A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3

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ABSTRACT Achondroplasia, the most common form of dwarfism in man, is a dominant genetic disorder caused by a point mutation (G380R) in the transmembrane region of fibroblast growth factor receptor 3 (FGFR3). We used gene targeting to introduce the human achondroplasia mutation into the murine FGFR3 gene. Heterozygotes for this point mutation that carried the neo cassette were normal whereas neo– homozygotes had a phenotype similar to FGFR3– deficient mice, exhibiting bone overgrowth. This was because of interference with mRNA processing in the presence of the neo cassette. Removal of the neo selection marker by Cre/loxP recombination yielded a dominant dwarf phenotype. These mice are distinguished by their small size, shortened craniofacial area, hypoplasia of the midface with protruding incisors, distorted brain case with anteriorly shifted foramen magnum, kyphosis, and narrowed and distorted growth plates in the long bones, vertebrae, and ribs. These experiments demonstrate that achondroplasia results from a gain-of-FGFR3–function leading to inhibition of chondrocyte proliferation. These achondroplastic dwarf mice represent a reliable and useful model for developing drugs for potential treatment of the human disease.

Four fibroblast growth factor receptors (FGFRs) are known (1), and >50 mutations in three of them (FGFR1, 2, and 3) recently have been implicated in congenital skeletal and cranial disorders (reviewed in refs. 2–4). Achondroplasia, the most common form of dwarfism, was shown to be linked to a single point mutation, G380R, in the transmembrane region of FGFR3 (5, 6). FGFR3 is expressed mainly by developing bones, brain, lung, and spinal cord (7, 8), and FGFR3–deficient mice show enhanced endochondral bone growth, expansion of their growth plate, and increased chondrocyte proliferation (9, 10). Thus, FGFR3 is a negative regulator of bone growth. Several experiments at the cellular level indicated that the Ach mutation (G380R) results in a constitutive activation of the receptor in a ligand–independent manner (11–13). It was suggested that this is because of stabilization of receptor dimers, a prerequisite for signal transduction in these receptors (14). This is also consistent with the constitutive activation by dimer formation described for an erbB2 (neu) receptor mutant, which carries a Val–to–Glu mutation in an analogous position to that of the FGFR3 variant in its transmembrane region (15). It is likely that, in many of the other mutations in FGFR1–3, the underlying mechanism of receptor activation is also through stabilization of receptor dimers because many of these mutations result in unpaired cysteines that may enhance inter–receptor disulfide bonds (2–4).

To generate an animal model for this type of mutation and to study the role of the mutated FGFR3 in vivo, we used gene targeting to introduce the achondroplasia mutation (G380R) into murine FGFR3. This resulted in a dominant dwarf phenotype that exhibits many of the features of human achondroplasia. This is an indication from in vivo data for the gain–of–function nature of this mutation as the cause of achondroplasia. The dwarf achondroplastic mouse represents a useful model to study the development of this disorder and to search for treatment of the human disease.

MATERIALS AND METHODS

Construction of Targeting Vector. A single phage was isolated from a mouse 129/SV fix DNA library (Stratagene) and contained the entire FGFR3 gene (16). To construct the targeting vector, three separate gene fragments were subcloned into pBluescript II KS(+) (Stratagene): (i) A 4.25-kilobase (kb) HindIII/XbaI fragment containing exons 10–19 was used to generate the G/A mutation at codon 374. The mutation was introduced into a HindIII/SmaI subclone containing exon10 by using the four–oligonucleotide PCR mutagenesis method. The sense (5′–GGCGCTCTCAGCTACAG–3′) and antisense (5′–GACCACCGT1AGCT–GAGGACGC 3′) oligonucleotides both contained the mutation (underlined), and the T3 and T7 primers were from the pBluescript II KS(+) flanks. The product was cut with HindIII and SmaI and was replaced the homologous fragment in subclone 1. (ii) The 3.0-kb EcoRI/HindIII fragment encompassing exons 5–9 then was joined to subclone 1 to generate plasmid 1+2. (iii) The 1.8-kb KpnI/EcoRII fragment containing exon 4, and part of exon of 5, was used to insert a polyclinker containing the sites: 5′–XhoI–HindII–BglII–3′ into the unique BglII site. The targeting construct was generated in the vector Osdupdel, which contains MCI neo flanked by loxP and thymidine kinase under control of 3′ phosphoglycerate kinase (PGK) (a gift from O. Smithies, Univ. of North Carolina), as follows: the KpnI/BamHI (1.3-kb) fragment from subclone 3 was ligated to the KpnI/BamHI sites of the vector downstream to the neo gene to form the 5′ region of homology. The XhoI/EcoRII fragment (0.3-kb) from subclone 3 and the EcoRI/XbaI fragment from subclone 1+2 (6.7-kb) were ligated to the XhoI/Nhel sites between the neo and the thymidine kinase genes to form the 3′ homology region. The transcription orientation of the FGFR3 gene and the neo marker are opposite. We noted that the neo marker had a deletion of 53 bp at the 3′ end, which included the polyadenylation signal.

