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FGF4 And its Novel Splice Isoform Have Opposing Effects on the Maintenance of Human Embryonic Stem Cell Self Renewal

Yoav Mayshar[1], Eran Rom[2], Irina Chumakov[2], Achia Kronman[1], Avner Yayon[2] and Nissim Benvenisty[1*]

[1]Department of Genetics, Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, Israel; [2] ProChon Biotech Ltd, 7 Golda Meir St., Weizmann Science Park, Nes Ziona 70400, Israel

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Abstract

Human embryonic stem cells (HESCs) are unique in their capacity to self renew while remaining pluripotent. This undifferentiated state must be actively maintained by secreted factors. In order to identify autocrine factors that may support HESC growth we have taken a global genetic approach. Microarray analysis identified FGF4 as a prime candidate for autocrine signaling. Furthermore, the addition of recombinant FGF4 to HESCs supports their proliferation. We show that FGF4 is produced by multiple undifferentiated HESC lines along with a novel FGF4 splice isoform (FGF4si) that codes for the amino-terminal half of FGF4. Strikingly, while FGF4 supports the undifferentiated growth of HESCs, FGF4si effectively counters its effect. We further show that FGF4si is an antagonist of FGF4, shutting down FGF4 induced Erk1/2 phosphorylation. Expression analysis shows that both isoforms are expressed in HESCs and early differentiated cells. However, while FGF4 ceases to be expressed in mature differentiated cells, FGF4si continues to be expressed after cell differentiation. Targeted knock down of FGF4 using siRNA increased differentiation of HESCs, demonstrating the importance of endogenous FGF4 signaling in maintaining their pluripotency. Taken together these results suggest a growth-promoting role for FGF4 in HESCs and a putative feedback inhibition mechanism by a novel FGF4 splice isoform that may serve to promote differentiation at later stages of development.

INTRODUCTION

One of the key issues in human embryonic stem cell (HESC) research involves their unique capacity to self renew while retaining pluripotency [1-3]. Research into the molecular basis of self renewal has been carried out extensively in mice. However, even though several core molecular features have been shown to be conserved between mouse and human embryonic stem (ES) cells, such as the importance of embryonic stem cell specific OCT4 and NANOG expression [4-8], the molecular mechanisms governing the maintenance of the undifferentiated state seem to be markedly different. Predominantly, the two key ligands that have been shown to promote mouse ES cell self renewal, leukemia inhibitory factor (LIF) [9, 10] and bone morphogenetic protein 4 (BMP4) [11], have been respectively shown to be either ineffective or even to induce differentiation in HESCs [12-16]. Although the mechanisms governing HESC self renewal remain largely unknown, recent reports have demonstrated that high doses of basic fibroblast growth factor (FGF2) can support feeder free growth of HESCs [14, 16-18]. Other studies

*Corresponding Author: Nissim Benvenisty, Department of Genetics, The Life Sciences Institute, The Hebrew University, Jerusalem 91904, Israel, Tel: 972-2-6586774, Fax: 972-2-6584972, E-mail: nissimb@mail.ls.huji.ac.il; Received December 18, 2007; accepted for publication December 27, 2007; first published online in Stem Cells Express January 10, 2008. 1066-5099/2008/$30.00/0 doi: 10.1634/stemcells.2007-1037

have suggested TGFβ/activin/nodal signaling to be similarly effective [18-21]. In this study we examine whether growth factors produced by HESCs could be involved in the regulation of their own growth.

Previously, we have analyzed the expression of receptors and secreted factors in HESCs using DNA microarrays. We have shown broad expression of FGFRs as well as a variety of FGFs in HESCs [22]. In this study we expand this analysis and show that among the FGFs expressed in HESCs, FGF2 alone continues to be expressed even after prolonged *in vitro* differentiation. We propose that another FGF family member, FGF4, may play a more physiologically relevant role in the maintenance of HESC self renewal.

FGF4 has been shown to be transcriptionally regulated by a heterodimer of SOX2 and OCT4 transcription factors [23, 24] and ceases to be expressed following induced differentiation. Initially, FGF4 has been identified as an oncogene from stomach cancer and Kaposi sarcoma capable of transforming fibroblasts *in vitro* [25, 26]. The highly restricted expression pattern of FGF4 in the developing embryo [27], along with its roles in embryonic survival and patterning [28, 29], have established FGF4 as a key player in early development. Two major signaling pathways have recently been found to be crucial for HESC growth and maintenance. Using chemical inhibitors it has been shown that the PI-3K/Akt pathway is important for survival and growth of HESCs [30]. Similarly, MEK/Erk signaling was shown to be critical in HESCs, as its inhibition causes differentiation of HESC colonies [31, 32]. Given the robust potential of FGFs in general and FGF2 in particular in activating Erk1/2 [31-33] we set to examine the role of FGF4 in this context.

Here we show that FGF4 is secreted from undifferentiated HESCs along with a newly identified FGF4 splice isoform (FGF4si). Furthermore, while FGF4 promotes the undifferentiated growth of HESCs, FGF4si effectively counters its effect. Expression analysis shows that both isoforms are expressed in HESCs and early differentiated cells. Interestingly, while FGF4 ceases to be expressed in late differentiated cells, FGF4si does not. Taken together these results suggest an autocrine growth-promoting role for FGF4 in HESCs that is restrained by a novel FGF4 splice isoform that may serve to promote differentiation at later stages of development.

MATERIALS AND METHODS

Cell culture. Human ES cells were cultured on Mitomycin-C treated mouse embryonic fibroblast (MEF) feeder layer (obtained from 13.5 day embryos) in 85% KnockOut DMEM medium (GIBCO-BRL), supplemented with 15% KnockOut SR (a serum free formulation) (GIBCO-BRL), 1mM glutamine, 0.1 mM β-mercaptoethanol (Sigma), 1% nonessential amino acids stock (GIBCO-BRL), Penicillin (50 units/ml), Streptomycin (50 μg/ml), ITSX100 (insulin-transferrin-selenium) in a 1:300 dilution (GIBCO-Invirogen Corporation), and 4 ng/ml basic fibroblast growth factor (FGF2, GIBCO-Invirogen Corporation). Cells were passaged using trypsin-EDTA (Biological industries, Israel). To obtain feeder free cultures, cells were plated on laminin (1μg/cm^2, Sigma) or gelatin (0.1%, Merck) coated plates and grown with medium conditioned by MEFs (CM). Differentiation in-vitro into embryonic bodies (EBs) was performed by typsinizing the cells to a near single cell suspension, withdrawal of FGF2 from the growth media and allowing aggregation in petri dishes.

