Delayed Onset of Igf2-Induced Mammary Tumors in Igf2r Transgenic Mice

Thomas L. Wise and Dimitrina D. Pravtcheva

Department of Human Genetics, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York

Abstract
The insulin-like growth factor-II (IGF-II) receptor (IGF2R) regulates the level or activity of numerous proteins, including factors that control growth and differentiation. Frequent loss or inactivation of this receptor in a diverse group of tumors indicates that it may act as a tumor suppressor, but it is not known which functions of this receptor are selected against in the tumors. Lysosomal targeting and degradation of the growth-promoting IGF-II has been proposed as a mechanism for the tumor suppressor effects of IGF2R. As a genetic test of this hypothesis in vivo, we have produced Igf2r transgenic mice that ubiquitously express the transgene and have crossed these mice with mice that develop mammary tumors as a consequence of Igf2 overexpression. Our findings indicate that the presence of the Igf2r transgene delays mammary tumor onset and decreases tumor multiplicity in Igf2 transgenic mice. These findings are relevant to human tumors and preneoplastic conditions accompanied by altered IGF2 expression.

(Cancer Res 2006; 66(3): 1327-36)

Introduction
The insulin-like growth factor-II (IGF-II) receptor (IGF2R), also known as the cation-independent mannose 6-phosphate receptor, binds a diverse group of mannose 6-phosphate–tagged proteins, including lysosomal hydrolases (1), granzyme B, CD26, the latent form of transforming growth factor-β (TGF-β; ref. 2), leukemia inhibitory factor (3), and proliferin. Receptor binding to some of these proteins mediates lysosomal targeting and clearance (1, 3), whereas binding of TGF-β results in its conversion from latent into active form (2). The IGF2R also binds through a distinct region of its extracellular domain the nonglycosylated IGF-II (1), which facilitates its lysosomal clearance. In addition, IGF2R binds with high-affinity retinoic acid, which enhances the known primary functions of this receptor (4). Inactivation of the Igf2r gene causes increased embryo size and late fetal or early postnatal death in most mouse strains (5–7), whereas increased dosage of the IGF2R results in decreased body or organ size (8, 9). The gene encoding this receptor (IGF2R/Igf2r in mice/humans) is imprinted and maternally expressed in mice (as well as in marsupials and many eutherian mammals) but is biallelically expressed in primates (10–12).

Many human tumors (including breast carcinomas) show loss or mutation of one copy of the IGF2R gene (13–16), in some cases accompanied by loss or mutation of the single remaining copy (13, 15, 16). Decreased levels of IGF2R in tumor cell lines increased the rate of tumor growth, whereas increased IGF2R levels had the opposite effect (17–19). Regression of rat mammary tumors after treatment with monoterpenes occurred only if IGF2R levels were raised by this treatment (20). All of the diverse activities of this receptor would be affected by the changes in its dosage (21); thus, their individual contribution to its apparent function as a tumor suppressor is unknown. Analysis of missense mutations in the IGF2R indicates that in most cases the mutations decrease IGF-II binding (22), suggesting a selective advantage of higher IGF-II supply for the tumor. Direct assessment of the tumor suppressor activity of the IGF2R in an in vivo tumor model has not been reported.

Control of IGF-II levels is one of the most important functions of the IGF2R in normal development. The IGF-II, encoded by the Igf2/IGF2 gene in mice and humans, is a member of the insulin-like peptide family in mammals, which also includes insulin and IGF-I. In both humans and mice, the gene is imprinted and expressed predominantly from the paternal allele (23). In addition to IGF2R, IGF-II binds to the IGF-I receptor (IGF1R; encoded by the Igf1r/IGF1R gene in mice/humans; ref. 24), the insulin receptor isoform A (IR-A), and hybrid IR-A/IGF1R (25, 26). These receptors mediate the growth-promoting and antiapoptotic effects of IGF-II (24, 27). Mice with an inactive paternal Igf2 are ~40% smaller than their normal littermates (28), whereas mice with biallelic expression of IGF-II are larger (29). Higher levels of IGF-II in mice are often associated with perinatal lethality (30, 31), and the late fetal or early postnatal lethality of mice with no active Igf2r genes is due to the increased IGF-II levels (7, 32). Two active copies of IGF2 (due to loss of imprinting, paternal disomy, or paternal duplication) have been found in a significant portion of patients with Beckwith-Wiedemann syndrome (33), which is characterized by increased body and organ size and tumor predisposition. The tumorigenic effects of IGF-II have been shown in several mouse models. Transgenic mice that overexpress Igf2 in the mammary gland develop mammary tumors (34, 35), and high serum levels of IGF-II were associated with a general increase in tumor incidence in older mice (36). Elevated local levels of IGF-II increased the incidence of intestinal tumors in ApcMin/+ mice (37, 38), and loss of Igf2 imprinting was a required step in the progression of pancreatic tumors induced by the SV40 large T antigen (39). Loss of imprinting of IGF2 has been described in many human tumors, including breast cancer (40–44). Elevated levels of IGF-II in the tumor cells or surrounding stroma are also a frequent finding in many human and experimental tumors, including breast tumors (45). It is therefore of particular interest to examine in an in vivo model whether the ability of the IGF2R to bind and target for degradation IGF-II will be associated with suppressive effect on the tumorigenic effects of this growth factor.

