Role of Fibroblast Growth Factor-2 Isoforms in the Effect of Estradiol on Endothelial Cell Migration and Proliferation


Abstract—Both 17β-estradiol (E2) and fibroblast growth factor-2 (FGF2) stimulate angiogenesis and endothelial cell migration and proliferation. The first goal of this study was to explore the potential link between this hormone and this growth factor. E2-stimulated angiogenesis in SC Matrigel plugs in Fgf2+/+ mice, but not in Fgf2−/− mice. Cell cultures from subcutaneous Matrigel plugs demonstrated that E2 increased both migration and proliferation in endothelial cells from Fgf2+/+ mice, but not from in Fgf2−/− mice. Several isoforms of fibroblast growth factor-2 (FGF2) are expressed: the low molecular weight 18-kDa protein (FGF2lmw) is secreted and activates tyrosine kinase receptors (FGFRs), whereas the high molecular weight (21 and 22 kDa) isoforms (FGF2hmw) remains intranuclear, but their role is mainly unknown. The second goal of this study was to explore the respective roles of FGF2 isoforms in the effects of E2. We thus thus generated mice deficient only in the FGF2hmw (Fgf2hmw−/−). E2 stimulated in vivo angiogenesis and in vitro migration in endothelial cells from Fgf2hmw−/− as it did in Fgf2+/+ mice. E2 increased FGF2hmw protein abundance in endothelial cell cultures from Fgf2+/+ and Fgf2hmw−/− mice. As shown using siRNA transfection, these effects were FGFR independent but involved FGF2-Interacting Factor, an intracellular FGF2hmw partner. This is the first report for a physiological role for the intracellular FGF2hmw found to mediate the effect of E2 on endothelial cell migration via an intracrine action. (Circ Res. 2004;94:1301-1309.)

Key Words: mouse • estradiol • growth factor • endothelium • migration

Endothelium, being uniquely positioned at the interface between the blood and the vessel wall, plays a crucial role in the physiology of circulation by performing multiple functions.1,2 It is involved in the regulation of coagulation, leukocyte adhesion in inflammation, transvascular flux of cells, liquids, and solutes, vessel tone, and vascular smooth muscle growth. Endothelium also constitutes a target for the sex hormone, 17β-estradiol (E2). Using a series of experimental models, E2 has been reported to promote angiogenic activity and endothelial cell migration and proliferation.3 The angiogenic effect is mediated through the estrogen receptor α (ERα).4 However, the mechanisms involved downstream of ERα remain unclear.5

Fibroblast growth factor-2 (FGF2) is an important mitogenic and angiogenic factor that stimulates endothelial cell growth and migration. Therefore, FGF2 could be a good candidate to be a partner of E2. However, FGF2 expression is complex because at least four (18, 22, 22.5, and 24 kDa) in human and three (18, 21, and 22 kDa) FGF2 isoforms in mouse are synthesized through the alternative use of translation initiation codons.6–9 These isoforms differ only in their NH2 extremities, which confer a nuclear localization to the high molecular weight CUG-initiated (22, 22.5, 24 or 21, and 22 kDa) isoforms (FGF2hmw), whose function is mainly unknown. In contrast, the smaller 18-kDa AUG-initiated FGF2 isoform (FGF2lmw) is predominantly cytoplasmic, excreted, and stored in the extracellular matrix.10 It stimulates cell proliferation and migration through binding to high-affinity transmembrane tyrosine kinase receptors (FGFR) and low-affinity receptors (heparan sulfate-containing proteoglycans), activating the mitogen-activated protein kinases (MAPKs) and/or phospholipase C-dependent pathways. Interestingly, E2 has been reported to stimulate MAPK activity via an autocrine loop involving FGF2.11 To achieve its proliferative activity, FGF2 needs to be internalized and translocated into the nucleus12 during the G1 phase of the cell cycle.13 In contrast, with the exception of human HSP 27-transformed endothelial cells,14 FGF2hmw remains intranuclear and interacts with several partners, such as FGF2 interacting factor (FIF)15 and survival of motor protein (SMN).16 These nuclear FGF2hmw promote cell proliferation independently of the cell surface receptors17 and are involved in tumor progression and metastatic dissemination.18

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A. Production of FGF<sub>lmw</sub><sup>-/-</sup> knock-in mice

