**FGF2 Signaling Is Required for the Development of Neuronal Circuits Regulating Blood Pressure**

Rosanna Dono,* Jörg Faulhaber,* Antonella Galli, Aimée Zuniga, Tilmann Volk, Gemma Texido, Rolf Zeller, Heimo Ehmke

**Abstract**—Fibroblast growth factor 2 (FGF2) signaling is involved in angiogenesis, vascular contractility, and cardiac hypertrophy. Mice lacking a functional FGF2 gene (FGF2^−/−) are hypotensive, but the primary physiological role of FGF2 in cardiovascular homeostasis remained unknown. Using a chicken FGF2 (cFGF2) transgene under control of the Wnt-1 promoter, we selectively re-expressed FGF2 in the developing nervous system of FGF2^−/− (transgenic FGF2 mutant) embryos. Expression of the cFGF2 transgene in the developing nervous system, including its autonomic region, was limited to the period between embryonic day 9.5 and 14.5. Significantly, no FGF2 re-expression was detected in developing heart and blood vessels. Pharmacological analysis revealed a normalization of the blood pressure response to isoproterenol-induced vasodilation in adult transgenic FGF2 mutant mice. In addition, the hypotensive phenotype was rescued in 1 line (of 2) transgenic FGF2 mutant adult mice having expressed higher levels of cFGF2 proteins during nervous system development. These genetic studies indicate that FGF2 signaling is essential for complete development of the neural circuitry required for central regulation of blood pressure, whereas it appears dispensable for blood pressure control in the healthy adult. The full text of this article is available at http://www.circresaha.org. *(Circ Res. 2002;90: e5-e10.)*

**Key Words:** blood pressure ■ fibroblast growth factor-2 ■ hypotension ■ mouse ■ transgenic

Fibroblast growth factor-2 (FGF2) is a signaling molecule with important functions for growth and differentiation, particularly in the central nervous system (CNS). From neurulation onwards, FGF2 is expressed at high levels in the CNS,1 and mice lacking a functional FGF2 gene show distinct defects in the developing cerebral cortex and adult spinal cord.2–4 Several observations indicate that FGF2 is also involved in cardiovascular homeostasis. In the adult, FGF2 is expressed in smooth muscle and endothelial cells.5 Infusion of FGF2 causes vasodilation and hypotension in rats, dogs, and humans6–9 via opening of ATP-sensitive K^+^ channels and enhanced release of nitric oxide.6 Endogenous release of FGF2 from vascular smooth muscle cells is triggered by an increase in mechanical strain above normal levels,10 possibly causing transient elevations in cell membrane permeability.11 Together these studies suggest that FGF2 functions in an antihypertensive vasodilator cascade.

Surprisingly, targeted inactivation of the FGF2 gene (FGF2^−/−) in mice leads to chronically decreased blood pressure levels in adults.2,12 FGF2^−/−^ mice display a markedly attenuated myocardial growth response to mechanical overload,13 raising the possibility that impaired left ventricular function may contribute to the hypotension. Furthermore, the spontaneous contractile activity of isolated portal veins from FGF2^−/−^ mice is reduced.12 This may cause alterations in blood volume distribution and cardiac filling pressure. In addition, if this defect affects directly the contractile apparatus, it may also affect arterial resistance vessels. Finally, the neural control of blood pressure is impaired in FGF2^−/−^ mice.2 However, the relative importance and possible contributions of these various deficits to the observed hypotensive phenotype was unclear. Accordingly, the primary physiological role of FGF2 in blood pressure homeostasis has remained obscure.

During CNS development, FGF2 is expressed by progenitor cells of neuronal circuits involved in the central regulation of blood pressure, for example in the myelencephalon2 and the intermediolateral neurons in the spinal cord.14 Furthermore, FGF2 is also expressed by migrating trunk neural crest cells,15 which give rise to peripheral cardiovascular nerves. This strongly suggests an essential role of FGF2 during the normal development of cardiovascular reflex control. If the hypotensive phenotype of FGF2^−/−^ mice were
Indeed primarily caused by a congenital malformation of neuronal circuits regulating blood pressure, then restricted re-expression of FGF2 during nervous system development should rescue the defects in cardiovascular homeostasis in adult mice. To directly test this hypothesis, we used a chicken FGF2 (cFGF2) transgene under the control of the Wnt-1 promoter to target FGF2 re-expression specifically to the developing nervous system in FGF2 mutant embryos.