We have described several lines of transgenic mice that express the mouse Igf2 gene under the control of the H19 enhancer (30, 35). The transgenes are expressed in several adult tissues, including the mammary glands, and induce mammary

Requests for reprints: Dimitrina D. Pravtcheva, Department of Human Genetics, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314. Phone: 718-494-5229; Fax: 718-494-1072; E-mail: dimitrina.pravtcheva@omr.state.ny.us.

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-05-3107
adenocarcinomas with a high incidence in parous females (ref. 35; http://www.compmed.ucdavis.edu/bcancerdc/); the Igf2-induced tumors can metastasize. We have produced transgenic mice that carry a large genomic clone of the mouse Igf2 gene (46) and express the transgene in all tested tissues. We crossed these mice with Igf2 transgenic mice and monitored the female progeny for mammary tumor development. Igf2 transgenic mice that also carried a (maternally inherited) Igf2 transgene showed a delay in mammary tumor development compared with Igf2 transgenic females without the Igf2 transgene. These findings support the role of the IGFR2 as a tumor suppressor in vivo, with respect to tumors that develop as a consequence of Igf2 overexpression.

Materials and Methods

Preparation of the microinjection construct. A yeast artificial chromosome (YAC) clone containing the entire mouse Igf2 gene (ICRF y903G011; ref. 47) was obtained from the Resource Centre of the German Human Genome Project. This clone extends up to the Sod-2 gene 5′ of the Igf2 gene and includes the Slc22a1 (formerly Lxl) gene 3′ of Igf2 (47). To distinguish the Igf2 transgene from the endogenous gene, we abolished an AvrII site in the nontranslated portion of the last exon of Igf2 (exon 48) by homologous recombination in yeast. We subcloned a 1,678-bp MseI genomic fragment (which contains Igf2 exon 48) from BAC 116p19 (Invitrogen, Carlsbad, CA) into pPCR-Script (Stratagene, La Jolla, CA). An AvrII site within this fragment was abolished by digestion with AvrII, filling in with Klenow, and religation (Fig. 1A). This fragment was excised from pPCR-Script by SalI/NotI digestion and transferred to the yeast replacement vector p680 (a gift from Dr. Philip Hieter, University of British Columbia, Vancouver, British Columbia, Canada), also digested with SalI and NotI. p680 carries the LEU2 and CHY2 markers, which allow positive/negative selection for homologous recombinants. YAC y903G011 was transferred from the original AB1380 yeast strain (MATα ura3-52 tryp1 ade2-1 his3 lys2-1 can1-100) to YPH925 (MATα ura3-52 tryp1 lys2-801 ade2-101 his3-d20 leu2-d1 cyh2 karl-d15), obtained from American Type Culture Collection (Manassas, VA), to make possible the positive and negative selection required for the two-step replacement protocol. A yeast transformation was done by cis-1– mating (48). UraA-Trp+Cyp8 colonies were selected on AHC +Cyc plates, and diploids were distinguished from YPH925 YACdau1ants by mating type locus PCR. Primers and conditions for the PCR were as described (48). In addition, selected colonies were analyzed by PCR for the retention of Mas1, Slc22a1, Igf2 exon 1, and exon 48. YPH925 cells carrying an intact YAC y903G011 were then transformed with the p680 clone containing the exon 48 AvrII MseI fragment (48), which was linearized with NsiI before transformation (Fig. 1A). Initial selection for clones retaining p680 was carried out on SC-Leu plates, whereas selection for homologous recombinants that have lost the plasmid sequences was carried out on AHC +Cyc medium. Colonies surviving the selection were analyzed by PCR to identify clones that carry the modified AvrII site. These clones were in addition analyzed by PCR for the retention of Igf2 exon 1, Mas1, and Slc22a1. To remove from the YAC genes irrelevant to the present study and to facilitate the isolation of the recombinant clone, we transferred the sequences between Slc22a1 and Mas1 (Fig. 1B) to the yeast bacterial shuttle vector pClasper (49) by homologous recombination in yeast. A 596-bp fragment from the last exon of Mas1 was amplified with a 5′ BamHI linker and a 3′ NruI linker. The PCR product was then polished and cloned into the SfiI site of pCRScript Amp SK+(Stratagene). A 496-bp fragment from Slc22a1 was also amplified with a 5′ SalI linker and a 3′ HindIII linker and cloned into the same vector. The BamHI/NruI Mas1 fragment was then excised from pPCR-Script and inserted into BamHI/NruI cut pClasper. The SalI/HindIII SfiI/Slc22a1 fragment was similarly inserted into the SalI/HindIII cut Mas1–pClasper clone. The Mas1–Slc22a1 pClasper clone was then linearized with NruI/SalI and used to transform yeast cells carrying the ΔAvrII YAC y903G011. Positive clones were selected on SC-Leu plates. Recombination between the linearized Mas1–Slc22a1 pClasper vector and the YAC results in transfer of the ~170-kb genomic fragment between Mas1 and Slc22a1 to the pClasper vector. Clones in which this transfer had occurred were identified by PCR with two primer pairs bracketing the Mas1 and Slc22a1 junctions that would be created if such transfer had occurred. DNA isolated from agarose plugs of the positive yeast clones was then used to transform bacteria. Transformed clones were tested for retention of the entire Igf2 gene by PCR with primers from selected Igf2 exons. The Igf2 genomic segment was separated from the pClasper vector by digestion with 1-Pmol followed by pulsed field gel electrophoresis (Fig. 1C). The ~170-kb genomic fragment was electroeluted from the gel, dialyzed, and microinjected into zygotes. The primers used for amplification were CCGGATCCGCACTAGTGGAGAGGCTCATAGC and GGTGTTCCGCCGAGCTCTCAAGTACAGTTG for Mas1, CGGTGGACCCGTAATCCCAACCACTGTCCATCT and CCAAGGTGGCCCGCCGACCAACTTCCACAGTCTCGCTC for Slc22a1, GGGCGGAAAAAGGCTATATCA (pClasper) and GGATTGGAACGAGCTCCTG (Slc22a1) and ATACAGCTATCGGCTTGA (pClasper) and CCACGTGTTTCTGTGACACTA (Mas1) for testing for recombination between YAC and pClasper, CTGCAACA- GGGCTCAAGTCC and GGGCGGCGCCGCAACCTTC for Igf2 promoter exon 1, CACATGGAGGCTGTGCACT and CCGAGTCTTCTGTCTTATTAG for Igf2 exon 2, AAGTTCAGGCGATACCTC and CCCACACAGAAGATG TAATGC for Igf2 exon 3, and ATAGATGGAGACTGGCGCTTAC and AACTGCGGCAAGCGGTTTC for Igf2 exon 48.