Levin and coworkers<sup>19</sup> reported that transfection and expression of the 27-kDa human heat shock protein in bovine arterial endothelial cells double the rate of estrogen-induced FGF2 secretion, preferentially inducing the release of FGF2<sub>hmw</sub>. The secreted FGF2 is mitogenic to breast adenocarcinoma cells cultured in the conditioned medium obtained from the transfected endothelial cells. In contrast, decreasing the level of the endogenous heat shock protein homolog with an antisense vector markedly decreases FGF2 release.<sup>19</sup> The same group reported later that although 18-kDa FGF2 promotes cell motility, FGF2<sub>hmw</sub> inhibited migration of endothelial cells by 50%, even in the presence of unrelated mitogens such as vascular endothelial growth factor (VEGF) and IGF-1 that promote cell migration.<sup>20</sup> Using specific antibodies, they localized the inhibition of migration to the amino-terminal end and stimulation of growth to the 18-kDa domain of 24-kDa FGF2. They concluded that 24-kDa FGF2 affects cell behavior differently than 18-kDa FGF2.

The goal of the present work was to analyze the role of FGF2 in E2-induced angiogenesis, and possibly, the specific involvement of the molecular isoforms of this growth factor. In a first set of experiments, we explored the angiogenic effect of E2 in vivo in a mouse model of Matrigel plugs. The role of the FGF2 in the effect of E2 on the migration and proliferation of the microvascular endothelial cells provided by the Matrigel plugs grown in culture was then studied. Using knockout mice disrupted for the Fgf2 gene (Fgf2<sup>-/-</sup>), we demonstrated that endogenous FGF2 is absolutely required for the effects of E2. We also observed that E2 affected the abundance of FGF receptors and FGF2 isoforms.

Using mice specifically deficient in the 18-kDa FGF2<sub>hmw</sub> (Fgf2<sub>hmw</sub><sup>-/-</sup>), we demonstrated that FGF2<sub>hmw</sub> was not required for the effects of E2. Indeed, this novel mouse model revealed that E2, effectively induced angiogenesis despite the absence of FGF2<sub>hmw</sub>. In addition, we found that E2 stimulated the migration, but not the proliferation, of microvascular endothelial cells from Fgf2<sub>hmw</sub><sup>-/-</sup>. The role of the two partners of FGF2<sub>hmw</sub>, SMN and FIF, in the action of E2 were finally investigated.

B. FGF2 expression on FGF<sub>lmw</sub><sup>-/-</sup> mice protein extracts.

![Western blot analysis of brain, aorta, lung, and endothelial cells extracts](image)

Figure 1. Characterization and phenotype of Fgf2<sub>hmw</sub><sup>-/-</sup> mice. A, Fgf2<sub>hmw</sub><sup>-/-</sup> mice were generated by targeted mutation of the ATG translational start site of the 18-kDa FGF2 isoform. B, Western blot analysis of brain, aorta, lung, and endothelial cells extracts did not show any degradation of the HMW FGF2 into lower molecular weight proteins.

Materials and Methods

Animal Studies

FGF2<sup>-/-</sup> mice were obtained as previously described.<sup>9</sup> To produce the Fgf2<sub>hmw</sub> allele, the Hprt minigene in the tagged Fgf2<sup>+</sup> allele was “exchanged” by gene targeting with a mutant exon 1 with the sequence CTGCAG replacing the wild-type CCATGC (Figure 1A). The mutant sequence encodes an alanine GCA in place of the methionine ATG translational start site of the 18-kDa FGF2 isoform. The adjacent upstream wobble base was also changed from C to T so that the composite of these three base changes generated a Psil site that is diagnostic for the exchanged allele. Cells with the exchanged allele were selected for the loss of HPRT function using 5 µg/mL 6-thioguanine (Sigma), and the resulting ES cell line was injected into C57BL/6 blastocysts to generate germ-line chimeras. The germ-line chimeras were intercrossed with Black Swiss mice to generate Fgf2<sub>hmw</sub><sup>-/-</sup> mice. The colony was maintained as an advanced intercross line on a mixed 129/SvEv×Black Swiss background in order to avoid inadvertent recombinant inbreeding that can alter FGF2-deficient phenotypes.<sup>21</sup> Fgf2<sub>hmw</sub><sup>-/-</sup> mice were fertile and showed no obvious developmental defects. As expected, brain, aorta, and lung homogenates from Fgf2<sub>hmw</sub><sup>-/-</sup> mice expressed exclusively FGF2<sub>hmw</sub> isoforms (Figure 1B). The background of Fgf2<sup>+/+</sup>, Fgf2<sup>-/-</sup>, and Fgf2<sub>hmw</sub><sup>-/-</sup> mice was a mixed genetic background 129/SvEv×Black Swiss. The resulting ES cell line was injected into C57BL/6 blastocysts to generate germ-line chimeras. The germ-line chimeras were intercrossed with Black Swiss mice to generate Fgf2<sub>hmw</sub><sup>-/-</sup> mice. The colony was maintained as an advanced intercross line on a mixed 129/SvEv×Black Swiss background in order to avoid inadvertent recombinant inbreeding that can alter FGF2-deficient phenotypes.<sup>21</sup> All mice (Fgf2<sup>+/+</sup>, Fgf2<sup>-/-</sup>, Fgf2<sub>hmw</sub><sup>-/-</sup>, ER<sup>α</sup><sup>-/-</sup>, and C57Bl/6) were ovariectomized at 4 weeks of age and implanted either with 60-day time-release E2 pellets (0.1 mg E2, Innovative Research of America, i.e., releasing 80 µg/kg per day) SC into the back of the animals using a sterile trochar, or with placebo-releasing pellets.