Western blot analysis. HESCs were grown for one passage without feeders on gelatin coated plates with MEF conditioned medium. Semi confluent plates were washed several times with phosphate buffered saline (PBS) and medium was substituted with a serum replacement free medium supplemented with ITSx100 at 1:150 dilution, but otherwise similar to the regular growth medium used. Following 24hrs incubation medium was collected and mixed

with a single wash of PBS. The medium sample was separated on a heparin affinity column using an identical NaCl elution gradient. Resulting fractions were pooled according to the elution step as unbound, low and high (0.2M NaCl, 0.5M NaCl and 2M NaCl respectively). Pooled fractions were precipitated using 80% trichloroacetic acid (TCA) overnight and resuspended and boiled in sample buffer. Samples were resolved on 12% SDS polyacrylamide gel, and stained using a polyclonal anti-FGF4 antibody (Peprotech). Specificity was validated using recombinant FGF4si and FGF4 proteins and by preincubation of the antibody with recombinant FGF4. For phosphorylation assays cells were grown as above only an additional medium change was performed 3 hours prior to induction and after a PBS wash. Phospho-Erk1/2 were detected using Sigma M8159 monoclonal antibody, and total Erk1/2 by M5670 antibody (Sigma).

Growth analysis. Cells were seeded in 96 well dishes coated with 0.1% gelatin in a density of ~$2X10^4$ cells per cm^2 (6000 cells/well), and medium changed daily. Recombinant human growth factors, midkine, pleiotrophin, FGF2 and FGF4 were obtained from Peprotech, Inc. Cell densities were determined by fixating the cells with 0.5% glutardialdehyde (Sigma), staining with Methylene Blue (Sigma) dissolved in 0.1M boric acid (pH=8.5). Color extraction was performed using 0.1M hydrochloric acid and the staining (which is proportional to cell number) was quantitated by measuring absorbance at 650nM.

RNA extraction and RT-PCR analysis. RNA was extracted using TRI-reagent for total RNA isolation according to the manufacturers' instructions (Sigma). cDNA was synthesized using random hexamer primers (Roche). Amplification was performed on the cDNA using Takara Ex-Taq. All PCR reactions for FGF4 and FGF4si were performed in the presence of 8% DMSO. GAPDH was used as a house-keeping gene to evaluate and compare quality of different cDNA samples. Primers for GAPDH (62°C annealing, 25 cycles); forward 5'AGCCACATCGCTCAGACACC3', reverse 5'GTACTCAGCGGCCAGCATCG3'. Primers for OCT4 (64°C annealing, 30 cycles); forward 5'CTCACCCTGGGGGTTCTATT3', reverse 5'CTCCAGGTTGCCTCTCACTC3'. Specific primers for FGF4 (60°C annealing, 40 cycles); forward 5'TTCTTCGGGCCATGAGCAG3', reverse 5'CCGAAGAAAGTGCACCAAGG3'. Specific primers for FGF4si (60°C annealing, 40 cycles); forward 5'GACACCCTTCTTCACCGATG3', reverse 5'CTCCAGGTTGCCTCTCACTC3'. Final products were examined by gel electrophoresis on 2% agarose ethidium-bromide stained gels. For sequencing of the complete FGF4 gene products, nested PCR was performed. Outer primers (60°C annealing, 40 cycles); forward 5'TCCTCAGAGTCCCAGCTCCA3', reverse 5'CTCCAGGTTGCCTCTCACTC3'. Inner primers (60°C annealing, 40 cycles); forward 5'TCCATGCAGCCGGGGTAGA3', reverse 5'ACCAAGGTGACCCTCGCACT3'.

DNA microarray analysis. Total RNA was extracted according to the manufacturers protocol (Affymetrix). When extracting RNA from undifferentiated HESCs the cells were grown for one passage on gelatin coated plates with conditioned medium to avoid contamination by feeder cells. Hybridization to U133A/B DNA microarrays, washing and scanning were performed according to the manufacturer's protocol, and expression patterns were compared between samples. Microarrays were analyzed using the affymetrix MAS5 probe condensation algorithm. Results were normalized according to the trimmed mean (95%) of the entire array and multiplied by a factor of 100. Absent calls (non-specific labeling) as judged by MAS5 have been collectively given a value of 10.

Surface-Antigen Expression. Cells were dissociated to single cell suspension using Trypsin-EDTA. Cells were then incubated for 1hr with TRA-1-60 antibody (kind gift of Prof. Peter Andrews and Santa Cruz sc-21705). As a

secondary antibody, Cy3 conjugated rabbit anti-mouse IgM (Jackson ImmunoResearch, dilution 1:200) was used. FACS analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson).

Cloning, expression and purification of hFGF4si. The FGF4si open reading frame (ORF) was amplified by PCR using the following primers and reaction conditions:

forward
5'CATGCCATGGCATCGGGGCCAGGGACG GC3',

reverse
5'ACGTGGATCCGATCGGTGAAGAAGGGT GTCGCGGGTGTCCGCGTG3'

The PCR product was digested with NcoI and BamHI and cloned in a similarly resricted pET32b (pET32b hFGF4si). BL21 bacteria were transformed with the resulting, sequence verified, construct. A single colony was picked and cultured O.N. The next day, bacteria were diluted 100 times and cultured to $O.D_{600}$=0.6. IPTG (0.1mM) was then added for additional O.N. incubation. The O.N. culture pellet was resuspended in phosphate buffer, sonicated and the cleared lysate was loaded on a Ni^{++} column. Phosphate buffer containing increasing concentrations of imidazol was used for washing and eluting the purified hFGF4si. Typically, rhFGF4si eluted at 200mM imidazol. Prior to use rhFGF4si was dialyzed against PBS O.N. at 4^0C.

siRNA knockdown experiments. HESCs were harvested to single cell suspension and plated at low density on gelatin coated plates in presence of MEF CM (5% serum replacer). Transfection of siRNA was carried out the following day using Oligofectamine reagent (Invitrogen) according to the manufacturers' instructions. Specifically, oligofectamine reagent was used at a final concentration of 1:350. siRNA oligos were used at a final concentration of 45nM. siRNA for FGF4 was obtained from Dharmacon (ON-TARGETplus SMARTpool). siRNA for GFP (IDT, Coralville,IA) was synthesized according to previously described sequences [34].