Pulsed field gel electrophoresis. High molecular weight DNA embedded in agarose plugs using LIDS (48) was run on a 1% agarose gel in 0.5× Tris-borate EDTA in a Bio-Rad (Hercules, CA) CHEF-DR II pulsed field gel apparatus. For identification of the 170-kb Igf2 genomic fragment, the gel was run at 230 V with a 1- to 25-second pulse time for 24 hours.

Embryo microinjection. Purified DNA was microinjected into FVB/N embryos (30). Injected embryos were cultured overnight, and those that had divided were implanted into day 1 pseudopregnant fosteres.

Mice. Unless otherwise indicated, all mice in this report were produced and maintained on a FVB/N background. The Mas1 and Slc22a1 probes used for Southern analysis were the fragments amplified for the YAC–pClasper transfer. The Igf2 transgenic line uniH1degfMu2- (30, 35) was used in the crosses with Igf2 transgenic mice. This Igf2 transgene contains a 17-kb EcoRI Igf2 genomic fragment (which includes part of the noncoding exon 1, exons 2-6, and 3′ flanking sequences) under the control of the H19 enhancers (30, 35). For functional tests of the Igf2 transgenes, transgenic mice were crossed with mice that carry the 7th deletion (strain C57S.AK7TH; The Jackson Laboratory, Bar Harbor, ME). Mice were removed when they were ~10% of the weight of the host, showed ulceration or the mouse looked sick.

DNA analysis. DNA was extracted from tails as described (30). Restriction digests, blotting, and hybridization were carried out by standard protocols. Routine screening of progeny was carried out by PCR followed by AvrII digestion.

Expression analysis. Transgene expression was analyzed by reverse transcription-PCR (RT-PCR) and Northern blotting. Total RNA was prepared from tissues with Trizol reagent (Invitrogen, Carlsbad, CA). For RT-PCR, total RNA (250 ng) was reverse transcribed with SuperScript II or III (Invitrogen). cDNA (2 μl) was amplified with Igf2 exon 48 primers under standard conditions with the addition of 1 mol/L betaine. PCR product (15 μl) was digested with AvrII and run on a 1.5% agarose gel. For Northern blots, total RNA (20 μg) was run on a 2.2 mol/L formaldehyde gel and transferred to a nitrocellulose or Nytran membrane.