At the time of euthanasia, serum was kept for hormone concentration measurement. Radioimmunoassay kits for E2 were used following manufacturer instructions (Sorin Biomedica, Italy).

Angiogenesis

On day 10 after ovariectomy and placebo or E2 treatment, the mice received a subcutaneous injection of Matrigel (0.5 mL) with 250 ng/mL FGF2.<sup>23</sup> After 7 more days, the animals were killed, and the Matrigel plugs were cautiously removed from the envelope wrapping them, to prevent fibroblast contamination, and incubated in 1.5 mL of Matrisperse cell release solution (BD Biosciences) during 2 hours at 4°C. After dispersion from the gel, cells were counted using Malasey cell. Their characterization as endothelial cells is indicated in the next section.
Endothelial Cell Cultures

Microvascular endothelial cells isolated as described were plated onto 5 µg/cm² fibronectin-coated 35-mm dishes and cultured in Phenol-red-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 mmol/L L-glutamine and 10% charcoal-stripped fetal calf serum (CS-FCS) in a 10% CO₂ atmosphere. All studies were performed on cells before any passage. Cultured cells were characterized using three markers of endothelial cells: rabbit polyclonal anti-CD31 (BD Pharmingen), rabbit polyclonal anti-vWF (DAKO, Via real, California), or isolectin Bandeiraea simplicifolia B4 conjugated to FITC (Sigma-Aldrich). The three techniques constantly showed that endothelial cells represented >90% of the primary culture (data not shown).

When necessary, 5 × 10−5 mol/L of raloxifen (Eli Lilly), or 5 × 10−8 mol/L of ICI 182780 (Zeneca Pharmaceuticals) were incubated 30 minutes before E₂ treatment. Untreated control culture received only ethanol (1:1000 final).

Cell Migration Assays

Cells were plated onto 35-mm dishes and cultured to 90% confluence in 2 mL phenol-red-free DMEM, 10% CS-FCS. Scratch injury was applied using a tip. The debris were removed by washing the cells with PBS, and the cells were incubated for 8 hours in phenol-red-free DMEM 2% CS-FCS supplemented with E₂ (1 nmol/L) or FGF2 (3 ng/mL). The percentage of cell migration in vitro (ratio of the area that was recovered by migrating cells on area initially wounded, photographed with a Leica microscope) was determined using Leica Qwin standard V2.3 system. At least three experiments were performed.

Mitogenic assay, DNA synthesis analysis, reverse transcription, and real-time quantitative PCR are described in the expanded Materials and Methods section available in the online data supplement at http://circres.ahajournals.org.

RT-PCR, Enzyme Analysis, and Southern Blotting of FGFRs

RT were performed with a common oligonucleotide of FGFRs: 5'–GAGATGGAGATGTGAAA–3’. PCR were performed using another set of oligonucleotide for FGFR1: 5’– primer 5’– AGTTGGAGCTACACC, 3’– primer 5’–TGAGGTCATACCATCCTG, and for FGFR2 as previously described. 24 Fifty microliters of the PCR mixture were added along with the specific restriction enzymes for the following: FGFR1 BstI, BseMI; FGFR1 IIIc, AclI; FGFR2 IIIb, AvdI; and FGFR2 IIIc, EcoRV. Southern blotting were performed as previously described. 24,25 The intensity of hybridization signals were quantified by densitometry using Phosphor Imager.

Transfection of Endothelial Cells With siRNA Duplexes

The 21-nucleotide siRNA duplexes were synthesized and purified by Dharmacon Research. The siRNA sequence targeting mouse Fj1 and Fgfr1 corresponded respectively to the coding region 951 to 972 and 1504 to 1525 relative to the first nucleotide at the start codon. The siRNA control was the scramble one from Dharmacon. The transfection was performed with 10 µmol/L of siRNA and oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions.