RESULTS

FGF4 is a putative autocrine self renewal promoting factor in HESCs. Previously, we have shown that several growth factors are expressed in HESCs in conjunction with their respective receptors [22]. Here we expand this analysis to examine the factors' temporal expression during spontaneous differentiation and maturation as embryoid bodies (EBs). The 18 growth factors shown in our previous study to be expressed in HESCs, fall into eight different families. Of these, the fibroblast growth factor (FGF) family displays the most dramatic down regulation during late differentiation (Supplementary Fig.1). Notably, FGF4, a known stem cell marker, is among those down regulated in 30 day old EBs (Figure 1A), correlating with the reported down regulation of the embryonic transcription factor OCT4 which is among its activators. In addition to the FGF family, another family of growth factors, the secreted PTN/MK factors and their respective receptors are highly expressed in HESCs (Figure 1C and D). In contrast to the FGF family, both members of the PTN/MK heparin binding factor family (Midkine –MDK and Pleiotrophin - PTN) remained highly expressed throughout differentiation. This suggests that FGF signaling but not PTN/MK signaling would be important for HESCs self renewal.

In order to test this hypothesis, a competitive growth assay was performed to measure the factors' contribution to HESC proliferation. In this assay, cells were plated at low densities in gelatin coated 96 well plates and grown for three days under defined conditions. Subsequently, cells were fixed and cell number was estimated by a methylene blue based colorimetric assay. The results of this experiment show a significant enhancement of cell number following treatment with FGF4 relative to untreated controls and in

contrast to treatment with either PTN or MDK, for which no significant induction was measured (Figure 1E). This increase is however, modest (1.6 fold relative to basal medium control), and significantly lower than that achieved by the addition of mouse embryonic fibroblast (MEF) conditioned medium (CM) (2.6 fold, data not shown). This activity of exogenous FGF4 was observed even after only a short period of time. Given the background of basal exogenous FGF2, and supposed presence of endogenous FGFs, the activity suggests a potential biological significance of FGF4 as an autocrine growth regulator.

A novel FGF4 splice isoform is co-expressed with FGF4 in HESCs. PCR amplification of the entire FGF4 cDNA, revealed, in addition to the expected FGF4 PCR fragment, an additional low molecular weight band which was identified by sequence analysis to be a novel FGF4 splice isoform lacking the second exon (Figure 2A). Isoform specific RT-PCR showed that both FGF4 isoforms are co-expressed in all five HESC lines tested; HES7 and HES9 [35], I3 and I6 [36], (Figure 2B) and H9 [3] (Figure 2C). Additionally, both isoforms were found to be expressed in mouse ES cells but not in MEF (Figure 2B). Sequence analysis revealed that following the excision of the second exon, the third exon is decoded at a +1 frame, resulting in the generation of a premature stop codon. In order to verify the PCR results, and test whether this putative splice isoform is translated, we performed Western blot analysis using a polyclonal FGF4 antibody. According to its nucleotide sequence the putative splice isoform peptide is 118aa long with an expected MW of ~12 kDa as compared to the full length FGF4 protein containing 206aa (MW~22kDa) . Examination of the heparin-binding fraction of HESC conditioned medium reveals two specific bands at 25kDa and 14kDa corresponding to both FGF4 and the FGF4 splice isoform (FGF4si), respectively (Figure 2A). These results demonstrate that both FGF4 and FGF4si are translated and actively secreted by HESCs.

FGF4si displays a distinct expression pattern relative to FGF4 and continues to be expressed in late differentiated HESC derivatives. In light of the discovery of the novel splice isoform a re-examination of the developmental regulation of FGF4 expression was required. As the microarray probes do not discriminate between the two isoforms, PCR amplification was performed at different time-points during HESC differentiation using specific primers for FGF4 and FGF4si (Figure 2C). Interestingly, a distinct pattern of gene expression was observed for each isoform. Whereas full length FGF4 is downregulated following long-term differentiation, FGF4si continues to be expressed at this stage. The continued expression of FGF4si at a developmental stage in which FGF4 ceases to be expressed indicated that FGF4si might have a unique biological role rather than being a simple by-product of FGF4.

FGF4 helps maintain the pluripotent state of HESCs and is antagonized by FGF4si. To examine the possible effect of FGF4si on the growth of HESCs, we first produced recombinant FGF4si. Parallel experiments were set, in which an identical number of cells were plated onto laminin-coated plates. The cultures were allowed to grow for five days with daily replacement of either basal medium, MEF-CM, or media containing either FGF4 with or without FGF4si, or FGF2. HESCs treated with either 50ng/ml or 100ng/ml FGF4 or 100ng/ml FGF2 grew as large and defined colonies characteristic of undifferentiated cells (Figure 3). The addition of 100ng/ml FGF4si to FGF4 treated cells caused a reduction in colony size with more differentiated cell morphologies, very similar to the morphology of the untreated control (Figure 3). To further investigate the nature of the observed effect, the fractions of undifferentiated cells in the different cultures were assessed by FACS using Tra-1-60 antibody. The observed percentage of undifferentiated cells was then multiplied by the total cell count for each culture condition to yield the total number of undifferentiated cells per culture. A dramatic

increase in undifferentiated cell numbers in FGF2 and FGF4 treated cultures were observed relative to basal medium control (3.2 fold increase for FGF2 100ng/ml and 2.7 and 2 fold increase for 50ng/ml and 100ng/ml FGF4, respectively) (Figure 4A). The effect of FGF4 was abolished when FGF4si was added to the medium. Thus FGF4si caused a significant reduction in the number of undifferentiated HESCs in the cultures treated with FGF4 ($p<0.005$) (Figure 4A). Taken together these results clearly demonstrate a significant effect of FGF4 in the undifferentiated growth potential of HESCs, an effect countered by FGF4si.

In order to asses the autocrine potential of FGF4 in HESCs we used an siRNA strategy to knock down FGF4 expression. HESCs constitutively expressing GFP were co-transfected with siRNA against both FGF4 and GFP. After several days in culture the differentiation levels of the GFP negative cells were evaluated in relation to GFP positive ones. FACS analysis showed a modest but significant increase in differentiation in the FGF4 knock down cells as measured by the loss of the TRA-1-60 marker (Figure 4B).