Western blot analysis of IGFR2. Protein extracts from frozen tissues were prepared as described (Santa Cruz Biotechnology Western blotting protocol, Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Protein (40 μg) was run under reducing conditions on a 3% to 8% Tris-acetate criterion gel (Bio-Rad). The protein was blotted onto a polyvinylidene difluoride membrane in a Transblot semidyry transfer cell (Bio-Rad) with Towbin transfer buffer (without methanol) for 30 minutes at 10 V. The membrane was cut in two and blocked for 1 hour in 5% nonfat dry milk, 0.1% Tween 20 in TBS at room temperature. The membranes were incubated for 2 hours with either anti-IGF2R antibody (top of gel; Santa Cruz Biotechnology) or anti-actin antibody (bottom of gel; Sigma, St. Louis, MO) at room temperature and then for 45 minutes at room temperature.
with alkaline phosphatase–conjugated anti-goat IgG antibody (Santa Cruz Biotechnology). The membranes were washed thrice for 20 minutes each in wash buffer [10 mmol/L Tris (pH 9.5), 10 mmol/L NaCl, 1 mmol/L MgCl₂] and antigen-antibody complexes were detected with CDP-Star reagent (New England Biolabs, Ipswich, MA). For quantitation of band intensity, the X-ray film was scanned and analyzed with Aida image analysis software.

**Statistical analysis.** The time of first tumor appearance was used to make Kaplan-Meier survival curves that were compared with the log-rank test. Tumor multiplicity comparisons were analyzed with the one-tailed Student’s *t* test. Results are mean ± SE.

**Results**

**Generation of Ig2r transgenic mice.** For reliable assessment of the antitumor effects of IGF2R in vivo, it was desirable to achieve widespread, nonmosaic transgene expression. A ubiquitously

---

**Figure 1.** *Igf2r* microinjection construct and production of transgenic mice. **A,** structure of the *Igf2r* gene (from ref. 46) and a magnified view of exon 48 with the position of relevant restriction sites. Vertical bars, exons; filled portion of exon 48, translated sequences; filled circle in intron 2, position of the differentially methylated region 2, which controls the transcription of the antisense transcript Air (50); asterisk, modified AvrII site in exon 48. **B,** gene content of YAC y903G011 (47) and transfer of the genomic fragment between MasI and Slc22a1 to pClasper. Distances between genes and relative size of pClasper and YAC clones are approximate. **C,** pulsed field electrophoresis of ΔAvrII-*Igf2r*-pClasper after digestion with I-PpoI, showing separation of insert from vector. Lane U, undigested DNA; lane M, midrange I-PpoI markers (New England Biolabs). **D,** genotyping of *Igf2r* transgenic founders. Top, PCR analysis. Tail DNA was amplified with *Igf2r* exon 48 primers followed by AvrII digestion. Numbers above the lanes, individual founder mice. Lane C, ΔAvrII-*Igf2r*-pClasper DNA (control); lane 0, without DNA; lane M, molecular weight markers. Founder mice 66, 70, and 72 contain the *Igf2r* transgene. Bottom, Southern analysis. DNA was digested with the indicated restriction enzyme, blotted, and hybridized with the probe indicated underneath. A novel fragment different from the endogenous gene is detected in each *Igf2r* transgenic line. Numbers above the lanes, transgenic line; NT, nontransgenic.
expressed transgene would intercept IGF-II regardless of the exact cell type expressing this growth factor. Large genomic constructs are more likely to contain the regulatory elements required for proper transgene expression. The Igf2r gene contains 48 exons, spread over 88 kb (Fig. 1A; ref. 46; http://www.ncbi.nlm.nih.gov/mapview). The Mas1 gene is located ~65 kb upstream, and the Slc22a1 gene is located ~34 kb downstream of the Igf2r gene on YAC Y903G011 (47). A very large (>100 kb) nonspliced