Western Blotting

Proteins (30 µg) were boiled for 2 minutes in a Laemmli’s sample buffer solution and loaded onto 12.5% polyacrylamide gels containing SDS. They were transferred onto PVDF membrane and blocked in 3% nonfat milk TBST. Specific antibodies were used at a dilution of 1:1000. Polyclonal anti-FGF antibodies were generated as previously described. 19 Mouse monoclonal anti-FGF2 (Ab3) was from Oncogene Science, and rabbit polyclonal against ERK2 (C14) mouse monoclonal against the phospho p44/p42 MAPK (E10) were purchased from Cell Signaling. Monoclonal anti-β-actin (AC-15) was from Sigma Aldrich, and monoclonal anti-SMN was from BD Biosciences. HRP-labeled secondary antibodies were revealed by the ECL system.

Specific ELISA for FGF2

FGF2 in supernatants of endothelial cells were quantified using standard sandwich ELISA (Quantikine FGF basic, R&D Systems Inc) following the manufacturer’s instructions. The sensitivity of FGF2 assays was 10 pg/mL.

MAPK Activation

Endothelial cells were kept for 18 hours in serum-free medium. Cells were left untreated or stimulated with 3 ng/mL FGF2 during 10 minutes and then frozen before protein analysis. Proteins were separated on a 12.5% SDS-Polyacrylamide gel and subjected to immunoblotting with the indicated antibodies.

Statistics

Results are expressed as mean ± SEM. To test the respective role of E₂ treatment and of FGF2 genotypes, a two-factor ANOVA was performed (comparison of four groups). A value of P<0.05 was considered as statistically significant. When an interaction was observed between the two factors, the four groups were compared using a 1-factor ANOVA. The results of this test is also indicated by an asterisk when P<0.05 to facilitate the reading of the figures.

Role of ERα and FGF2 in the Effect of E₂ on In Vivo Angiogenesis

As previously described in an angiogenic model of exogenous FGF2-loaded Matrigel plugs, 3,4 we found that E₂ increased about 2-fold the number of endothelial cells in plugs from both C57Bl/6 and Fgf2−−/− mice, confirming the angiogenic effect of E₂ in this model (Figure 2). The angiogenic effect of E₂ was abolished in ERF−/− mice, 3 confirming in another model of ERF gene inactivation 22,26 that ERF mediates the angiogenic effect of E₂. The angiogenic effect of E₂ was abolished in Fgf2−/− mice (P=0.41, NS). In contrast, the angiogenic effect of E₂ on Fgf2−−/− mice was similar to that observed in Fgf2−/− mice (Figure 2).

Role of ERα and FGF2 in the Effect of E₂ in Endothelial Cell Culture Experiments

E₂ (1 nmol/L) significantly increased both the migration and cell proliferation of cultured endothelial cells obtained from the subcutaneous Matrigel plugs of Fgf2−−/− mice (Figures 3A and 3B). Raloxifen and ICI 182780 demonstrated a pure antagonist activity, and completely blocked the effect of E₂ on endothelial cell proliferation and migration. Endothelial cells from ERF−/− mice were completely unresponsive to E₂,
confirming again, that this ER isoform mediates the effect of E₂ on both migration and proliferation (Figure 3).

Also, E₂ did not increase significantly (P=0.16, NS) the migration (Figure 4A) and the ³H-thymidine incorporation (Figure 4B) of endothelial cells obtained from Fgf2⁻/⁻ mice. In contrast, FGF2 (3 ng/mL) and VEGF (20 ng/mL) stimulated both the proliferation and the migration of cells from Fgf2⁻/⁻ mice, although the effect of the former was attenuated on cell migration (53% compared with cells from Fg⁻/⁺ mice) (Figure 4A).

Interestingly, the effect of E₂ on cell migration (Figure 4C), but not on ³H-thymidine incorporation (Figure 4D), was intact in cells obtained from Fgf2⁻/⁻ mice as well as the effect of exogenously added FGF2 and VEGF on both migration and ³H-thymidine incorporation in these cells.

Effect of E₂ on FGF2 Isoforms, FGF Receptors, and FGF Signalization

The effect of E₂ on FGF2 expression was then studied. As shown by Western blot analysis, untreated Fgf2⁻/⁻ endothelial cells expressed mainly the FGF2 lmw isoform, whereas E₂ treatment induced the FGF2 hmw protein expression (Figure 5A). As previously reported, most of FGF2 hmw was located in the nuclear fraction of Fgf2⁻/⁻ endothelial cells, and as expected, FGF2 hmw was more abundant in nuclei from E₂-treated endothelial cells than in untreated control (online Figure, available in the online data supplement). ELISA did not reveal the presence of FGF2 in the supernatant of either E₂-treated or untreated cells (data not shown).