FGF4si inhibits FGF4 induced Erk1/2 phosphorylation in HESCs. To obtain insight into the underlying mechanism of FGF4si action we set to investigate its effect on FGF4 induced signal transduction. It was previously shown that FGF stimulation causes robust phosphorylation of the mitogen-activated protein kinases Erk1/2 [31, 37]. In order to test the activated levels of Erk1/2, HESCs were starved and then stimulated with FGF2 or FGF4 with or without FGF4si. Two cell lines were thus tested, H9 (Figure 5A) and HES9 (Figure 5B). Both FGFs strongly induced Erk1/2 phosphorylation as shown by Western blot analysis. The stimulation of Erk1/2 phosphorylation by FGF4 was significantly reduced by FGF4si in both cell lines. However, the effect on Erk1 was more prominent as its phosphorylation was virtually eliminated in the presence of FGF4si. The specificity of FGF4si antagonistic action was demonstrated by the relatively weak inhibition of FGF2 stimulated MAPK.

DISCUSSION

With the recent derivation of HESCs using mouse ES cell based technique, it could have been presumed that these two cell types will be highly similar. Indeed, several mouse stem cell markers were also shown to be expressed in HESCs and to have similar function [1, 5, 6, 22]. However, the mechanisms governing the active maintenance of the pluripotent state were found to be remarkably different. Notably, leukemia inhibitory factor (LIF), that in mouse works in conjunction with BMP4 in maintaining ES cell self-renewal, has been found to be ineffective in human [12, 13], while BMP4 induced differentiation to trophoblast [15]. Concurrently, FGF signaling has been shown to promote the differentiation of mouse ES cells [11, 38-41], while in HESCs FGF2 has been shown to help maintain pluripotency [14, 16-18]. Clearly, understanding the molecular basis of this essential trait will lead to significant improvement in culture technique and deepen our understanding of one of the most primal events of embryonic development.

In contrast to the majority of studies hitherto published, attempting to characterize growth factors important for self renewal in HESCs, we decided to focus on the identification of endogenous growth factors that could affect HESC fate. Thus we performed DNA microarray analysis of HESC samples, along with samples of *in vitro* differentiated embryoid bodies of successive stages (2, 10 and 30 day EBs) [22]. This analysis identified eighteen growth factors, where both ligand and receptor are expressed in undifferentiated HESCs. Of these, seven showed significant down-regulation in their expression upon differentiation. Marked among the downregulated genes are those of the FGF family in which the expression level of three out of four expressed members are dramatically reduced upon late differentiation. Given the broad expression of FGF receptors (FGFRs) in

HESCs [22, 31] it seems plausible that FGFs could function as autocrine signals in HESC cultures.

Using a short term proliferation assay with low density cultures seeded as single cells, we identified FGF4 as a factor capable of enhancing the growth potential of HESCs under these conditions. Subsequent cloning of the gene revealed a novel alternative splice variant, termed FGF4si. Analysis of additional HESC lines as well as a mouse ES cell line showed FGF4si to be expressed in all of them but not in MEF. FGF4si lacks the second exon as verified by sequence analysis of the RT-PCR products, the absence of which, results in a ribosomal frameshift leading to premature translation termination. Thus FGF4si shares with FGF4 the first 113 N-terminal amino acids yet lacks a substantial portion of the proteins C-terminus, including several predicted heparin and receptor binding sites [42]. Western blot analysis using an FGF4 polyclonal antiserum identified both isoforms of FGF4 in the heparin bound fraction of HESC conditioned medium. Thus, we demonstrated that both proteins are actively secreted by HESCs and bind heparin. Indeed at least one heparin binding site remains intact in this splice isoform [42]. Interestingly, we show that the expression pattern of FGF4si differs from that of FGF4, in that it remains expressed even following late differentiation *in vitro*.

The continued expression of FGF4si in the absence of FGF4 prompted us to investigate the possible role of serine/arginine-rich (SR) proteins which are known to modulate alternative splicing [43]. Thus, we examined in HESCs and EBs the expression of four SR proteins with known recognition motifs, namely; SC35, SRp40, SRp55, and SF2/ASF. Comparison of DNA microarray data shows that SC35 and SF2/ASF genes are overexpressed in HESCs relative to EBs (Figure 6A). Subsequent prediction of recognition motifs for the SR proteins [44] identified numerous such sites for SC35 and SF2/ASF along the second exon and flanking introns. However, recognition sites for SRp55 and SRp40 that were expressed at similar levels in HESCs and EBs were less abundant and only one site displayed the same level of confidence (Figure 6B). Taken together these results suggest that a general shift in SR proteins expression during differentiation of HESCs may specifically cause the transition from coexpression of both the FGF4 isoforms to the continued expression of FGF4si alone.

In order to test whether FGF4si has a distinct biological role, we generated a recombinant form of the predicted protein. By adding recombinant FGF4si to the growth medium of undifferentiated HESCs, we show that FGF4si opposes FGF4 activity. We demonstrate that FGF4 works to actively maintain the undifferentiated cell population indistinguishably from FGF2. However, FGF4si acts to counter this effect. Thus, cultures grown in the presence of FGF4 and FGF4si together grew similarly to control cultures by all parameters tested; morphology, cell number, and percentage of undifferentiated cells. However, it remains to be investigated which aspect of HESC growth is affected in this system. Elevated cell numbers may be the result of an increase in cell survival in the initiation of clonal growth, increased proliferation rate of HESCs or inhibition of differentiation. Any of these effects may give rise to the observed changes in culture.

Following the results using recombinant proteins we decided to evaluate the role of FGF4 as a potential autocrine signal. Using an siRNA mediated knock down approach we could show a significant increase in differentiation in cells receiving siRNA for FGF4. This effect was however modest and may be an underestimation of the true importance of the FGF4 signal. This may be due to redundancy of FGF signals in HESCs, compensation of FGF4 by untransfected cells, or due to the knock down of both FGF4si and FGF4 resulting from sequence identity that precluded us from using isoform specific siRNA.

In light of the biological effects of FGF4 and FGF4si we examined their effect on the

downstream MAPKs Erk1/2. Previous studies have shown robust phosphorylation of both Erks in response to FGF2 stimulation in HESCs [31, 32]. Interestingly while the activation of Erk signaling has been implicated using chemical inhibitors to be necessary for HESC self renewal [32], studies in the mouse have shown that Erk activity inhibits self renewal and promotes differentiation [37, 45]. Similar to FGF2 stimulation we could see robust activation of Erk1/2 by FGF4. Most importantly however, we could clearly show an almost complete elimination of FGF4 stimulated Erk1/2 phosphorylation with the addition of FGF4si. Moreover, we could show that this antagonistic effect is preferentially directed against FGF4, as FGF2 stimulation of Erk1/2 was less affected by FGF4si. An additional downstream effector of receptor tyrosine kinases (RTKs) is the PI-3K/AKT pathway which has been shown to promote survival of HESCs in response to neurotrophins [30]. However, Western bolt analysis with anti-phospho-AKT antibodies showed no effect of either FGF tested on AKT stimulation (data not shown).

