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Disproportionate growth in mice with *Igf-2* transgenes

(insulin-like growth factor II/organ hyperplasia/skin/uterus/colon)

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ABSTRACT **Injection transgenesis was used to study the long-term effects of excess insulin-like growth factor II on mouse growth and differentiation. By using a construct in which the coding region of the mouse insulin like growth factor II gene (*Igf-2*) was placed under the control of a keratin gene promoter, four transgenic lines were established, all of which displayed overgrowth of the skin as judged by wrinkling. In addition to high levels of expression in the skin, transgene transcripts were also present in the alimentary canal and uterus. At most of the sites of transgene expression the cell number (DNA content) was greatly increased, indicating a local action of the excess insulin-like growth factor II on cell multiplication. Adult total live weight was slightly increased and there was no macroscopic evidence of tumor formation. The characteristics of these transgenic mice indicate distinct local and systemic actions for insulin-like growth factor II.**

We have investigated the long-term effects of excess insulin-like growth factor-II (IGF-II) on mammalian growth and differentiation, using transgenic mice. In contrast to humans (e.g., ref. 1), rodent tissue and plasma IGF-II levels fall rapidly after birth, adult rodents have low levels of this growth factor, and they provide a natural test bed for its particular effects (2, 3). The consequences of IGF-II excess were unknown because it has been difficult to produce transgenic mice overexpressing this growth factor (4, 5) and because minipump infusion can be applied only for limited periods. No substantial growth effects were detected following IGF-II infusion into hypophysectomized rats (6) or fetal sheep (7) or in adult rodents with IGF-II-producing tumors (8).

The absence of a functional gene encoding IGF-II (*Igf-2*) in the mouse did not alter growth rate or body proportions after birth (9, 10). There was also evidence that IGF-II countered some of the actions of IGF-I (refs. 8 and 11; reviewed in ref. 7) and thus might act as a competitive inhibitor of the growth hormone/IGF-I axis of growth promotion (12, 13). The only indication that IGF-II might promote postnatal growth was the work of Van Buul-Offers *et al.* (14), who observed organ specific growth promotion when IGF-II was repeatedly injected into pituitary-deficient Snell dwarf mice. The success of this procedure was attributed to the relative immaturity of these animals, and the general view was that IGF-II was a poor substitute for IGF-I (reviewed in ref. 15).

The difficulty in producing transgenic mouse lines which overexpress IGF-II may be due, at least in part, to the extreme sensitivity of the developing fetus to levels of this growth factor (refs. 9, 10, 16, and 17; reviewed in ref. 18). Consequently, we used a promoter normally active in limited regions of the late fetus and the adult. The bovine keratin 10 (bovine keratin VI, BKVI; ref. 19) promoter had been shown to direct transgene expression to the suprabasal layer of skin and the forestomach (20, 21), while the keratin encoded by the equivalent mouse gene was detected as a protein in the forestomach, the upper parts of the alimentary canal, and the skin after embryonic day 15 (E15) (22, 23). We describe the overgrowth of organs in which BKVI promoter/*Igf-2* transgenes are expressed.

MATERIALS AND METHODS

Transgenesis. The transgene construct was formed in the pUC19 vector by placing a 4.5-kb fragment, containing the BKVI gene promoter (19), upstream of a 5-kb segment of the *Igf-2* gene, encompassing all three coding exons (Fig. 1). This *Igf-2* segment was subcloned as a *Kpn* I fragment following partial digestion of the cosmid clone cosIGF4 (24). The resulting fusion was excised as a *Sal* I–*Eco*RI fragment and purified from plasmid sequences before pronuclear microinjection. Standard microinjection methods were used (25), and the zygotes were from an intercross of F_1 (C57BL/6 × CBA) mice. The resulting mice were screened for the presence of the transgene by Southern blot analysis of DNA prepared from tail biopsy tissue. (Unless stated otherwise, nucleic acid manipulations were carried out essentially as described in ref. 26.)