E₂ treatment also increased the expression of FGF2 isoforms in cultured endothelial cell homogenates
A. FGF2 isoform abundance in Fgf2<sup>+/+</sup> endothelial cell extracts.

B. FGF2 HMW expression on Fgf2<sup>LMW−/−</sup> endothelial cell extracts.

C. Fgf2 mRNA abundance in Fgf2<sup>+/+</sup> endothelial cells.

from FGF2<sup>LMW−/−</sup> mice to the same extent as in Fgf2<sup>+/+</sup> cells (Figure 5B).

Unexpectedly, the abundance of Fgf2 mRNA was decreased by E<sub>2</sub> treatment 2.5-fold in cultured endothelial cells (Figure 5C).

We then investigated the effect of E<sub>2</sub> on FGF receptor expression. mRNA from endothelial cells were reverse-transcribed using a common oligonucleotide. The sequence coding the third immunoglobulin-like domain was amplified and restriction analysis was used to distinguish between the IIIb and IIIc variant of receptors (Figure 6A). We found that both FGFR1 and R2 are expressed in our primary cell cultures but essentially FGFR1 presented the splicing variant coding the FGF2 high affinity receptor isoform (FGFR1 IIIc) (Figure 6A). Using real-time PCR analysis, we showed that E<sub>2</sub> increased FGFR1 IIIc mRNA 1.8-fold in Fgf2<sup>+/+</sup> and 2-fold in Fgf2<sup>LMW−/−</sup> endothelial cells, whereas it has no effect on FGFR1 IIIc expression in Fgf2<sup>−/−</sup> endothelial cells (Figure 6B).

After determining the time course of FGF2-stimulated ERK phosphorylation in endothelial cells (Figure 7A), we found that FGF2-sustained ERK phosphorylation could be efficiently prevented by siRNA Fgfr1 transfection (Figure 7B), demonstrating the functional efficacy of FGFR blockade and the predominance of FGFR1 in the signal transduction of FGF2<sup>LMW</sup>. The Fgfr1 mRNA was significantly reduced after siRNA Fgfr1 transfection of endothelial cells (Figure 7C), but its blockade did not alter the E<sub>2</sub>-stimulated migration (Figure 7D), demonstrating the independence of E<sub>2</sub>-elicited migration from FGFR.

SMN was recently shown to specifically interact with FGF2<sup>LMW</sup>. SMN mRNA and protein were found to be expressed in cultured endothelial cells but no change of SMN expression could be detected after E<sub>2</sub> exposure (Figure 8A and 8B). FIF was also recently found to be an intracellular partner of the HMW FGF2. The abundance of Fif mRNA and protein were then assessed in cultured endothelial cells, and both were found to be increased on E<sub>2</sub> exposure (Figure 8C and 8D). The induction of FIF protein expression by E<sub>2</sub> was also altered by siRNA, resulting in 80% inhibition, whereas scramble siRNA had no significant effect (Figure 8D). The partial suppression of Fif induced an attenuation of the stimulating effect of E<sub>2</sub> on Fgf2<sup>+/+</sup> cell migration and an
abolition of the effect in \( \text{Fgf}^2\text{lmw}^-^-^-^- \) (Figure 8E), this result being in the line with the role of \( \text{FGF}^2\text{hmw} \).

**Discussion**

It is now clear that the effects of \( \text{E}_2 \) are not limited to the field of reproduction but have many extrareproductive effects, in particular in the pathophysiology of atherosclerosis\(^27\) and in angiogenesis.\(^3,28\) Johns et al\(^4\) have shown that \( \text{ER}^\alpha \) mediates the stimulating effect of \( \text{E}_2 \) on angiogenesis. In the present work, the angiogenic effect of \( \text{E}_2 \) was abolished in \( \text{ER}^\alpha^-^-^-^- \) mice,\(^22\) a model of complete \( \text{ER}^\alpha \) gene inactivation,\(^26\) confirming that the \( \text{ER}^\alpha \) is required to mediate the angiogenic effect of \( \text{E}_2 \), and the results of angiogenesis obtained in both transgenic models are valid. In addition, we found that the stimulating effect of \( \text{E}_2 \) on endothelial cell migration and proliferation was mediated by \( \text{ER}^\alpha \) and completely antagonized by \( \text{raloxifene} \) and \( \text{ICI} 182780 \).\(^29\)