Generation of FGFR3 Mutant Mice. Culture and selection of embryonic stem (ES) cells (R1 cell line of 129/SV mouse) and the screening of the targeted clones were performed as described (17) by using negative (ganciclovir) and positive

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(G-418) selection. Cell clones were screened by Southern blot analysis by using BglII digestion and probing with the 1.5-kb 5′ probe derived by PCR from sequences upstream to the KpnI site in intron 3 (see Fig. 1). This analysis was also verified by HincII-digested DNA by using both the 5′ probe and 3′ probe based on the HincII site inserted into the polylinker at intron 3 (see Fig. 1). This analysis also was verified by HincII-digested DNA by using both the 5′ probe and 3′ probe based on the HincII site inserted into the polylinker at intron 3 (see Fig. 1). This analysis also was verified by HincII-digested DNA by using both the 5′ probe and 3′ probe based on the HincII site inserted into the polylinker at intron 3 (see Fig. 1). This analysis also was verified by HincII-digested DNA by using both the 5′ probe and 3′ probe based on the HincII site inserted into the polylinker at intron 3 (see Fig. 1). This analysis also was verified by HincII-digested DNA by using both the 5′ probe and 3′ probe based on the HincII site inserted into the polylinker at intron 3 (see Fig. 1).

**RESULTS**

**Generation of Mice with Mutant FGFR3.** To generate a mouse model for achondroplasia, we isolated the mouse FGFR3 gene and introduced a G-to-A point mutation, changing Gly (GGG) to Arg (AGG) in codon 374 (the ortholog of human codon 380), which is located in exon 10. This point mutation (FGFR3<sup>G374R</sup>) was incorporated into a targeting construct, including a floxed neo cassette in intron 4. Other than this mutation, the coding sequence in the construct, which included exons 4–19 (16), was unchanged, but new restriction sites 3′ to the neo cassette were introduced to facilitate molecular analysis (Fig. 1). After homologous recombination in ES cells, heterozygotes were derived that had phenotypes 4 (Fig. 1B). The HincII digest also was used to detect the mutant allele after the Cre-mediated deletion of the neo (Fig. 1D). Clones that carried homologous recombinant containing the mutant FGFR3 were aggregated with eight-cell embryos (18) of MF1 mice. The resulting chimeric mice were crossed with MF1 females, and heterozygotic mutants were identified by Southern blot analysis of tail DNA. Heterozygous mice were mated with PGK-Cre<sup>em</sup> mice (19) to delete the neo marker. The mouse genotypes were identified by Southern blots of tail DNA.

**Skeletal and Histological Analysis.** Skeletal and histological preparations were produced as described (20). Biometry was performed electronically. The brain was observed after perfusion with 2.5% paraformaldehyde. Paraffin sections, staining, alkaline phosphatase activity determination, and in situ hybridization were performed as described (21). Animal care was in accordance with the Weizmann Institute guidelines.
indistinguishable from the wild type. Surprisingly, FGFR3<sup>G374R</sup> homozygotes (abbreviated as neo<sup>+/neo</sup>) exhibited a phenotype very similar to the targeted loss-of-function mutation of FGFR3 (9, 10). They were viable and fertile with kinky tails and dorsal kyphosis, exhibited overgrowth of the long bones, and displayed a greatly expanded growth plate (Fig. 2). DNA sequence analysis of exon 10 verified the presence of the G-to-A mutation in codon 374.

Northern blot analysis revealed that neo<sup>+/neo</sup> animals lacked normal size FGFR3 mRNA (4.2 kb) but contained a novel high molecular weight RNA (~10 kb) that hybridized with FGFR3 as well as with neo (Fig. 3A). These results suggest that the neo cassette may have interfered with the proper splicing of the transcript from the recombinant gene. We conclude that this transcriptional interference caused a loss of FGFR3 expression in the homozygote, resulting in a phenotype similar to the targeted inactivation of FGFR3 (9).