Taken together, these results indicate an important role for FGF4 signaling in HESC growth and differentiation. Our study furthermore describes a novel endogenous control mechanism that regulates FGFR signaling by inhibition of FGF4 mediated Erk1/2 activation. We suggest a model, in which FGF4, expressed in undifferentiated cells in conjunction with FGF4si, is prevalent, and actively helps maintain HESCs in their undifferentiated state. During differentiation, with the decline of FGF4 expression and the continued expression of FGF4si, this balance shifts possibly due to global changes in SR proteins expression, shutting down remaining FGF4 signaling and effectively promoting differentiation.

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Figure 1. FGF4 is a potential autocrine regulator of HESC growth. (A) Shown is the RNA expression profile of the members of the FGF family expressed in HESCs, and of the PTN/MK growth factors (C) during *in vitro* differentiation as embryoid bodies (EBs) for the indicated number of days. Additionally, the expression levels of the corresponding receptors in undifferentiated cells are shown (B and D respectively). Analysis was performed using the Affymetrix U133 DNA microarray set and each experiment performed in triplicate. Expression levels were normalized and centered to an average value of 100. Error bars represent s.e.m.

(E) Shown are relative cell numbers of treated HESC cultures. Cells were seeded in gelatin coated 96 well plates ($6x10^3$ cells/well) and grown in the presence of the indicated factors at two doses for three days. Cells were Methylene Blue stained and absorbance (650nm) was used as an indication of relative cell number. The values shown represent the relative cell number in respect to control wells, and are the average of three independent experiments, each performed in triplicate. FGF4, (17 or 50ng/ml), enhanced the number of cells in the cultures significantly in respect to controls (*P<0.05). Error bars represent s.e.m.

Fig 1

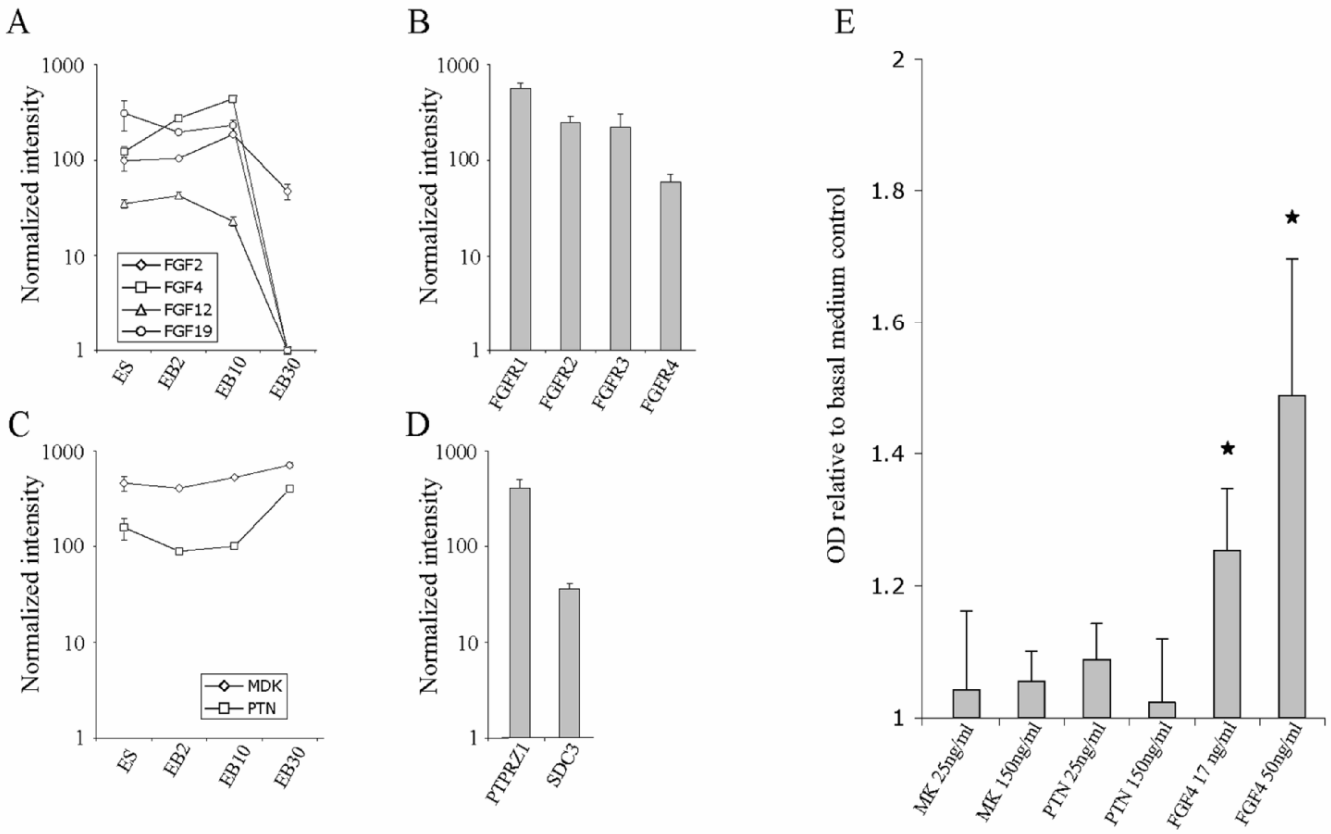


Figure 2. A novel splice isoform of FGF4 is expressed and secreted by HESCs. (A) RT-PCR amplification of the entire FGF4 transcript using primers specific for the RNA untranslated regions reveals two distinct products in HESCs. The bands were sequenced and found to correspond to the expected full-length transcript (upper band) and to a novel splice isoform (FGF4si) lacking the second exon (lower band). The cartoon depicts both splice events and illustrates the introduction of a premature stop codon by frameshift, caused by the loss of the second exon in FGF4si (stop codons indicated by flags). HESC conditioned medium (CM) was analyzed by immunobloting using a polyclonal antibody directed against recombinant human FGF4. Prior to analysis the sample was run on a heparin affinity column. Shown is the high salt elution fraction (2M NaCl). Recombinant human FGF4 (182aa, 19.7kDa) 2ng and 10ng, served as control. Arrow heads on the left indicate specific labeling.
(B) Expression of FGF4 and FGF4si was examined in several HESC lines (I3, I6, HES7, HES9), in a line of mouse ES cells (E14Tg2a) and MEF. (C) The expression pattern of FGF4 and FGF4si during differentiation was examined by RT-PCR in H9 HESCs. Three stages of differentiation were thus tested: Undifferentiated HESCs (ES) and 2 and 30 day old embryoid bodies (EB2 and EB30 respectively). RT-PCR was performed using specific primers for each transcript. The temporal expression of the FGF4 isoforms was compared to the expression pattern of OCT4, a known regulator of FGF4 transcription and a marker of undifferentiated cells. GAPDH was used as a positive control.