Because no growth factor downstream of the \( \text{ER}^\alpha \) have been clearly identified to explain this effect, we investigated, in the present work, the role of \( \text{FGF}^2 \), a potent angiogenic factor produced and secreted by endothelial cells, which promotes their migration, proliferation, chemotaxis, and protease production. Interestingly, in this model of Matrigel loaded with exogenous \( \text{FGF}^2 \), basal angiogenesis was similar in \( \text{Fgf}^2\text{hmw}^-^-^-^- \) and in \( \text{Fgf}^2\text{lmw}^-^-^-^- \) mice, endogenous \( \text{FGF}^2 \) not being required. In contrast, endogenous \( \text{FGF}^2 \) appears absolutely required for the \( \text{E}_2 \)-induced acceleration of this process. We thus showed for the first time, that the regenerative properties of \( \text{E}_2 \) are dependent on endogenous \( \text{FGF}^2 \).

We then explored the effect of \( \text{E}_2 \) on \( \text{FGF}^2 \) isoforms. \( \text{FGF}^2 \) isoforms are synthesized through an alternative use of translation initiation codons.\(^7-^9\) The smaller AUG-initiated \( \text{FGF}^2 \) (\( \text{FGF}^2\text{lmw} \), 18 kDa) is predominantly cytoplasmic, excreted, and stored in the extracellular matrix.\(^10\) In the present work, we found that \( \text{E}_2 \) increased \( \text{FGF}^2\text{hmw} \), but not \( \text{FGF}^2\text{lmw} \), abundance in cultured mouse endothelial cells, and we were unable to detect secreted \( \text{FGF}^2 \) in the supernatant of these cells. This suggests that \( \text{E}_2 \) specifically induces the expression of \( \text{FGF}^2\text{hmw} \). At the same time, \( \text{Fgf}^2 \) mRNA concentration decreases. Such a discrepancy has been previously reported by us in \( \text{3T3} \) cells\(^30\) and others in erythroid cells.\(^31\) This strongly suggests a regulation at the translational level. Indeed, when the pool of silent mRNA is mobilized by the ribosomal machinery, its translation could activate its instability, possibly by removing masking-protein able to protect the instability region.\(^31\) Such a region has been clearly identified within the \( 3' \) end of \( \text{FGF}^2 \) mRNA.\(^32\) Furthermore, we have previously identified a translational enhancer located

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**Figure 6.** Expression of \( \text{FGFR}^1 \) and \( \text{FGFR}^2 \) in lung and endothelial cells. A, Expression of \( \text{FGFR}^1\text{Ilb} \), \( \text{FGFR}^1\text{Ilc} \), \( \text{FGFR}^2\text{Ilb} \), and \( \text{FGFR}^2\text{Ilc} \) in endothelial cells studied by RT-PCR, restriction enzyme analysis, and Southern blot hybridization of \( \text{FGFRs} \). Restriction enzymes \( \text{Bst} \) and \( \text{Acc} \), which recognize sequences \( \text{FGFR}^1 \) exon Ilb and Ilc, and restriction enzymes \( \text{Ava} \) and \( \text{Eco} \), which recognize sequences \( \text{FGFR}^2 \) exon Ilb and Ilc, respectively, were used to distinguish between exon Ilb-containing and exon Ilc-containing cDNA in the PCR. Size of products resulting from Southern blot hybridization after restriction enzyme treatment is indicated. B, Real-time PCR showed a significant increase of \( \text{Fgfr}^1 \) mRNA in endothelial cell extracts of \( \text{Fgf}^2\text{Ilb}^-^-^-^- \) and \( \text{Fgf}^2\text{lmw}^-^-^-^- \) mice, whereas this effect was abolished in \( \text{Fgf}^2\text{Ilc}^-^-^-^- \) endothelial cells. *\( P<0.001 \) vs respective untreated control.
at the end of the 3’ UTR of the Fgf2 gene, the activation of which promotes a preferential increase of the FGF2\textsubscript{hmv} forms.\textsuperscript{33} These two phenomena involving the role of the 3’ UTR of Fgf2 mRNA could explain the effect observed here after stimulation by E\textsubscript{2}.

FGF2\textsubscript{hmv} is thought to stimulate proliferation and migration through tyrosine kinase receptors (FGFR), activating the MAPKs and/or phospholipase C-dependent pathways. The complex then translocates into the nucleus during the G1 phase of the cell cycle\textsuperscript{13} by a mechanism in which translokin appears to play a key role\textsuperscript{12} and is very distinct from the translocation of FGF2\textsubscript{hmv}, which is nuclearized by the importin machinery.\textsuperscript{8} Indeed, the nuclear localization sequence of the FGF2\textsubscript{hmv} is responsible of their nuclear localization, but their function is mainly unknown.