Transgene Expression. Steady-state levels of *Igf-2* mRNAs were determined by RNase protection assays (27). The probe allows transgene and endogenous *Igf-2* mRNAs to be distinguished and was derived from the region of the transgene which spans the BKVI promoter/*Igf-2* sequence junction (Fig. 1) subcloned as a *Stu* I–*Hin*cII fragment, into the *Hin*cII site of pGEM-4Z (Promega). This subclone was linearized with *Eco*RI and used to generate an antisense RNA probe uniformly labeled with ^{32}P by the use of bacteriophage T7 RNA polymerase. Total RNA was prepared from whole embryos at E14.5 and E16.5, from the skin and the body (after removal of the head and tail) of 7.5-day-old mice, and from dissected organs at later stages (28). Samples containing approximately 1 μg of total RNA were analyzed both from transgenic animals and from nontransgenic littermates, after the genotype was established by Southern blot analysis (as before, except that the DNA source was yolk sac in the case of E14.5 embryos). In all cases, samples were hybridized with the *Igf-2* probe together with a second probe, used as a loading control, which was specific to mRNAs encoding murine glyceraldehyde-3-phosphate dehydrogenase (mGAP; ref. 29). An organ was said to lack transgene transcripts only if the mGAP signal was obvious and there was no signal from the transgene. Controls used in all RNase protection experiments included an RNA sample prepared from NIH 3T3 cells (cultured murine fibroblasts which express *Igf-2* mRNA) and a reaction mixture in which cellular RNA was replaced with tRNA from bakers' yeast. Sizes of protected fragments were routinely checked by comparison with parallel loadings of

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Abbreviations: IGF, insulin-like growth factor; BKVI, bovine keratin VI (synonym, keratin 10); mGAP, murine glyceraldehyde-3-phosphate dehydrogenase; E*n*, embryonic day *n*.

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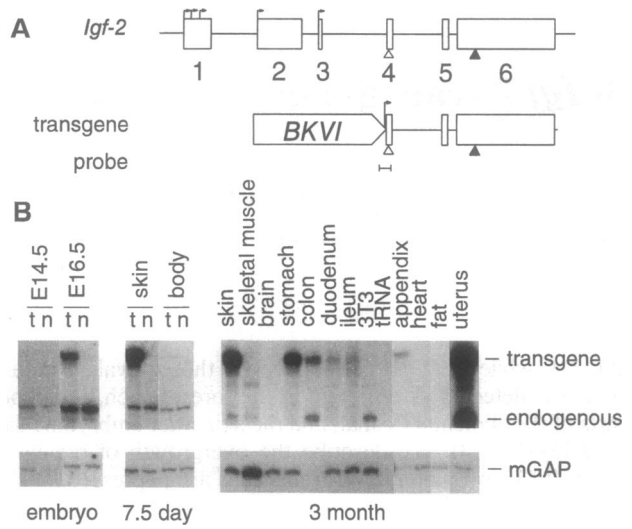

FIG. 1. Transgene expression. (*A*) Structure of the *Igf-2* gene (24). Exons are shown as boxes, and transcript initiation sites are marked with arrows. The region encoding IGF-II is contained in exons 4–6, and translational start (ATG; open triangle) and stop (TGA; filled triangle) codons are indicated. For analysis of transgene expression by RNase protection assays, a uniformly labeled antisense RNA probe was derived from the region which spans the BKVI promoter/*Igf-2* sequence junction. (*B*) RNase protection analysis using total RNA prepared from embryos at E14.5 and E16.5 and from 7.5-day-old and 3-month-old animals. Embryonic and 7.5-day samples were from heterozygous transgenic mice (t) and nontransgenic (n) littermates; for 3-month-old mice, only the transgenic samples are shown. Positions of the major protected fragments representing the *Igf-2* transgene, endogenous *Igf-2*, and mGAP mRNA species are indicated. Less abundant protected fragments, seen in samples from tissues expressing the transgene at high levels, are thought to reflect heterogeneity in the sites of transcript initiation, which has been documented for the BKVI promoter (19). Controls were RNA from 3T3 cells and tRNA (see *Materials and Methods*). The fat sample was from the epididymal fat pads, and skeletal muscle was from the hindlimbs.

both undigested probe and products of dideoxynucleotide sequencing reactions.

Mice, Organ Weights, DNA Measurements, and Protein Analysis. Mice were maintained under a 14 hr light/10 hr dark regime (light 04:00–18:00), at 20 ± 2°C and relative humidity of 55 ± 10%. Both water and the Porton combined diet (Special Diet Services, Witham, Essex, U.K.) were available ad libitum. On the day of birth, newborns were weighed to an accuracy of 0.01 g, sexed, and numbered by toe-clipping. Live weight measurements were repeated either at 1, 2, 3, 4, 8, and 12 weeks or only at 8 and 12 weeks. The transgene status was usually established from tail biopsy samples taken at 3–4 weeks of age, and statistical analysis is described in the table legends. Methods for the measurement of IGF proteins in serum samples were as described (30).