Figure 2. Igf2r expression and imprinting in ΔAvrl-Igf2r transgenic mice. Expression was analyzed by RT-PCR using primers that bracket the AvrI restriction site (Fig. 1). RT-PCR products were digested with AvrI and run on an agarose gel. The genealogy of each analyzed mouse is shown above the corresponding panel. Filled symbols, Igf2r (transgene) homozygotes; half-filled symbols, Igf2r hemizygotes; empty symbols, nontransgenic mice. Low expression after paternal inheritance is due to imprinting. Expression of transgenes inherited from maternal grandfathers (lines 51, 66, 70, and 72) indicates that inactive transgenes are reactivated after passage through the female germline.
antisense transcript (Air) is initiated in intron 2 of the Igf2r gene and extends into the last intron of the gene (Fig. 1). The promoter of this transcript (region 2) is methylated on the maternal chromosome, silencing the antisense transcript and allowing transcription of the maternal allele (50, 51). On the paternal chromosome, this promoter is not methylated, Air is transcribed, and the paternal Igf2r allele is silenced. Whereas the nature and location of regulatory elements required for proper Igf2r expression has not been defined, an active transgene (whose reading frame was disrupted in the first exon) was properly expressed after truncation at the Mars1 locus (51). Therefore, we used a genomic clone containing the entire mouse Igf2r gene as well as flanking sequences up to the neighboring Mars1 and Slc22a1 genes (Fig. 1). The starting point for this construct was YAC ICRF y903G011 (47, 51). An AvrII site in the nontranslated portion of Igf2r exon 48 was abolished by homologous recombination in yeast (Fig. 1A; see Materials and Methods), and the YAC was truncated at the Mars1 and Slc22a1 loci by homologous recombination and transfer to the shuttle vector pClasper (Fig. 1B). The 170-kb Igf2r insert from this BAC was used for microinjection. Ten transgenic founders were identified by PCR with primers from Igf2r exon 48 that bracket the modified AvrII site. AvrII digests of the 496-bp PCR product of the endogenous gene yield two fragments (355 and 141 bp; Fig. 1D), whereas the PCR product of the transgene remains uncut. Four of the lines (51, 66, 70, and 72) showed normal transmission, proper expression, and imprinting (see below); these were used in the crosses described in this report. Southern analysis of these four lines with the Slc22a1 probe detected novel fragments in all of the lines, indicating the retention of the 3′ flank of the injected Igf2r gene in the transgenic mice. The Mars1 probe detected novel fragments in lines 51, 70, and 72 and an increased intensity of a fragment migrating with the endogenous Mars1 band in line 66, indicating that the 5′ flank of the injected Igf2r gene is also present in all of the transgenic lines (Fig. 1D). An exon 48 probe detected a band with increased intensity corresponding to the endogenous fragment in all transgenic lines (data not shown).

Transgene expression and imprinting. Expression was analyzed by RT-PCR followed by AvrII digestion (Fig. 2). Adult mice that had inherited the Igf2r transgene from their mothers expressed the transgene in all tested tissues (similar to the endogenous gene). Mice that had received the transgene from their father showed little transgene expression, with the exception of kidney and brain. The endogenous mouse Igf2r gene is also biallelically expressed in the brain (52). Low-level expression in additional tissues was detected after paternal transmission in lines 51 and 72. The genealogy of the mice whose tissues were used for the expression analysis indicates that an inactive transgene transmitted from the father is reactivated and expressed in the progeny of its daughters. All of the transgenes were expressed in the mammary gland. We also analyzed transgene expression in day 16 embryos of line 51 and day 17 embryos of line 72 by Northern analysis. Moderately increased levels of the Igf2r transcripts, between 1.4- and 2.6-fold, were detected in the transgenic progeny (Fig. 3). We have
Additionally examined the level of IGF2R expression in the Igf2r transgenic mice by Western blotting (Fig. 4). In all lines and in all tested tissues, including the mammary gland, we detected a moderate increase in IGF2R signal, generally in the 1.5- to 2.5-fold range.

**Functional tests of the Igf2r transgenes.** As an additional test of the Igf2r transgenes in these mice, we examined their ability to complement the lethal phenotype of mice that inherit the I^pr^ deletion from their mothers. The I^pr^ deletion removes a segment of proximal chromosome 17, including the endogenous Igf2r gene (refs. 10, 53; http://www.informatics.jax.org). Mice (from most strains) that inherit the deletion from their mothers die in the late prenatal period mainly as a result of the accumulation of nondegraded IGF-II (7, 32). Reactivating the paternal Igf2r allele (by preventing its imprinting) restores viability to these mice (9).

We crossed Igf2r transgenic females with males that carry the I^pr^ mutation (strain C3Sn.AK-1^pr^ from The Jackson Laboratory or F1 male progeny of a FVB/N × C3Sn.AK-1^pr^ cross) and identified female progeny that had inherited both the I^pr^ mutation and the Igf2r transgene. Carriers of the I^pr^ mutation were identified by the abnormal tail morphology and by Southern analysis of their DNA with the Bb-40 probe (53). Igf2r transgenic, I^pr^ mutant females were subsequently crossed with FVB/N males to produce one to two litters. Females from all four transgenic lines were able to transmit the I^pr^ mutation to their progeny, and in all cases, live mutant progeny also contained the Igf2r transgene. Therefore, we concluded that the transgenes in all four of the lines are producing a functional IGF2R, capable of mediating IGF-II degradation and counteracting the IGF-II-induced lethality. Production of a functional IGF2R protein was also suggested by the smaller size of Igf2r+ progeny compared with their Igf2r− littersmates.\(^1\)