Materials and Methods

Transgenic Mice
The generation of transgenic mouse lines expressing the 3 cFGF2 protein isoforms in the dorsal aspect of the neural tube has been described previously. Two transgenic lines expressing the transgene appropriately in the embryos (Tg and Tg); see reference 16) were used for all experiments in the present study. The transgenic mice were crossed to FGF2 mice to generate adult mice and embryos of all genotypes. All control and mutant mice were littermates of a mixed C57BL/6J/H11003 genetic background. Mice and embryos carrying the FGF2 loss-of-function allele and/or the cFGF2 transgene were genotyped by polymerase chain reaction (PCR) analysis of tail biopsies or embryonic yolk sac DNA using specific PCR primers.

In Situ Hybridization and Antibody Staining
Expression of the cFGF2 transgene was determined using digoxigenin-labeled riboprobe complementary to coding exon 2 of the chicken FGF2 gene. This probe does not detect endogenous mouse FGF2 transcripts. Embryos and embryonic CNS were dissected into PBS, fixed in 4% paraformaldehyde, and processed for whole-mount in situ hybridization. Alternatively, embryos were embedded in paraffin and 10-μm transverse sections were processed for RNA in situ hybridization. Parallel sections were processed for immunohistochemistry. Ectopic cFGF2 proteins were detected using affinity purified FGF2 antibodies. Endogenous mouse FGF2 proteins were not detected under the conditions used.

Immunoblot Analysis
Embryonic brains and spinal cords were dissected in PBS and immediately frozen in liquid nitrogen. Normalized protein extracts were prepared and analyzed as previously described. Samples were further normalized for protein content using monoclonal β-tubulin antibodies (Sigma 1:2000).

Cardiovascular Studies
All cardiovascular studies were performed in awake, unrestrained female mice (body weight 21 to 42 grams; age 5 to 10 months) in accordance with national guidelines for the care and use of research animals. Animals were anesthetized with ketamine (100 μg per g body weight IP) and xylazine-HCL (4 μg per gram body weight IP). Chronic catheters were implanted in the left femoral artery and vein under aseptic conditions as described previously. Blood pressure was measured in the abdominal aorta via the femoral artery catheter (transducer PRC-21K, amplifier MIO-0501, Föhr Medical Instruments) and continuously recorded at 500 Hz (80586, DAS-0216, Keithley-Metrabyte; LabTech Notebook pro 10.2,1, Labtech). Drugs were infused via the femoral vein catheter by using a calibrated pump (Precidor 5003, Infors AG). Baseline values of arterial blood pressure and heart rate were determined for 1 hour in each mouse in its own cage on day 2 or day 3 (ie, 48 to 72 hours) after surgery. To test the cardiovascular response to an acute hypotensive stimulus, on day 1 (ie, >24 hours) after surgery the β/β-adrenoceptor agonist isoproterenol (Sigma) was infused after a control period of 5 minutes at increasing doses (0.05, 0.1, 0.2, and 0.4 μg/min per gram body weight). Isoproterenol does not cross the blood-brain barrier, and therefore, has no direct effects on the central nervous system. Before each dose of isoproterenol, a baseline recording of 5 minutes was performed. Isoproterenol was infused at a constant rate for 3 minutes. Blood pressure and heart rate responses were averaged over each infusion period. Between each dose of isoproterenol, mice were allowed to recover until blood pressure and heart rate returned to their baseline levels over the entire experimental period.

Statistical Analysis
Values are expressed as mean±SEM. Statistical comparisons were made by 1-way ANOVA (1-hour baseline recordings) or 2-way ANOVA (infusion studies) followed by the Bonferroni’s Multiple Comparison test. An error level of P<0.05 was considered significant.

Results

Temporal and Spatial Expression of the cFGF2 Transgene
Chicken FGF2 (cFGF2) is functionally conserved to its mouse counterpart, and its ectopic expression does not alter morphology of the wild-type CNS. In the dorsal spinal cord and developing autonomic nervous system, the transgene distribution overlaps with that of endogenous FGF2 (Figure 1; compare to reference 2). Therefore, two independent cFGF2 transgenic lines (called Tg and Tg); see reference 16) were intercrossed to FGF2 mice to generate compound transgenic FGF2 mutant embryos and adult mice (FGF2 ; Tg and FGF2 ; Tg) for developmental and physiological analysis.