Comparisons were made during the first three or four generations that the transgene was progressively bred on to either a C57BL or a CBA background. The strain background did not appear to alter the phenotype. In most cases, comparisons were made between the phenotype of virgin transgene heterozygotes and their normal sex-matched littermates. All organ weights are the wet weight after removal of mesenteries and fat and internal contents (alimentary canal and uterus). Organs were dabbed on tissue paper to remove fluid. DNA was measured by the method of Labarca and Paigen (31). Most comparisons were by *t* test of matched pairs, with the two-sample *t* test used where indicated. Details of the matching are in the figure legends.

RESULTS

Transgenesis and Wrinkled Skin. Four of 43 mice were transgenic after microinjection of the BKVI promoter/*Igf-2* fusion gene: each transgenic founder had wrinkled skin and this character therefore does not depend on the integration site (Fig. 2). The degree of wrinkling corresponded roughly to the number of integrated copies of the transgene, and in each line the transgenes were transmitted as a single Mendelian factor, their restriction fragment patterns were consistent

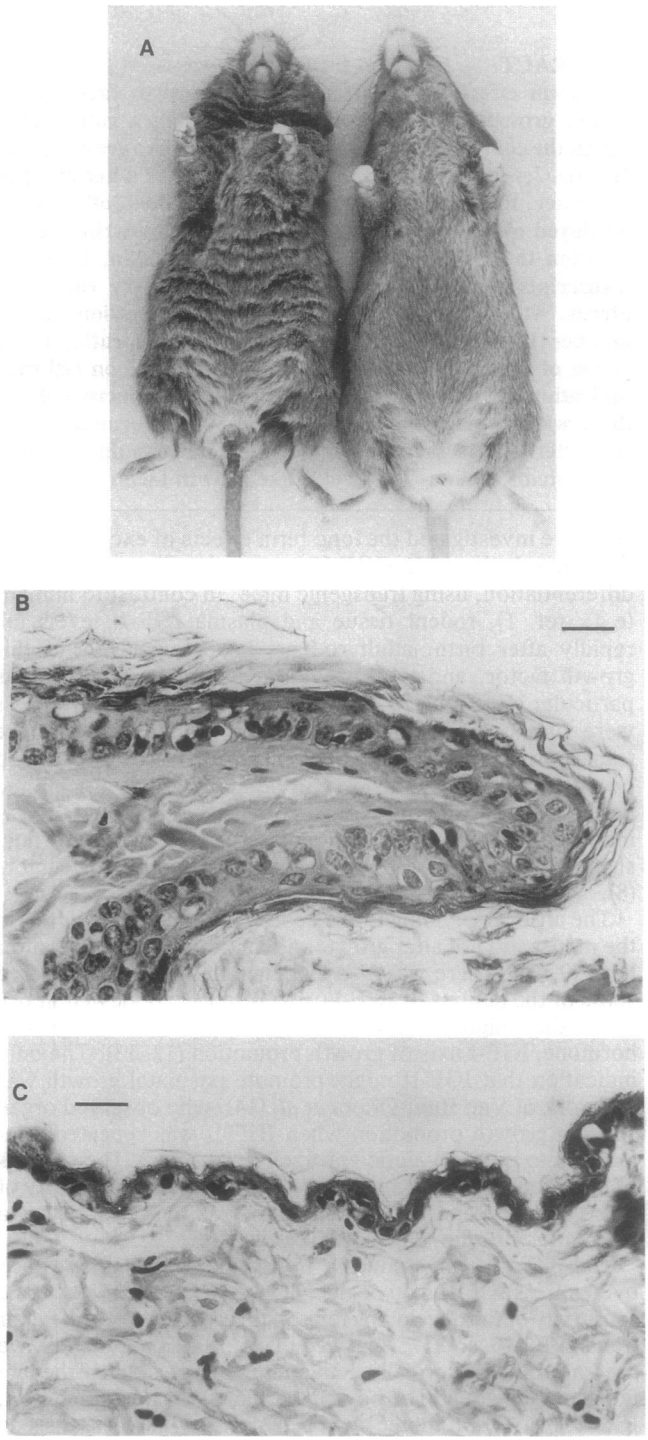

FIG. 2. Transgene effects on skin growth. (*A*) Wrinkled 9-month-old transgenic heterozygote female from the Blast line (left) compared with one of her normal sisters (*B* and *C*). Sections of skin from a 3-month-old Blast transgenic heterozygote female (*B*) and a normal female littermate (*C*). (Bars = 0.02 mm.)

with multiple integrations at single sites, and they were stable over at least three to six generations (heterozygote copy number: Blast line, 10–20; Beano line, 5–6; Bart line, 4; Basil line, 2).