**Igf2r transgenes delay tumor onset in Igf2 transgenic mice.** To assess the ability of IGF2R to counter the tumorigenic effects of IGF-II, we produced mice that carry the Igf2 transgene with or without the Igf2r transgene by crossing female mice from each of our four Igf2 transgenic lines with male mice from the H19egMlu line 2 (30, 35). Progeny were genotyped for the presence of the Igf2 and the Igf2r transgene using the ΔMlu1 and the ΔAvrII polymorphisms introduced into these transgenes. For uniformity, female mice were allowed to produce a single litter. Because tumor incidence in MMTV-c-myc transgenic mice was increased by early pregnancy (54), we tried to ensure that our groups were comparable with regard to the age at which they produced litters. The mice were monitored by weekly inspection and palpation for the development of mammary tumors. The tumors in these mice appear as firm nodules and can be unambiguously detected by palpation when they are only ~1 mm in size. The single initial tumors (whose appearance marked the age of tumor onset) are followed by the appearance of additional tumors at a later time. Mice that do not contain the Igf2 transgene had a low incidence of mammary tumors: 1 of 34 (2.9%) of Igf2r−, Igf2+ mice and 1 of 84 (1.2%) of Igf2−, Igf2+ mice developed mammary tumors at <1 year of age. Therefore, our report will focus on the comparison between Igf2−, Igf2+ and Igf2+, Igf2+ transgenic groups. Comparison between these two groups of mice was carried out separately for progeny of each line to control for intrauterine or postnatal care variables (e.g., nutrition) that may independently influence tumor development.

The mean age at litter delivery of the Igf2r+ mice was nearly identical in lines 51 and 66 and differed by 2 to 4 weeks in lines 70 and 72 (Table 1). The median age at tumor discovery (in weeks) of the mice in the Igf2r+, Igf2+ and Igf2−, Igf2+ groups was 39 versus 29 for line 51, 39 versus 33 for line 66, 39 versus 29 for line 70, and 37 versus 29 for line 72. The presence of the Igf2r transgene thus caused a statistically significant delay in the appearance of mammary tumors in all of the lines (Fig. 5).

**Tumor multiplicity in Igf2r+, Igf2− and Igf2−, Igf2+ transgenic mice.** Because of the different rate of growth of these tumors and our intention to look for effects of the Igf2r transgene on metastases, tumors were allowed to grow for as long as possible (see Materials and Methods). Tumor number was determined at the time of dissection. A summary of the data on tumor multiplicity for all lines combined is shown on Table 2. The data indicate that approximately one-fifth to one-fourth of the tumors that would have developed in the Igf2 transgenic mice were suppressed in the presence of the Igf2r transgene. The mean ± SD age in weeks at the time of dissection was 65.84 ± 12.40 for the Igf2r+, Igf2+ group and 57.22 + 11.60 for the Igf2−, Igf2+ group (P = 0.0003). Thus, Igf2r+, Igf2+ mice had a lower number of tumors despite being significantly older than the Igf2−, Igf2+ mice at the time of dissection. This age difference is partly due to the later age of tumor appearance in the Igf2r+ mice.

**Tumor histology and metastases in Igf2r+, Igf2− and Igf2−, Igf2+ transgenic mice.** Forty-five Igf2 transgenic mice that carried the Igf2r transgene and 54 Igf2 transgenic mice without the Igf2r were dissected and examined for metastatic involvement of lung, spleen, and liver (organs to which these tumors are known to spread; ref. 35). Mammary tumor tissue as well as liver, spleen, or lung of all mice that had macroscopically visible metastases or suspect lesions were analyzed by histology (11 Igf2r+, Igf2+ mice and 16 Igf2−, Igf2+ mice). The mammary tumors in these mice (Fig. 6) displayed papillary or tubuloacinar patterns, with the former being found more frequently in Igf2r+ tumors (45% versus

<p>| Table 1. Mean age at litter delivery of mice monitored for tumor development |
|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th><strong>Igf2r line</strong></th>
<th><strong>Igf2r+, Igf2+</strong></th>
<th><strong>Igf2r−, Igf2+</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. mice with recorded date of delivery</td>
<td>Mean ± SD age at delivery (wk)</td>
<td>No. mice with recorded date of delivery</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>51</td>
<td>20</td>
<td>12.85 ± 4.73</td>
</tr>
<tr>
<td>66</td>
<td>23</td>
<td>12.69 ± 5.81</td>
</tr>
<tr>
<td>70</td>
<td>24</td>
<td>13.00 ± 5.49</td>
</tr>
<tr>
<td>72</td>
<td>26</td>
<td>12.93 ± 8.74</td>
</tr>
</tbody>
</table>

