Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2

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ABSTRACT Basic fibroblast growth factor (FGF2) is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor that is expressed at low levels in many tissues and cell types and reaches high concentrations in brain and pituitary. FGF2 has been implicated in a multitude of physiological and pathological processes, including limb development, angiogenesis, wound healing, and tumor growth, but its physiological role is still unclear. To determine the function of FGF2 in vivo, we have generated FGF2 knockout mice, lacking all three FGF2 isoforms, by homologous recombination in embryonic stem cells. FGF2−/− mice are viable, fertile and phenotypically indistinguishable from FGF2+/+ littermates by gross examination. However, abnormalities in the cytoarchitecture of the neocortex, most pronounced in the frontal motor-sensory area, can be detected by histological and immunohistochemical methods. A significant reduction in neuronal density is observed in most layers of the motor cortex in the FGF2−/− mice, with layer V being the most affected. Cell density is normal in other regions of the brain such as the striatum and the hippocampus. In addition, the healing of excisional skin wounds is delayed in mice lacking FGF2. These results indicate that FGF2, although not essential for embryonic development, plays a specific role in cortical neurogenesis and skin wound healing in mice, which, in spite of the apparent redundancy of FGF signaling, cannot be carried out by other FGF family members.

FGF2 (basic fibroblast growth factor), is the prototype of the FGF family of structurally related proteins, which so far includes 15 members. Although FGFs have been implicated in a variety of physiological and pathological processes and FGF signaling clearly plays an important role in development, the specific function of each FGF in vivo is not yet clear (1–4).

FGF2 is a pleiotropic factor that induces proliferation of most mesoderm- and neuroectoderm-derived cells. FGF2 is also a potent chemotactic factor for fibroblasts and endothelial cells, can promote or inhibit cell differentiation, and is a potent angiogenic and neurotrophic factor (3). The biological activities of FGF2 are mediated by its binding to specific tyrosine kinase transmembrane receptors on the target cell surface (1–4). Among the four FGF receptors identified so far, FGFR1 and FGFR2 bind FGF2 with the highest affinity (5–7). However, there is a high level of redundancy in receptor utilization within the FGF family (7).

In contrast to other FGFs that have a restricted pattern of expression, FGF2 is present in the majority of tissues of both adult and embryonic origin, and it is produced by many cell types. An overwhelming variety of pharmacological effects have been reported for FGF2, both in vitro and in vivo (reviewed in ref. 3). FGF2 acts as a mesoderm inducer when applied to Xenopus embryonic caps, and it can substitute the apical ectodermal ridge and maintain proliferation of limb bud mesenchyme during limb development (8, 9). FGF2 also promotes differentiation of both endothelial cells and hematopoietic cells from dissociated quail epiblasts, which, along with its angiogenic activity, suggests a role for FGF2 in blood vessel development (10). In the hematopoietic system, FGF2 enhances myelopoiesis in long-term bone marrow cultures and is a potent stimulator of megakaryocytogenesis (11, 12). FGF2 is released by cardiomyocytes in response to mechanical load and causes myocardial hypertrophy (13). Systemic administration of FGF2 in rats decreases arterial blood pressure (14).

The activities of FGF2 in the central nervous system are also multiple. FGF2 promotes quiescent astrocytes to reenter the cell cycle and induces expression of glial fibrillary acidic protein, a marker of astrocyte differentiation (15). FGF2 maintains survival of isolated neurons, promotes neurite outgrowth of hippocampal and cortical neurons (16), and regulates expression of neurotransmitters like neuromodulatory peptide Y (17). FGF2 stimulates division of cortical multipotential stem cells and may also act on postmitotic neurons to promote differentiation and survival (18–20).

FGF2 acts as a survival factor in many models of cell and tissue injury (3). Topical application of FGF2 accelerates healing of skin wounds in animal models, as well as of eye, retina, and corneal wounds (3). Finally, FGF2 may also play a role in tumor growth and angiogenesis (2, 3).

This very wide range of biological activities, however, does not identify FGF2 as the natural effector of all these processes, because FGF2 could mimic the effect normally produced by another member of the FGF family, or perhaps by more than one FGF that can act on the same receptor.

FGF2 is also unusual because it lacks a signal peptide and is not secreted by a classical endoplasmic reticulum/Golgi-dependent mechanism, and thus is not released efficiently from producing cells, although it is detected in the extracellular environment in many tissues (3). Furthermore, the FGF2 mRNA contains additional translational start sites (CUG codons) upstream of the canonical initiator AUG. Translation starting at these upstream sites occurs efficiently both in vitro and in vivo and produces N-terminally extended, high molecular mass FGF2 forms that, in contrast to the AUG-initiated 18-kDa form, which is mostly cytosolic or found associated with the extracellular matrix, localize predominantly in the nucleus (3, 21, 22). Although the function of the nuclear FGF2 forms is still unclear, a dual mechanism of action for FGF2 has been proposed (23).