The wrinkled skin character was transmitted by both sexes, and there was no indication that this feature of transgene expression was subject to genomic imprinting. The skin wrinkling was readily detected at 10–15 days after birth and the ripples were obvious in older agouti animals (Fig. 2). The subsequent observations were made on the wrinkliest lines (Blast and Beano). Transgenic animals from these two lines had significantly raised plasma IGF-II levels: for the Blast line 53.1 ± 16.5 ng/ml and for the Beano line 31.25 ± 37.1 ng/ml, compared with 16.3 ± 13 ng/ml in normal animals from the Blast line (30). Skin hyperplasia, which was more pronounced in Blast than in Beano transgenic animals, was marked by hyperkeratosis and an overrepresentation of epidermis compared to dermis (Fig. 2 *B* and *C*).

Expression. We found a broader field of action of this keratin promoter than that expected from previous transgenic studies (20, 21). In the Blast line, transgene expression was first detected in the embryo at E16.5, was found in the whole body on the day of birth (data not shown), and was prominent in the skin at 7.5 days after birth (Fig. 1*B*). As expected, the transgene transcripts were at high levels in skin sampled from the flank and the tail at 3–6 months. Surprisingly, there were equally high levels in the virgin uterus. We anticipated and found lower levels of transgene mRNA in the stomach, but the transgene was also expressed along the alimentary canal in the duodenum, the ileum, the appendix, and the colon. Steady-state mRNA levels were lower in the Beano line, but once again we found transgene mRNA in the uterus, as well as in the alimentary canal and the skin (data not shown).

In both lines, transgene transcripts were not found in gross muscle samples (hindlimbs and heart) or in brain or the epididymal fat pads (Fig. 1*B*). No transgene mRNA could be detected in tongue, testis, or the cauda epididymis, and they were absent from visceral organs such as the liver and the kidney (data not shown).

Disproportionate Growth. Substantial differences in body proportions were found in comparisons of transgenic animals with their normal littermates (Table 1). An initial survey of organ wet weights from five matched pairs indicated that the major increases would be confined to the skin, the appendix, the colon, and the uterus. These organs were selected for further study (Figs. 3 and 4). There were no obvious changes in the wet weights of the brain, submaxillary and sublingual salivary glands, thymus, heart, lung, liver, spleen, adrenals, kidney, testis, stomach, and duodenum plus ileum of 7- to 8-month-old mice of the Blast line. This impression was confirmed with a similar set of samples from 3-month-old Beano mice. A significant decrease in fat content was observed at all sites examined in 2- to 4-month-old and 7- to 9-month-old transgenic Blast animals (30).

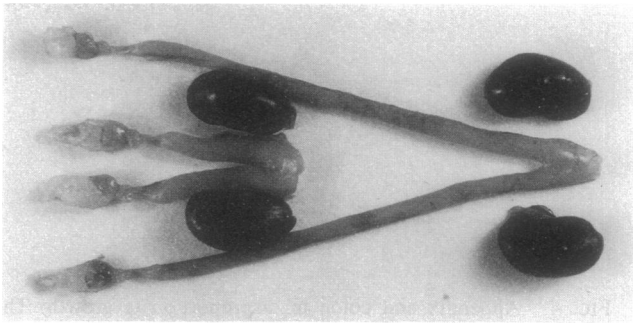

FIG. 3. Uterus and disproportionate growth. Organs from a 3-month-old heterozygous Blast female (right) and her sister, displaying the relative size of the uterus and kidneys. The disproportionate growth of the uterus can be appreciated because the kidneys did not express or grow in response to the transgene. The reduction in fat can just be seen in the depot around the ovaries, while the fat beside the kidney and on the mesentery of the uterus has been stripped off in this dissection.

In a more extended series of matched pairs, both the wet weight and the DNA content of the selected organs were measured. Disproportionate growth was established in all the organs which had shown an increase in wet weight (Table 1). Excess growth of the appendix plus colon was not found in 1-week-old Blast mice, and it was first detected at 2 weeks, when the uterus was also enlarged. By 7–9 months the cell