The temporal and spatial distribution of the cFGF2 transgene was analyzed in transgenic FGF2 mutant (FGF2; Tg) and wild-type littermate embryos (FGF2; Tg and/or FGF2; Tg) using cFGF2 specific probes and antibodies (Figure 1). cFGF2 transcript and protein distributions were identical for both transgenic lines (Tg, Tg) in wild-type and FGF2 mutant embryos (Figure 1 and data not shown). The cFGF2 transgene was expressed in the developing CNS from early neural fold stages onwards (data not shown; see also reference 17). By embryonic day 9.5, the cFGF2 transgene was abundantly expressed in the CNS and at lower levels in developing sympathetic ganglia (arrow, Figure 1A). In the CNS, the cFGF2 transgene was expressed mainly by the dorsal diencephalon, midbrain, myelencephalon, and spinal cord, although no expression was detected in the telencephalon (Figure 1A). Most relevant to the purpose of this study, the cFGF2 transgene was not expressed by the developing heart and blood vessels (embryonic day 9.5 to 16.5; see Figure 1A). From embryonic day 9.5 to 14.5, the cFGF2 transgene expression pattern remained the same in the CNS, whereas it was lost from differentiating sympathetic ganglia (data not shown).

During embryonic day 11, immature neurons of the developing autonomic nervous system separate from the ventral pool of motor neurons and migrate to the intermediate spinal cord layers, where they undergo terminal differentiation. The intermediolateral neurons of the thoracic spinal cord are a major component of the developing autonomic nervous system. Therefore, the cellular distribution of the cFGF2 transgene was determined in transverse sections of thoracic spinal cords at embryonic day 12.5 (Figures 1B and 1C). Highest levels of cFGF2 transcripts were detected in the dorsal aspect of the neural tube (Figure 1B) and a ventral region bordering motor neurons (arrow, Figure 1B). Using...
During later developmental stages, expression of the cFGF2 transgene is progressively downregulated. By embryonic day 16.5, expression in the brain was limited to the developing cerebellum (Figure 1D). In the spinal cord, both cFGF2 transcripts and proteins were restricted to the ependymal layer (Figures 1E and 1F). Interestingly, expression was no longer detected in regions containing differentiating autonomic neurons (indicated by brackets, Figure 1F). This significant decrease of cFGF2 transgene expression was confirmed by semiquantitative reverse transcriptase–PCR (data not shown). Finally, immunoblot analysis of normalized protein extracts prepared from dissected CNS (embryonic day 12.5) showed that all 3 cFGF2 protein isoforms are expressed (Figure 1G). It is important to note that cFGF2 protein levels were at least 2-fold higher in embryos carrying TgC (Figure 1G, lanes 3 and 5) than TgA (Figure 1G, lanes 2 and 4). In summary, these studies establish that expression of the cFGF2 transgene in the developing autonomic nervous system within the CNS is limited from embryonic day 9.5 to about 14.5. The transgene was never expressed outside the developing nervous system (Figure 1; see also references 16 and 17).

Rescue of Blood Pressure Regulation by the cFGF2 Transgene

The results shown in Figure 1 indicate that the cFGF2 transgenic lines are suited to determine whether re-expression of FGF2 in the developing nervous system of FGF2 knockout embryos rescues the defects in blood pressure regulation. Adult FGF2−/− mice fail to compensate a hypertensive challenge induced by intravenous infusion of the β/β-adrenoceptor agonist isoproterenol. Therefore, the effects of increasing doses of isoproterenol on mean arterial blood pressure and heart rate were comparatively analyzed in conscious, adult mice of all genotypes (Figure 2 and Table). Over the entire concentration range, FGF2−/− mice displayed significantly lower blood pressures than their wild-type littermates (Figure 2A). In contrast, FGF2 mutant mice carrying transgene C (FGF2+/−; TgC), or A (FGF2+/−; TgA), respectively, responded normally to increasing doses of isoproterenol (Figures 2B and 2C). This can best be seen by comparing the dose-response curves of transgenic FGF2 mutant mice to those of their wild-type (FGF2+/+) and transgenic wild-type (FGF2+/−; TgC and FGF2+/−; TgA; see Figures 2B and 2C).
littermates. Heart rates were not different between FGF2\(^{\text{+/+}}\), FGF2\(^{\text{−/−}}\), and FGF2\(^{\text{−/−}}\); Tg\(^{\text{A}}\) and their corresponding wild-type controls (FGF2\(^{\text{+/+}}\); Tg\(^{\text{A}}\) and FGF2\(^{\text{−/−}}\); Tg\(^{\text{A}}\); see Table). These results indicate that cardiovascular reflex control of blood pressure is normalized in adult FGF2 mutant mice, which as embryos expressed the cFGF2 transgene specifically in their developing nervous system (Figure 1).