Fig 2

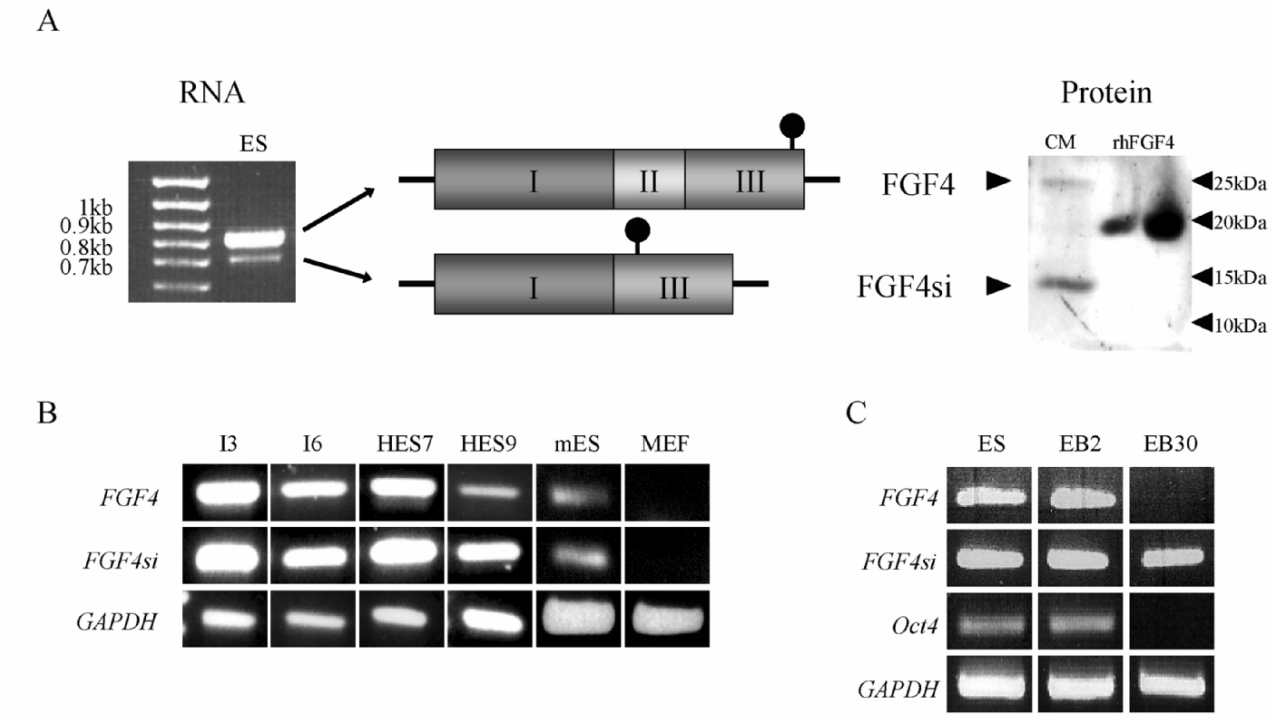


Figure 3. FGF4si blocks the self-renewal activity of FGF4. $2x10^4$ cells/cm^2 were seeded on laminin coated plates. Cultures were grown in basal medium with the addition of the indicated growth factors. Medium was replaced daily and representative fields photographed following four days.