The generation of mice with homozygous deletion of individual genes has highlighted new and specific roles of individual gene functions, as well as revealed complex redundancies in the function of molecules within given gene families. We have generated FGF2 knockout mice by homologous recombination in embryonic stem (ES) cells in an attempt to

Abbreviations: FGF, fibroblast growth factor; ES, embryonic stem. ||To whom reprint requests should be addressed.
elucidate the physiological role of FGF2 in vivo. The FGF2−/− mice are viable, indistinguishable from wild-type littermates by gross examination, survive to adulthood, and are fertile. However, the brains of the FGF2−/− mice show abnormalities in the cytoarchitecture of the neocortex and a significant reduction in the number of neurons in the motor-sensory area of the cortex. The mice also exhibit a significant delay in the rate of healing of full-thickness excisional skin wounds.

MATERIALS AND METHODS

Generation of Targeted ES Clones. A genomic clone containing the first coding exon of FGF2 was isolated from a 129SVJ mouse genomic library (Stratagene). The library was screened with fragments of the human FGF2 cDNA, and four positive clones containing the first FGF2 exon were isolated. The targeting vector was constructed in the pPNT plasmid (24). The XhoI and EcoRI sites of pPNT were used to clone the 2.4-kb 5′ arm and 6.6-kb 3′ arm of the targeting vector, respectively. The vector (20 µg) was linearized with NotI and electroporated into E14 ES cells. Colonies were selected in G418 (400 µg/ml) and 1(2′-deoxy-2′-fluoro-1β-D-arabinofuranosyl)-5-iodouracil (0.25 µM) on a feeder layer of neomycin-resistant mouse primary embryonic fibroblasts. Colonies were picked after 7 days in selection medium and expanded. Genomic DNA was isolated, and clones were screened by Southern hybridization.

Brain Histology and Immunohistochemistry. Mice were perfused with 4% paraformaldehyde, pH 7.4. Brains were dissected, postfixed in 4% paraformaldehyde for 2 hr at room temperature, incubated in 30% sucrose at room temperature overnight, embedded in M-1 #1310 embedding matrix and 6.6-kb 3′ arm of the targeting vector, respectively. The vector (20 µg) was linearized with NotI and electroporated into E14 ES cells. Colonies were selected in G418 (400 µg/ml) and 1(2′-deoxy-2′-fluoro-1β-D-arabinofuranosyl)-5-iodouracil (0.25 µM) on a feeder layer of neomycin-resistant mouse primary embryonic fibroblasts. Colonies were picked after 7 days in selection medium and expanded. Genomic DNA was isolated, and clones were screened by Southern hybridization.

Wound Healing. Adult mice, between 2 and 3 months of age, were anesthetized with an intraperitoneal injection of 2.5% avertin (15 µl/g). The back was shaved and disinfected with 70% ethanol. A circle, 6 mm in diameter, was drawn on the skin of the mid-dorsal region, and a full-thickness wound was created by excision of the area within with curved scissors. Wounds were considered healed when they were completely reepithelialized, without scabs, and the only evidence of wounding was a dermal scar.

Western Blot. Protein extracts were prepared by homogenizing the frozen tissues in 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 2 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride and clarified by centrifugation. The relaxed recombinant mouse primary embryonic fibroblasts. Coloni

RESULTS

Generation of FGF2−/− Mice. The murine FGF2 gene is a single-copy gene localized on chromosome 3 (human chromosome 4). The gene is approximately 40 kb in length and consists of three coding exons separated by two large introns. The murine FGF2 gene encodes three different proteins of 22-, 21-, and 18-kDa molecular mass, respectively, that result from alternative translation initiation codons (3). We designed a replacement-type vector to create a 200-bp deletion in the FGF2 gene that eliminates exon I sequences. The deletion extends from the NcoI site in exon I to the first XhoI site in the first intron and includes 59 codons of the FGF2 coding sequence, starting at the codon immediately downstream of the ATG initiation codon. The deletion also eliminates the splicing donor site of exon I, preventing the correct splicing of the FGF2 mRNA. The deleted sequences are replaced by the neomycin-resistance gene expressed under the control of the PGK-1 promoter and polyadenylation signal.