Potential rescue of the hypotensive phenotype in FGF2 mutant mice (Figure 3A; see reference 2)\(^1\)\(^2\) carrying either transgene was assessed by determining the mean arterial blood pressure in conscious, unrestrained adult mice over prolonged recording periods. Indeed, FGF2 mutant mice carrying transgene C (FGF2\(^{\text{+/+}}\); Tg\(^{\text{C}}\)) displayed normal blood pressure levels (Figure 3B), whereas FGF2 mutant mice carrying transgene A (FGF2\(^{\text{+/+}}\); Tg\(^{\text{A}}\)) remained hypotensive (Figure 3C; compare to Figure 3A). Interestingly, baseline heart rates did not differ between the genotypes (Table). The differential rescue of blood pressure may result from a lower level of cFGF2 protein expression in transgenic embryos of line A (Figure 1G), which is probably because of differences in transgene copy numbers and/or integration site. These results indicate that higher levels of FGF2 proteins are required to rescue the hypotensive phenotype in addition to cardiovascular reflex control.

**Discussion**

Although physiological, pharmacological, and genetic studies have demonstrated that FGF2 fulfills important functions in cardiovascular homeostasis, its primary role in blood pressure regulation has remained elusive. We now show that FGF2 signaling in the developing nervous system, but not in vascular smooth muscle cells or cardiac myocytes, is required to establish normal blood pressure control mechanisms.

In particular, these findings indicate that FGF2 signaling is essential for normal development of the neuronal regulatory circuits involved in central regulation of blood pressure. An essential role during embryonic development is in agreement with the essential role of FGF2 in the developing nervous system for normal development of the cardiovascular system.

### Baseline Heart Rates and Heart Rate Responses to Isoproterenol

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Baseline</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.4</th>
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<td>FGF2(^{\text{+/+}})</td>
<td>12</td>
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<td>66±23</td>
<td>78±23</td>
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<tr>
<td>FGF2(^{\text{−/−}})</td>
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<td>7</td>
<td>665±16</td>
<td>66±26</td>
<td>74±25</td>
<td>92±17</td>
<td>70±11</td>
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<tr>
<td>FGF2(^{\text{−/−}}); Tg(^{\text{C}})</td>
<td>8</td>
<td>630±14</td>
<td>73±17</td>
<td>74±12</td>
<td>75±12</td>
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<tr>
<td>FGF2(^{\text{−/−}}); Tg(^{\text{A}})</td>
<td>7</td>
<td>633±16</td>
<td>70±31</td>
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Data were obtained on days 1 through 3 after surgery in conscious, unrestrained mice and represent mean±SEM. Baseline refers to mean heart rates obtained in 1-hour recordings. Responses to isoproterenol denote changes in heart rate relative to 5-minute control recordings obtained immediately before the infusion of isoproterenol was started. n is the number of animals.
with the observed high expression of FGF2 during development of the autonomic nervous system in embryos. However, the specific target cells of FGF2 signaling in these determinative processes remain to be identified. Interestingly, neither baseline heart rates nor heart rate responses to isoproterenol were affected in FGF2 mutant mice and their wild-type littermates. This argues against the cardiovascular phenotype of FGF2 mice being caused by a major defect in regulation of heart rates. Because the slopes of the blood pressure response to isoproterenol are similar in all genotypes, an impairment of the afferent limbs of the baroreceptor reflex seems also unlikely. Rather, the physiological results would be compatible with a dysfunction of preganglionic intermediolateral neurons in the spinal cord. These neurons relay the central sympathetic outflow to the target organs. Their degeneration reduces baseline sympathetic tone and excitatory sympathetic reflexes and is associated with impaired baroreceptor reflex function and hypotension. However, the differential rescue of cardiovascular response to isoproterenol and baseline blood pressure in FGF2 mutant mice displaying transgene A (FGF2; Tg) and wild-type transgenic controls (FGF2; Tg) does not seem to be caused by major defects in blood pressure regulation mechanisms. This observation corroborates the proposal that short- and long-term blood pressure regulation depend on different neural reflex mechanisms. Further comparative physiological analysis of both transgenic FGF2 mutant mouse strains may help to further dissect these pathways. More importantly, the present study demonstrates that neural control mechanisms are an essential component of long-term blood pressure regulation. The identification of the precise nature of this FGF2-dependent pathway will greatly enhance our understanding of the interplay between neural, vascular, and renal mechanisms in controlling blood pressure.

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References


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