Fig 3

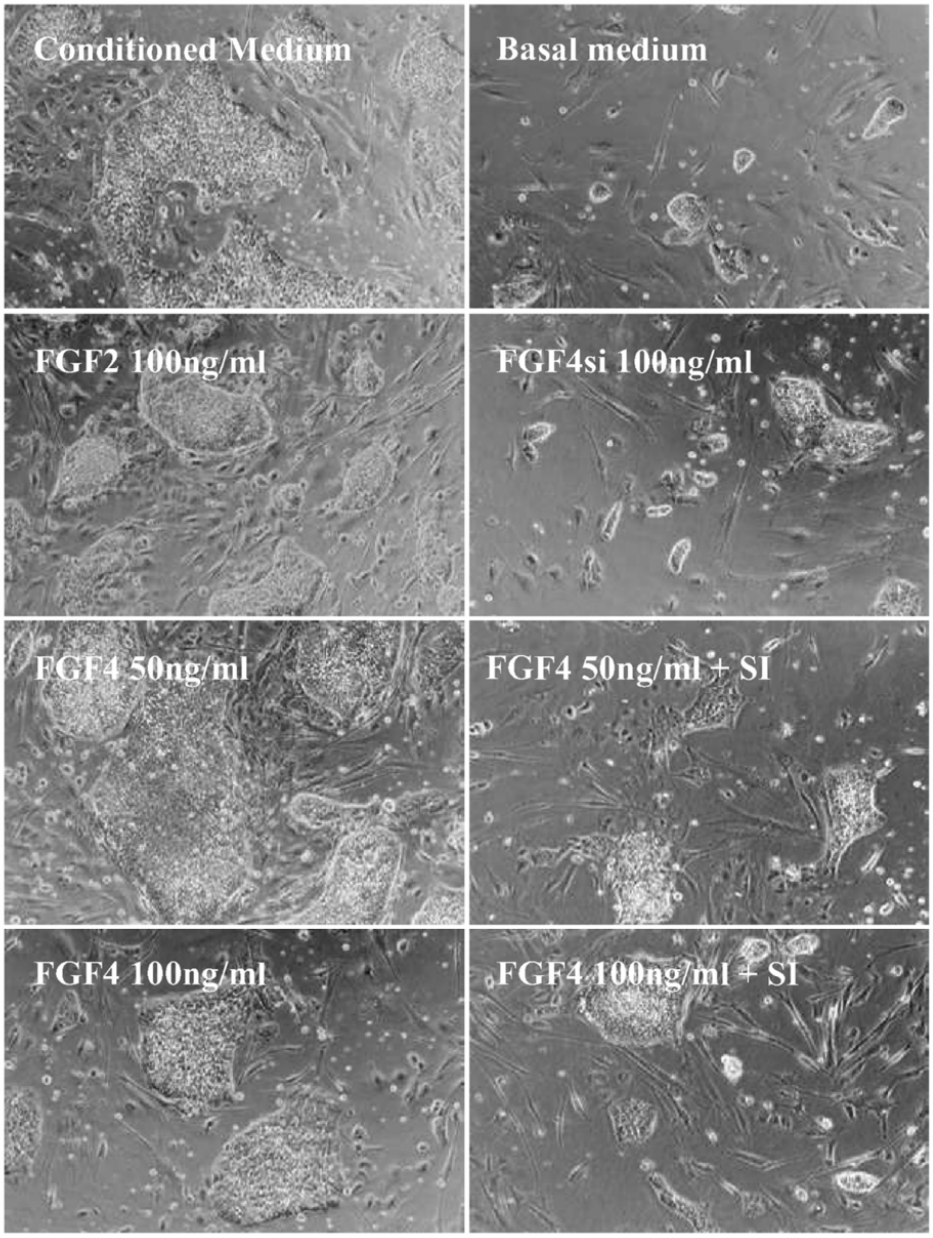


Figure 4. (A) FGF4 causes an increase in the number of undifferentiated cells in culture and is antagonized by FGF4si. $2x10^4$ cells/cm^2 were seeded on laminin coated plates. Cultures were grown in basal medium with the addition of the indicated growth factors and medium replaced daily. Following five days cells were analyzed by FACS using TRA-1-60 antibody. Shown are the results of multiplication of the percentage of TRA-1-60 positive cells with the total cell count. FGF4si (SI) added together with FGF4 effectively abolished the growth enhancement conferred by FGF4 alone (*P<0.005). Experiments were performed in triplicate. Error bars represent s.e.m. (B) Shown are the percentages of TRA-1-60$^-$ cells of successfully transfected HESCs as judged by GFP reporter knock down. GFP siRNA only, serves as control (*P<0.05).

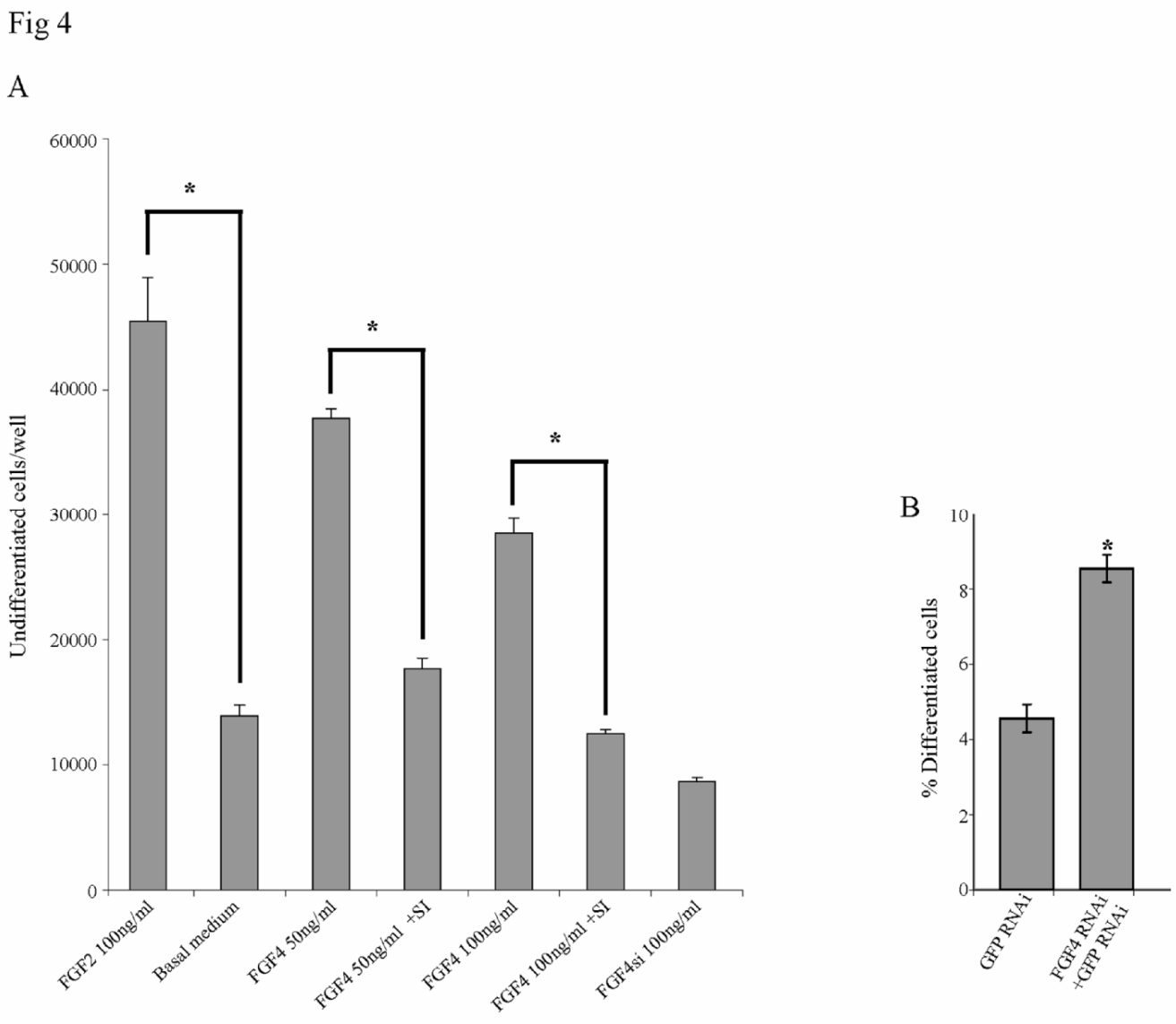


Figure 5. FGF4si antagonizes FGF4 induced Erk1/2 activation. H9 (A) and HES9 (B) HESCs were treated with either FGF2 or FGF4 (50ng each) with and without FGF4si (100ng). Cells were incubated for the indicated amount of time and phosphorylation levels of Erk1/2 determined by Western blot using phospho-specific anti-Erk1/2 antibody. Reprobing with anti-total Erk1/2 antibody serves as loading control.