The targeting vector was electroporated into E14-ES cells (25). Colonies were selected in the presence of G418 (positive selection) and 1(2′-deoxy-2′-fluoro-1β-D-arabinofuranosyl)-5-iodouracil (negative selection). A total of 120 colonies were expanded and screened for homologous recombination by Southern blotting by using initially a 5′ internal probe (Probe I). Homologous recombinants were confirmed by subsequent screening with 5′ external and 3′ internal probes (Fig. 1A and B). Out of 120 colonies, 2 were homologous recombinants. Targeted ES cells were injected into C57BL/6 blastocysts. One of the clones contributed to the germ line as assessed by transmission of the agouti coat color to the offspring after breeding male chimera to C57BL/6 females. As expected, 50% of the agouti offspring contained one mutated FGF2 allele. The heterozygous FGF2+/− mice were indistinguishable from their FGF2+/+ littermates and were bred to generate homozygous offspring (Fig. 1C). Homozygous FGF2−/− mutant mice were born at the expected Mendelian frequency of 1 in 4, indicating no embryonic lethality.

Western blot analysis was performed on protein extracts of various organs to confirm the absence of FGF2 protein in the FGF2−/− mice. As expected, all three FGF2 isoforms were absent in these tissues (Fig. 1D). Reverse transcription–PCR analysis confirmed the absence of FGF2 mRNA in brain and embryonic fibroblasts of FGF2−/− mice. We tested whether expression of the RNA for FGF1 and FGF2, the highest-affinity FGF2 receptors, or that of FGF1 RNA and protein was
up-regulated as a result of FGF2 gene inactivation. We found no changes in the levels of expression of these molecules in the brain, testis of FGF2−/− mice. Western blots of protein extracts concentrated on heparin-Sepharose beads. Blots were probed with a monoclonal anti-FGF-2 antibody (Transduction Laboratories). The same result was obtained with polyclonal antibodies for FGF2. The position of the three isoforms of FGF2 is indicated; 25 ng of human recombinant FGF2 was loaded as a control.

In the cerebral cortex these calcium-binding proteins are expressed in specific subpopulations of neurons. The calcium-binding proteins parvalbumin and calbindin are expressed in distinct subpopulations of neurons that therefore may be distinguished by specific calcium-dependent processes. In the cerebral cortex these calcium-binding proteins are expressed in distinct subpopulations of neurons.

FIG. 1. FGF2 gene targeting. (A) Partial map of the FGF2 gene (Top), gene-targeting vector (Middle), and FGF2-targeted allele (Bottom). The first exon of the FGF2 gene is shown as a black box. The neo- and hsvTK-expressing cassettes, containing the PGK-1 promoter and polyadenylation sequences, are shown as white boxes. The position of the relevant restriction sites for the enzymes BamH1 (B), EcoR1 (E), Xba1 (X), Nco1 (N), SalI (S), HindIII (H), and PstI (P) is marked. The NotI site was used to linearize the targeting vector. DNA fragments I, II, and III were used as probes in Southern blot hybridization. (B) Genomic DNA from ES cells was digested with EcoR1, Nco1, and BamHI and hybridized to the probes indicated. T, targeted allele. (C) Southern blot of tail DNA from the offspring of heterozygous FGF2+/− mutant parents. DNA was digested with EcoR1 and probed with probe I. (D) Absence of FGF2 in protein extracts from brain and testis of FGF2−/− mice. Western blots of protein extracts probed with polyclonal antibodies for FGF2. The position of the three isoforms of FGF2 is indicated; 25 ng of human recombinant FGF2 was loaded as a control.

FIG. 2. Defects in the motor area of the neo-cortex of FGF2−/− mice. Coronal sections of the FGF2−/− mutant cortex compared with those of wild-type FGF2+/+ cortex after cresyl violet (Nissl) staining (A), parvalbumin (B), and calbindin (C) immunohistochemistry. Reduced cell density is evident in layers V and VI in A-C.

In particular, the motor area of the frontal cortex (Fig. 2) showed some thickening, with less defined layers in the FGF2−/− mice. There was a slight expansion of all the cortical layers that was more pronounced in layers V and VI. The barrel field of the S1 area, the typical barrel structure of layer IV, consisting of a series of cylindrical hollow areas surrounded by dense rings of granular neurons, was reduced in the FGF2−/− mice (data not shown). Most important, the cell density was reduced in most layers of the motor cortex, with more space between cresyl violet-stained cells (Fig. 2). Table 1 shows the number of cresyl violet-stained cells (per area unit) of the different layers of the motor cortex. A statistically significant reduction in neuronal density in layers II–VI was observed in FGF2−/− mice. This reduction was most pronounced in layers V and VI but affected also layers II–IV.