\(^1\) Unpublished observations.
There was no evidence for decreased mitotic activity in the \textit{Igf2r}+ tumors: 55% of these tumors showed more than three mitotic cells per field, whereas 31% of the \textit{Igf2r}– tumors had more than three mitotic cells per field. Histologic analysis distinguished primary lung tumors (which are a strain characteristic of older FVB/N mice and are increased in frequency in \textit{Igf2} transgenic mice; refs. 35, 55) and mammary tumor metastases (Fig. 6). A summary of our findings is presented in Table 3. There was no evidence for an effect of the \textit{Igf2r} on metastases in these mice. However, the frequency of \textit{Igf2} transgenic mice with metastases in the present study is lower than the frequency we detected previously (35–40%; ref. 35). We attribute this difference to the fact that the mice described here were allowed to have only a single litter, whereas those in the previous report had multiple litters. The effect of the number of pregnancies on the metastatic frequency will be directly addressed in future studies.

\textbf{Discussion}

In the present study, we examined the effect of the IGF2R on mammary tumors induced by overexpression of an \textit{Igf2} transgene. Whereas it was our hypothesis that a reduced amount of IGF-II (as would be expected in the presence of the \textit{Igf2r} transgene) should reduce the number or delay the appearance of tumors in these mice, this outcome could not be anticipated with certainty. The exact mechanism of the tumor suppressor effects of the IGF2R and its effectiveness in this tumor model was unknown. The IGF2R level in our mice was increased very moderately. In addition, only 10% of the IGF2R is present at the cell surface (1). It was not clear if the small increase in the number of IGF2R molecules on the cell surface of these mice will have a measurable effect on the interaction of IGF-II with its numerous other binding partners, including the IGR1R, the insulin and hybrid receptors, and the family of IGF-binding proteins. In a previous report, increased levels of the soluble IGF2R decreased the size of most but not all organs expressing an \textit{Igf2} transgene (8). Although IGF2R levels increased to a similar extent in an \textit{in vitro} model were effective in suppressing tumor growth (17), we could not rule out that the tumor cells \textit{in vivo} would quickly adapt to the lowered levels of IGF-II, with no measurable effect on their growth and characteristics. These experiments thus represent the first \textit{in vivo} test of the functional significance of small variations in IGF2R levels, within the range that can be elicited by physiologic stimuli, with respect to tumor growth.

IGF-II activates mitogenic and antiapoptotic signaling pathways (24, 27) and affects the differentiation state of intestinal cells (38). The mouse \textit{Igf2} gene is expressed in the normal mammary epithelium before and during pregnancy, with a punctate pattern

### Table 2. Effect of \textit{Igf2r} on tumor multiplicity

<table>
<thead>
<tr>
<th>\textit{Igf2r}+ (all lines), \textit{Igf2}+</th>
<th>\textit{Igf2r}– (all lines), \textit{Igf2}+</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. mice examined</td>
<td>Mean ± SE no. tumors/mouse</td>
</tr>
<tr>
<td>52</td>
<td>3.63 ± 0.34</td>
</tr>
</tbody>
</table>

\( P = 0.018 \)
similar to the localization of dividing cells (56). It mediates the effects of prolactin on cyclin D1 in pregnancy-associated alveologenesis (57). Overexpression of prolactin or its receptor, Igf2 or cyclin D1 in mice has been associated with mammary tumor development (34, 35, 58–60). Human breast tumors more commonly express IGF2 in their stroma, whereas IGF1 is predominantly expressed by stromal cells in the normal breast (45). Breast cancer patients show loss of imprinting for IGF2 (40–43), which can be detected in benign breast disease (40) and in the normal tissue adjacent to the tumor (43). Early loss of imprinting has also been reported in human gastrointestinal tumors (44), indicating that reactivation of the maternal IGF2 allele precedes overt malignant growth. Thus, the results of this study are relevant to human preneoplastic and neoplastic conditions accompanied by altered IGF2 expression.