**neo**<sup>+</sup> Heterozygotes Display Dwarfism and Skeletal Defects.

Deletion of the neo cassette by mating neo<sup>+</sup> heterozygotes to PGK-Cre<sup>m</sup> “deleter” transgenic animals (19) resulted in heterozygotes distinguished by a novel phenotype. This cross yielded 50% dwarf and 50% normal offspring whereas mating the neo<sup>+</sup> homozygotes to PGK-Cre<sup>m</sup> transgenic mice resulted in 100% dwarf mice. The gross structure of FGFR3 gene in the homozygotes distinguished by a novel phenotype. This cross yielded 50% dwarf and 50% normal offspring whereas mating the neo<sup>+</sup> homozygotes to PGK-Cre<sup>m</sup> transgenic mice resulted in 100% dwarf mice. The gross structure of FGFR3 gene in the homozygotes (abbreviated as neo<sup>−/neo</sup>) was identical to that of the wild type, as judged by hybridization of FGFR3 cDNA to Southern blots of DNA digested with three different enzymes (data not shown). DNA digested with SpeI, which flanks almost the entire gene (10), demonstrated that the neo<sup>−</sup> heterozygote contains one wild-type allele (~18 kb), which hybridized only with the FGFR3 probe, and a larger fragment, which hybridized to both FGFR3 and neo, representing the recombinant allele. The size of this fragment (~24 kb), the only band that hybridized with the neo probe, was larger than expected from the addition of a single neo cassette (1.2 kb) to the recombinant allele. It is therefore likely that more than one neo cassette was incorporated into FGFR3. To analyze this possibility, we performed PCR on DNA derived from a neo<sup>+</sup> heterozygote mouse by using two oligonucleotides derived from the neo sequence in opposite orientation. This yielded a 3.9-kb PCR product flanked by neo fragments. Sequence analysis of this PCR product showed that it contains the 5′ KpnI-BanHI fragment from the targeting construct followed by the neo cassette and continues with the 3′ fragment of the construct, including exons 5, 6, and 7 and ending at the middle of intron 7. The results indicate that two such truncated fragments of the targeting construct were incorporated in tandem in a head-to-tail orientation in the recombinant allele. These insertions did not alter the size of the fragments detected by hybridization with the external probes. The Cre-recombinase removed all of these fragments together with the neo cassettes, yielding the wild-type size DNA fragment of FGFR3 (Fig. 3B, lane2). Sequencing of the DNA of dwarf mice in the region joining the 5′ and 3′ regions of homology of the construct revealed only a single loxP sequence, as expected from Cre/loxP recombination. The absence of neo cassettes in the DNA of the dwarf mice is correlated with the Northern blot analysis, which showed that the neo<sup>−</sup> heterozygotes contain only FGFR3 mRNA of wild-type size (Fig. 3A, lane2), in contrast to the

Fig. 3. Northern and Southern blot analysis of normal and mutant mice. (A) Northern blot of brain RNA analyzed by FGFR3 cDNA and neo probes. Lanes: 1 and 5, control wild-type mouse; 2 and 6, FGFR3<sup>G374R</sup> heterozygote dwarf mouse; 3 and 7, FGFR3<sup>G374R</sup> homozygote, showing both FGFR3 RNA and the high molecular weight RNA that also hybridized with neo; 4 and 8, FGFR3<sup>G374R</sup> homozygote RNA is devoid of normal size FGFR3 RNA. The blots also were hybridized with a β-actin probe to normalize RNA loading. (B) Southern blot of DNA digested with SpeI that cuts 3′ to the FGFR3 gene and in intron 2. Lanes: 1 and 4, control wild-type mouse; 2 and 5, FGFR3<sup>G374R</sup> heterozygote dwarf mouse; 3 and 6, FGFR3<sup>G374R</sup> homozygote. The results indicate that the neo<sup>−</sup> heterozygote contains a band that hybridized with both neo and FGFR3 (lanes 3 and 6). In the neo<sup>−</sup> heterozygote, this band migrates like wild-type FGFR3, and no hybridization with neo was detected (lanes 2 and 5). The bottom band represents a fragment containing exons 1 and 2. An FGFR3 cDNA probe was used (7).
neo\textsuperscript{+} heterozygotes, which contain both a high molecular weight mRNA species that hybridizes with both FGFR3 and neo and a normal-sized mRNA (Fig. 3A, lane3). Thus, removal of the neo cassettes resulted in a dominant dwarf phenotype that was associated with an FGFR3 transcript of normal size.

The phenotype of the neo\textsuperscript{−} heterozygotes that carried the G374R point mutation is a model for human achondroplasia (Fig. 4A). The weight of these mice was half or less of their wild-type littermates (Fig. 4B), and they could be distinguished by a rounded head (Fig. 4A, D, and G) by as early as 10 days.
of age. In addition, the incisors of the dwarf mice did not align normally (Fig. 4 D, G, and H) because of changes in the skull. To allow normal food intake, the teeth of the dwarfs were softened under anesthesia, and the pelleted mouse chow was provided an experimental system for the testing of drugs to provide an experimental system for the testing of drugs to restore normal bone formation.

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