucleus, both when added exogenously and when expressed endogenously. In the former case, nuclear uptake is cell cycle-dependent occurring only during G1 to S transition. In the latter case, only the larger (22-25kDa) isoforms are found in the nucleus while the 18kDa form remains cytoplasmic. Through cell fractionation studies Wiedlocha et al., clearly showed that WT FGF-1 was found in all fractions (membrane, cytoplasmic and nucleus) of the cell, S130A was found in the nuclear fraction whereas S130E was mainly in the cytosolic fraction. We wish to emphasize that since we have only analyzed localization of FGF-1 and FGF-2 by immunocytochemistry, it is possible that we are only able to detect small amounts of FGF-1 and FGF-2 in S130A and NLS/FGF-2 in the nuclei. Thus, a more sensitive method such as cell fractionation may be used in future studies.

Based upon previous studies from our group, we hypothesized that a niche in the form of stromal cells may increase the mitogenic activity of transduced cells and maintain stem cell activity. A role is now emerging for the ‘stem-cell niche’ to affect maintenance, differentiation and regulation of self-renewal of HSCs in vivo. Since stromal cells include a high proportion of fibroblast cells that respond to FGFs, especially FGF-2, we co-cultured WT FGF-1, S130E and S130A transduced cells with FBMD-1 stromal cells. Unfortunately, co-culturing of transduced cells with a stromal layer was ineffective as these cells failed to reconstitute the peripheral blood of recipients.

Heparin or heparin sulfate proteoglycans play a pivotal role in stimulating and stabilizing the interaction of FGFs to FGFRs. One possibility as to why no dramatic repopulation was observed in FGF-1 and FGF-2 overexpressing cells may be due to the lack of heparin in cultures. In previous studies involving exogenous FGFs, heparin was also added exogenously. In our current study, heparin was not added. Klingenberg et al., has shown that a correlation exists between mitogenic potency and heparin affinity of FGF-1 phosphorylated mutants. If the reason for the reduced repopulation ability of FGF-1, FGF-2, S130 mutants and NLS/FGF-2 was due to the
lack of heparin, future overexpression studies with FGFs should involve the addition of heparin.


Acknowledgements

We thank Professor C. Jordan from the University of Rochester for providing us with the MIEV vector. We also thank Geert Mesander and Henk Moes for their assistance with cell sorting and the staff of the animal facility for taking care of the animals. This work was supported by the National Institute of Health (R01-HL073710) and the Ubbo Emmius Foundation.
References