By using a large genomic construct, we were able to achieve widespread expression and proper imprinting of the Igf2r transgenes. Expression was analyzed by RT-PCR (which distinguishes between endogenous and transgene products; Fig. 2), Northern analysis (which indicated an increased level of Igf2r mRNA in the transgenic mice; Fig. 3), and Western blotting (which detected increased levels of the IGF2R protein; Fig. 4). The increase of Igf2r mRNA and protein in most tissues was between 1.5- and 2.5-fold. In this regard, the Igf2r transgenic mice differ from mice with a segmental chromosome 17 trisomy and two maternal copies of the Igf2r gene; these mice expressed both maternal copies of the Igf2r but showed no increase in Igf2r transcript levels in adult liver and heart and no increase greater than normal variation in E12.5 embryos (61). However, mice with a paternally inherited Igf2r allele unable to undergo imprinting did show increased Igf2r levels and corresponding effects on growth (9). The reasons why there seems to be dosage control of Igf2r transcript levels in some systems but not in others are unclear. The ability of our Igf2r transgene to rescue mice with a maternally inherited deletion of the endogenous Igf2r gene indicated that this transgene produces a functional IGF2R protein capable of reducing the lethal levels of IGF-II in these mice.

The Igf2r transgenes were expressed in the mammary gland (Figs. 2 and 4), which is necessary for countering the autocrine/paracrine tumorigenic effects of the Igf2 transgenes. In all four of our transgenic lines, the presence of the Igf2r transgene was associated with a statistically significant delay in the time of first tumor appearance (Fig. 5). Igf2r+/Igf2+ mice also had a statistically significant decrease in tumor multiplicity (Table 2) despite the much higher mean age of the mice of this group at examination. The most likely mechanism for the observed tumor suppressor effect is a reduction in IGF-II levels, although other mechanisms (e.g., TGF-β activation) can also contribute to these results. The effects of the Igf2r on tumor onset and multiplicity are particularly remarkable in view of the very modest increase in Igf2r expression detected by Northern and Western blotting. The Igf2r transgene did not seem to reduce the mitotic activity in the tumors or affect their metastatic ability. The number of mice with metastases in the current study, however, was low; therefore, this latter observation needs to be confirmed on a larger number of mice, particularly in line 51.

We conclude from these findings that the Igf2r (at the expression levels achieved in our study) affects the early stages

| Table 3. Metastases in Igf2r+, Igf2+ and Igf2r−, Igf2+ transgenic mice (to lung, spleen, or liver) |
|---|---|---|
| Igf2r line | Ratio (%) with metastases |
| Igf2r+, Igf2+ | Igf2r−, Igf2+ |
| 51 | 1/11 (9) | 3/13 (23) |
| 66 | 1/15 (7) | 1/18 (6) |
| 70 | 0/8 (0) | 0/6 (0) |
| 72 | 2/13 (15) | 2/17 (12) |
| 4/45 (9) | 6/54 (11) |

Figure 6. Histology of mammary tumors and neoplastic lung lesions in Igf2 transgenic mice. A, mammary tumor; B, lung metastasis of mammary carcinoma; C, bronchioalveolar adenoma. H&E staining.
of Igf2-induced tumor development (those that precede the appearance of palpable tumors). These results agree with findings in humans, where loss of heterozygosity for the IGF2R is already present in preinvasive stage breast cancer (canceroma in situ; refs. 13, 14) and in premalignant hepatic lesions (16), indicating selection against the IGF2R before the cells acquire invasive properties. The fact that loss of imprinting for IGF2 also seems to be an early event in the development of breast cancer (43) suggests that IGF2R loss of heterozygosity or mutation is selected for to increase the supply of IGF-II to the premalignant or early tumor cells. On the other hand, there is no evidence that IGF2 expression by the breast tumor stroma affects the clinical course of the disease, because it was found to have no independent effect on patient survival (62). Decreased reliance of the tumor cells on growth factors at later stages of their evolution has been proposed as an explanation (62).

In summary, we have produced transgenic mice with a large genonic construct of the Igf2r transgene to carry out genetic analysis of the role of this receptor as a tumor suppressor in vivo. Our findings indicate that the Igf2r slows down or suppresses the early stages of tumors that develop as a result of IGF2 overexpression. These findings would justly attempt to increase IGF2R levels through pharmacologic means as a way to delay or prevent the appearance of IGF-II-dependent tumors in humans and to counter other deleterious effects associated with IGF2 overexpression.

Acknowledgments
Received 8/30/2005; accepted 11/15/2005.

Grant support: NIH grant DK R0148936 (D.D. Pravtcheva) and New York State Office of Mental Retardation and Developmental Disabilities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Daniel Kerr and Jennifer Shen for excellent technical assistance, Dr. Suzanne Bradshaw for the pClasper vector and helpful information on using it, Dr. Phil Hieter for the p680 vector, and Dr. Yu-Wen Wang for helpful tips with Western blotting. Dr. Suellen Greco (Department of Comparative Medicine, Washington University) carried out pathology analysis of the mice.

References


Delayed Onset of \textit{Igf2}-Induced Mammary Tumors in \textit{Igf2r} Transgenic Mice

Thomas L. Wise and Dimitrina D. Pravtcheva


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/3/1327

Cited articles
This article cites 60 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/3/1327.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/66/3/1327.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.