We thus explored the effect of E\textsubscript{2} on the FGF receptors. We found that in mouse endothelial cells, FGFR1 IIIc and FGFR2 IIIb mRNA are expressed. E\textsubscript{2} increased 1.8-fold FGFR1 IIIc mRNA in Fgf2\textsuperscript{-/-} endothelial cells, whereas it had no effect in Fgf2\textsuperscript{+/+} endothelial cells. This suggests that an increase in FGFR1 signaling could have contributed to the effect of E\textsubscript{2} on angiogenesis, migration, and proliferation. However, as shown in Figure 7, treatment of endothelial cells by siRNA against Fgfr1 reduces the level of its mRNA and fully inhibits both cell response as well as ERK phosphorylation in response to FGF2 stimulation, showing the dominant role of FGFR1 versus FGFR2 for MAPK activation. In these conditions, E\textsubscript{2} is still active, demonstrating that the receptor-dependent pathways are not required to relieve the E\textsubscript{2} effect.

The specific role of the FGF2 isoforms had previously been studied in various cell culture models but these studies provided conflicting data as recently pointed out by Boilly.\textsuperscript{34} Estrogen was reported to promote proliferation and migration via the activation of p42/44 and p38 MAPK in porcine aortic endothelial cells\textsuperscript{29}; this activation was also reported to involve an autocrine loop by FGF2\textsubscript{lmw} in cultured human endothelial cells.\textsuperscript{11} In contrast, in a model of bovine aortic endothelial cells (BAECs) stably transfected with 27-kDa human HSP, but not in parental BAECs, FGF2\textsubscript{hmv} was found to inhibit migration under the influence of E\textsubscript{2} by a mechanism involving activated MAPK and phosphorylated estrogen receptor.\textsuperscript{14,20} However, HSP27 protein expression was not altered by E\textsubscript{2} in primary culture of mouse endothelial cells (B. Garmy-Susini, unpublished data, 2004). This goes along with the fact that FGF2\textsubscript{hmv} could not be detected in the supernatant of the cells.

\textbf{A. Time course of ERK phosphorylation after FGF2 treatment}

\textbf{B. MAP Kinase activation after FGF2 stimulation}

\textbf{C. Fgfr1 mRNA abundance in endothelial cells after siRNA FGFR1 transfection.}

\textbf{D. Migration of endothelial cells after siRNA Fgfr1 transfection.}

\textit{Figure 7.} Mechanisms of action of E\textsubscript{2} in endothelial cells: role of FGF2 receptors. A, Time course of MAPK activation after FGF2 stimulation. ERK1/2 phosphorylation was analyzed by Western blot analysis on FGF2-stimulated endothelial cell extracts. B, Effect of Fgfr1 siRNA transfection on ERK1/2 phosphorylation after FGF2 stimulation. C, Real-time PCR analysis showing the effect of Fgfr1 siRNA transfection on Fgfr1 mRNA abundance in E\textsubscript{2}-treated endothelial cells. D, Lack of effect of Fgfr1 siRNA transfection on the E\textsubscript{2}-stimulated migration of endothelial cells. *\textit{P}<0.05 vs respective control; §\textit{P}<0.05 vs untreated cells.
Until the present work, very little was actually known about FGF2 hmw mode of action. Thanks to the generation of mice deficient in FGF2 lmw, we were able to demonstrate for the first time that FGF2 hmw, is absolutely required for the stimulating effect of E2 on endothelial cell migration, whereas FGF2 lmw appears dispensable for this effect (Figure 4C). However, both the intracrine FGF2 hmw pathway and the classical auto/paracrine FGF2 lmw pathway (including FGFR1 IIIc signaling) appear required to mediate the stimulating effect of E2 on 3H-thymidine incorporation (Figure 4D).

FGF2 hmw has been shown to specifically interact with two mainly nuclear protein: FIF15 and SMN.16 SMN is essentially described as a key activator for snRNP assembly and recycling. The role of its association with FGF2 hmw is still unclear. In our hands, we did not detect any modulation of the cellular content of SMN after E2 stimulation. Conversely, FIF expression was stimulated by E2 and its proposed role is in accordance with our results: FIF has been previously identified as an antiapoptotic factor,35 whose expression allows the synthesis of some matrix metalloproteases and the repression of TIMP-2. The functional role of FIF in cell migration was demonstrated in this study by the partial and complete abolition of the E2 effect by Fif siRNA duplex in cells from Fgf2+/+ and Fgf2+/- mice, respectively.