To further investigate alterations in cell number, we examined the distribution of specific neuronal phenotypes. The calcium-binding proteins parvalbumin and calbindin are expressed in distinct subpopulations of neurons that therefore may be distinguished by specific calcium-dependent processes. In the cerebral cortex these calcium-binding proteins are expressed in distinct subpopulations of neurons that therefore may be distinguished by specific calcium-dependent processes.
FGF2 mice. The healing rate of age-matched FGF2 mice did not become significantly more pronounced in older mice. The number of calbindin- or parvalbumin-stained neurons in the striatum and cerebellum (Purkinje cells) was less than only 50% that of controls. There was an approximately 3-day delay in the time required by FGF2 mice to achieve 50% healing (Fig. 3).

### Table 1. Neuronal cell density in the motor cortex of FGF2 null mice

<table>
<thead>
<tr>
<th>Layer</th>
<th>FGF2+/+</th>
<th>FGF2−/−</th>
<th>Δ, %</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5.42 ± 1.38, n = 20 (103)</td>
<td>5.68 ± 1.70, n = 20 (108)</td>
<td>20</td>
<td>P &gt; 0.1</td>
</tr>
<tr>
<td>II</td>
<td>30.70 ± 5.51, n = 20 (614)</td>
<td>24.78 ± 6.37, n = 20 (471)</td>
<td>20</td>
<td>0.001 &lt; P &lt; 0.005</td>
</tr>
<tr>
<td>III</td>
<td>15.98 ± 4.26, n = 60 (991)</td>
<td>14.43 ± 3.73, n = 56 (823)</td>
<td>10</td>
<td>0.01 &lt; P &lt; 0.05</td>
</tr>
<tr>
<td>IV</td>
<td>17.50 ± 4.50, n = 64 (1103)</td>
<td>15.54 ± 4.37, n = 64 (979)</td>
<td>12</td>
<td>0.005 &lt; P &lt; 0.01</td>
</tr>
<tr>
<td>V</td>
<td>15.97 ± 2.90, n = 44 (719)</td>
<td>10.55 ± 2.18, n = 36 (388)</td>
<td>34</td>
<td>P &lt; 0.0005</td>
</tr>
<tr>
<td>VI</td>
<td>18.37 ± 2.80, n = 64 (1151)</td>
<td>14.41 ± 4.15, n = 64 (894)</td>
<td>22</td>
<td>P &lt; 0.0005</td>
</tr>
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Data show cresyl violet-positive cells per area unit of layers I–VI of the motor cortex of FGF2−/− compared with FGF2+/+ mice. Mean values ± SD are shown. n, total number of areas counted. The total numbers of cells counted are shown in parenthesis. Stained cells were counted with a Zeiss Axioplan-2 microscope by using a 10 × 10 micrometer grid and a ×40 objective. Three different depths of field were counted, and each count included only cells that were in sharp focus. Counts were obtained from four sets of matching sections, corresponding to four FGF2+/+ and four FGF2−/− animals, respectively. Each animal was equally represented in the counting. Δ, decrease in cell density in the FGF−/− sections. The data were analyzed statistically by using a two-sample (pooled) t test.

By immunostaining with anti-calbindin and anti-parvalbumin antibodies we also observed a 30% reduction in the number of calbindin- and parvalbumin-positive cells in the motor cortex (Fig. 2). Therefore, the reduction in cell number is not specific for a particular subtype of neurons. Furthermore, the decreased neuronal density detected in 8-week-old animals did not become significantly more pronounced in older mice. The number of calbindin- or parvalbumin-stained neurons in the striatum and cerebellum (Purkinje cells) was similar in both FGF2−/− and FGF2+/+ mice (not shown).

**Delayed Wound Healing in the FGF2−/− Mice.** To study the role of FGF2 in skin wound healing, we created single full-thickness excisional skin wounds in the mid-dorsal region of the mice. The healing rate of age-matched FGF2−/− or FGF2+/+ mice was monitored in various ways. Initially, 11 FGF2+/+ and 12 FGF2−/− adult mice, 8–11 weeks of age, were examined macroscopically and photographed every other day to analyze the healing progress (Fig. 3). No significant difference between FGF2+/+ and FGF2−/− mice was observed in the gross appearance of the wounds between days 1 and 7. By day 7 all wounds were covered with a dry scab that remained adherent to the wound of FGF2−/− mice until day 10, when it began to detach in FGF2+/+ mice. After 12 days approximately 20% of the FGF2+/+ wounds were completely healed, with no sign of residual skin defect, and by day 14 more than 50% of the wounds were closed (Fig. 3B). However, by day 14 only 10% of the FGF2−/− wounds were healed, and 50% healing was not achieved until day 17. Thus, there is a 3-day delay in the time required by the FGF2−/− mice for complete healing of excisional wounds.