Fig5

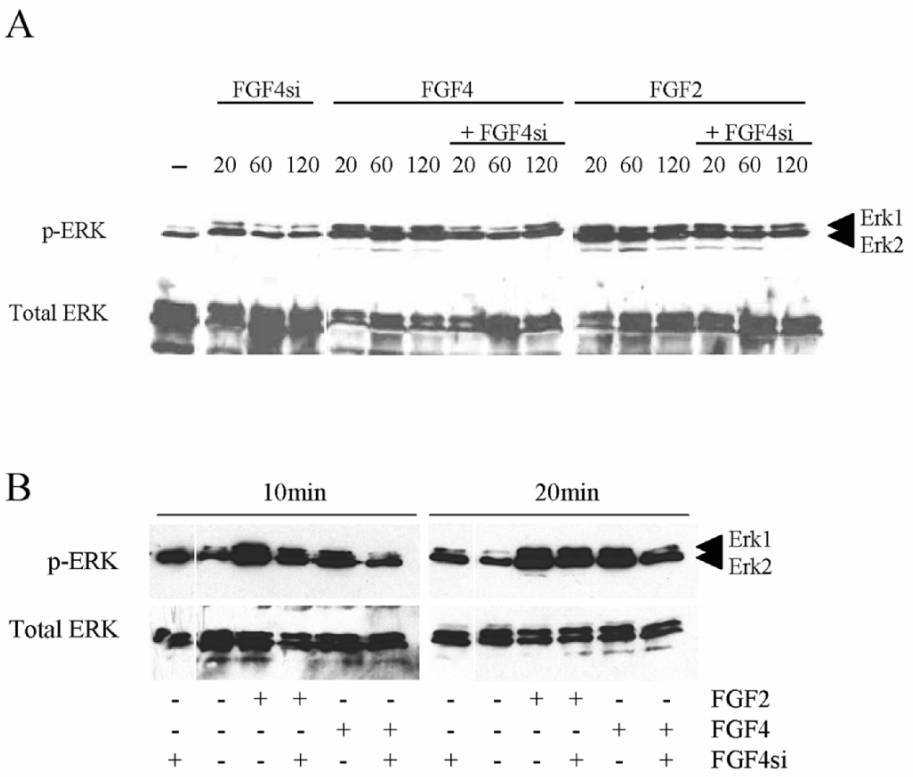
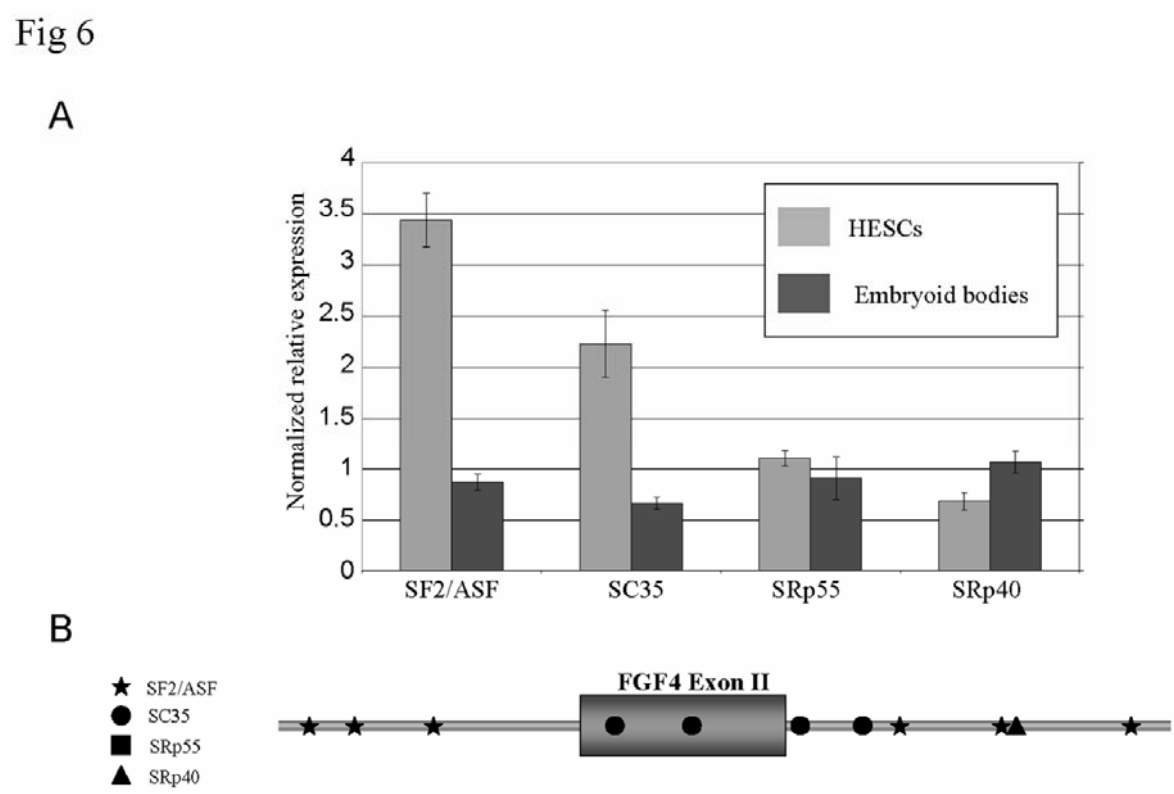


Figure 6. Changes in SR proteins levels may explain shift in expression of FGF4 isoforms during differentiation. (A) RNA expression levels of four SR proteins were examined using DNA microarrays. Expression values were normalized to a baseline of averaged normal human adult tissues [46]. (B) Recognition motifs of SR proteins were predicted using ESEFinder 3.0 online resource [44, 47]. Shown are recognition motifs identified above a threshold of 4, along exon2 and 200bp flanking introns.



FGF4 And its Novel Splice Isoform Have Opposing Effects on the Maintenance of Human Embryonic Stem Cell Self Renewal

Yoav Mayshar, Eran Rom, Irina Chumakov, Achia Kronman, Avner Yayon and Nissim Benvenisty

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