The roles of FGF2 in many different systems, from brain and bone development, to arterial restenosis, vessel remodeling, differential functions of FGF2 and VEGF, fibrosis, wound healing, etc, coupled with the fact that there are FGF2 isoforms the functions of which are completely unknown but obviously important because they are conserved across species and their expression is differentially regulated across tissues, all make this gene and the function of its isoforms of considerable importance.

The present work demonstrates a novel link between FGF2 isoforms and estradiol in angiogenesis and endothelial cell migration and proliferation. Although the phenotype of Fgf2+/- mice suggest a dispensable role of E2-dependent angiogenesis in the domains of reproduction and development, the present data should be taken into consideration in pathophysiological circumstances such as atherosclerosis,36 collagen disease,37 diabetic angiogenesis,28 and possibly breast cancer,38 where both FGF2 and E2 have been suggested to be involved.

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References


Role of Fibroblast Growth Factor-2 Isoforms in the Effect of Estradiol on Endothelial Cell Migration and Proliferation

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Supplemental Data:

FGF2 isoform protein abundance in Fgf2+/+ nuclear and cytoplasmic extracts from endothelial cells treated (E2) or not (Ctrl) by 10⁻⁹M E2 for 8 hrs.
Mitogenic Assay. Microvascular endothelial cells were initially seeded at 1.5x10^4 cells per well of 12-well tissue culture plates (BD), stimulated for 24hrs in DMEM 2% CS-FCS with or without 10^{-9}M E2. After trypsinisation, cell number was determined by using a Coulter counter.

For DNA synthesis analysis, cells were stimulated or not with E2 (1nM) or FGF2 (3ng/mL) for 24hrs in 1 ml phenol-red-free DMEM 2% CS-FCS. One µCi of [methyl-3H] thymidine (1 mCi/ml, 20 Ci/mmol, NEN Life Science Products, Boston, MA) was added for the last 8 hrs. At the end of incubation period, cells were washed twice with PBS, incubated with 0.5 ml of 10% trichloroacetic acid (TCA) for 15 min, washed with 5% TCA, 95% ethanol, and lysed in 0.1M NaOH. Samples were harvested, mixed with liquid scintillation cocktail (Ecoscint H, National Diagnostics, Atlanta, GA), and the incorporated radioactivity was counted (cpm/min) in a liquid scintillation counter (Beckman LS 6500).

Reverse Transcription. Total RNA was isolated from endothelial cells of Fgf2^{+/+} Fgf2^{-/-} and Fgf2^{lw-/-} mice with the RNAble method (Eurobio, Les Ulis, France). They were reverse transcribed (RT) with Superscript II (Invitrogen, Groningen, The Netherlands) following the manufacturer’s instructions, using 2.5 µM Random Hexamer probe for real-time quantitative PCR experiments, or an oligonucleotide common to the different molecular isoforms of FGFRs (5’-GAGATGGAGATGATGAAA-3’) for southern blotting analysis.

Real-time Quantitative PCR. PCR was performed with an ABI PRISM 7700 Sequence Detector and SYBR Green reagents (Applied Biosystems). Specific primers for Fgfr1, Fift, and Smn were designed for the same cycling conditions (50°C for 2 min to permit uracil N-glycosylase cleavage, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min). TaqMan RNA control reagents (Applied Biosystems), designed for ribosomal S18 RNA amplification, were used as a reference to normalize the results.
<table>
<thead>
<tr>
<th>Gene</th>
<th>SENSE</th>
<th>ANTI-SENSE</th>
</tr>
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<tbody>
<tr>
<td>Fgf2</td>
<td>5'-CACCAGGCCACTCAAGGA-3'</td>
<td>5'-GATGGATGCGCAGGAAGAA-3'</td>
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<tr>
<td>Fgfr1</td>
<td>5'-GTAAGATCGGGCCAGAAC-3'</td>
<td>5'-CCATTTCCTTGTCGGTGGTATT-3'</td>
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<tr>
<td>Fif</td>
<td>5'-ACAGTAGCAACCTAAGAGATGGG-3'</td>
<td>5'-TCCTTTGCCATTAGTCAGCCTTAAC-3'</td>
</tr>
<tr>
<td>Smn</td>
<td>5'-GAATGCCACAACTCCCTGAA-3'</td>
<td>5'-GCAGCGGTCTTGTGACCAA-3'</td>
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