In an independent experiment, wound healing was analyzed histologically at different times after wounding in a genotype-blind study (Fig. 4). Mice were sacrificed at 5, 11, and 16 days, and frozen sections were prepared through the center of the wounded area. Wound diameter, scab thickness, percentage of reepithelialization, and collagen deposition were assessed as described in Materials and Methods. During the wound healing the wound diameter and scab thickness progressively decrease while the epithelium is reconstituted so that the wound is completely reepithelialized. This is accompanied by deposition of collagen to reconstitute the dermis as a scar. All of these processes were delayed in the FGF2−/− mice (Fig. 4). At 11 days after wounding the mean wound diameter in the FGF2−/− mice was almost twice that of controls, the scab thickness was 30% greater, the percentage of reepithelialization was only 60% of controls, and the collagen deposition was only 50% that of controls. There was an approximately 3-day
FGF2 has long been thought to play a role in tissue repair and differentiation. The absence of any dramatic phenotype in FGF2−/− mice is the consequence of an early developmental defect. It is also possible that the dramatic wave of apoptosis that occurs in the brain of newborn animals is increased or prolonged in FGF2−/− mice because of the absence of this antiapoptotic factor.

FGF2 has been shown to stimulate in vitro proliferation of telencephalic neocortical cells with characteristics of multipotential stem cells (35, 36). Specifically, cortical progenitor cells also have been shown to proliferate in vitro in response to FGF2. It has been proposed that FGF2 and NT-3 cooperate in the regulation of corticogenesis in vivo (18). Qian et al. (19) also have shown that FGF2 influences the development of cortical progenitor cells into neuronal or glial lineages in a dose-dependent manner. FGF2 mRNA is present in the mouse neuroepithelium as early as day E9.5 and is the only FGF detected in the neuroepithelium at that early stage (37).

These observations are consistent with the hypothesis that the neuronal deficiency observed in our FGF2−/− mice is the consequence of an early developmental defect. It is also possible that the dramatic wave of apoptosis that occurs in the brain of newborn animals is increased or prolonged in FGF2−/− mice because of the absence of this antiapoptotic factor.

FGF2 has long been thought to play a role in tissue regeneration and skin wound healing. FGF2 is present in normal skin together with other members of the FGF family, FGF1, FGF5, and FGF7, and its expression level increases upon injury (39–41). A mechanism has been proposed by which stored endogenous FGF2, bound to
heparin sulfate proteoglycans in the extracellular matrix, could be released by matrix-degrading proteases present at the wound site and could participate in wound healing (42).

In line with these hypotheses, we have detected a small but significant defect in skin wound healing of FGF2−/− mice. This resulted in a temporary delay for the complete healing of skin wounds and appeared to affect several parameters of wound healing, rather than a single, specific process. It is interesting to note that mice which do not produce FGF7, another FGF that is up-regulated in wounds and is specific for keratinocytes, do not have wound-healing defects (43). It will be also interesting in the future to determine whether in pathological situations leading to wound healing defects, such as diabetes, the requirement for FGF2 is more pronounced than in normal animals.

In conclusion, the results presented here show that FGF2 is not essential for embryonic development and its absence only causes a modest defect in wound healing and a decrease in neuronal density in the motor cortex, whose functional importance remains to be determined. Because FGF signaling has been shown to be essential for many processes of development and tissue formation, these findings probably do not imply that FGF2 is a superfluous gene product, but rather that its function can be fulfilled by other members of the FGF family. A variety of considerations (lack of a signal peptide and inefficient secretion, production of nuclear forms, widespread expression) suggest that the FGF most likely to duplicate FGF2 function is FGF1. FGF1 has a very broad affinity for FGF receptors and is present in many tissues and cells where FGF2 is also expressed (2). The generation of FGF1 knockout mice, which is now in progress in our laboratory, as well as the breeding of heterozygous FGF1 and FGF2 knockout mice to produce doubly homozygous mutants, will verify whether widespread developmental and adult defects are produced by the simultaneous absence of both of these growth factors.

As this manuscript was being completed, Zhou et al. (44) also reported the creation of FGF2 null mutant mice. Although these authors also showed that mice lacking FGF2 are morphologically normal and fertile, they focused their investigation on the cardiovascular system and observed low blood pressure accompanied by a reduction in vein spontaneous contractility. We had also observed a slight decrease in blood pressure in our FGF2−/− mice, but we did not pursue this observation further.

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