Table 1. Effects of *Igf-2* transgenes on body proportions

Line	Organ	% difference in transgenics (*n*)		
		Wet weight	DNA	Live weight
Blast				
7 days (♂ and ♀)	Appendix + colon	ND	−5† (*n* = 42)	−7.6
13–16 days (♂ and ♀)	Appendix + colon	+39*** (6)	+27* (6)	−2.3
	Uterus	+72* (4)	+14 (4)	−1.0
	Kidney	−4 (7)	−5 (7)	−2.1
7–9 months (♂ and ♀)	Skin	+55*** (12)	+72*** (6)	+0.5
	Appendix	+67*** (12)	+49*** (12)	+0.5
	Colon	+95*** (12)	+30* (12)	+0.5
	Uterus	+772** (7)	+143** (7)	+4.0
	Kidney	+13 (7)	−2 (7)	+0.7
Beano				
3 months (♂ and ♀)	Skin	ND	+25* (10)	+1.1
	Uterus	ND	+10 (7)	−1.3
	Kidney	ND	+1 (11)	−2.9

To determine percentage differences in body proportions, the wet weights and DNA contents of selected organs were compared between animals heterozygous for *Igf-2* transgenes and their normal littermates. In most cases, the comparison was by the paired *t* test (paired for litter and sex) on males and females which had never mated, the paired animals were of similar live weights (the mean difference in total live weight for the animals used in each comparison is indicated), and the increases in organ size therefore represent major alterations in body proportions. The number of paired comparisons are in parentheses.

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ND, not determined.

†Student's *t* test was used because the transgenic animals consistently had less mass, making it impossible to pair with comparable normal mice.

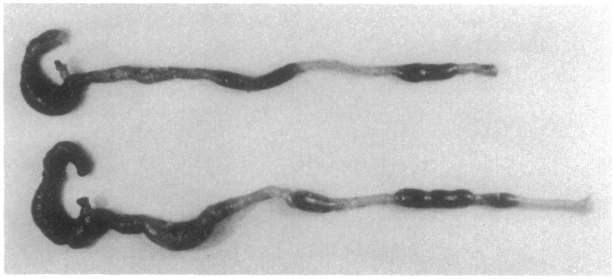

FIG. 4. Appendix and colon in disproportionate growth. The appendix and colon were dissected from a 3-month-old heterozygous Blast female (lower) and her normal littermate (the animals in Fig. 3). Both organs express the transgene, contain more cells, and are longer.

numbers of the skin, appendix, colon, and uterus were greatly increased in the Blast line. At this age the extended colon was frequently associated with a prolapse, and the external protrusion was not included in the samples because it was difficult to detect where the colon ended and the anus began. The uterus was enlarged to such an extent that it could have been confused with the gut in 6-month-old females. These females mated but rarely bore litters. Sections of the enlarged uterus and colon revealed that tissue organization was not grossly distorted, at least in animals up to 6 months old (data not shown). The uterus tended to be edematous, a feature consistent with the major discrepency between wet weights and DNA content in this organ. Beano animals were less distorted at 3 months, with only the skin showing a significant increase in cell number, and the females were fertile.

In no line did the kidney express the transgene or increase in DNA content. Only the stomach and the ileum plus duodenum expressed the transgene and did not increase in wet weight, as judged by the preliminary survey. We do not know whether minor weight gains would have been observed in a larger sample of these organs, and it is certainly possible that they are insensitive to excess IGF-II. The general correspondence between the sites where the transgene was expressed and disproportionate overgrowth suggested that the products of the transgene had a local action.

Whole Body Growth. The transgene had age-related effects on whole body growth (Table 2). From birth until 1 month of age the weight of transgenic Blast animals was low, whereas it was higher than normal at 2 and 3 months of age. The action of the Beano transgene showed the same age dependence, but only the weight gain at 2 months was significant.

Table 2. Effects of *Igf-2* transgenes on live weight

	Blast line			Beano line		
Age	*n*	Mean, g	Difference, %	*n*	Mean, g	Difference, %
1 day	139	1.41	−4.10**	199	1.39	+1.71
1 week	131	3.60	−8.48***	178	3.77	−0.26
2 weeks	114	5.91	−4.42**	168	6.18	−1.20
3 weeks	111	8.18	−3.20	166	8.26	−2.08
1 month	107	11.85	−2.07	167	12.37	−0.13
2 months	178	21.32	+4.17*	449	21.48	+1.54*
3 months	189	24.55	+7.96***	439	24.50	+1.24

Live weights were recorded for comparisons of transgenic (heterozygous) with normal animals of the specified ages. Most of the effects of the transgene were significant in the Blast line, and while the Beano line displayed a similar trend, the effect of the transgene was rarely significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). At each age, the contribution of sex and transgene status to the variation in weight was analyzed, allowing for litter-specific effects (Minitab, version 8, GLM command; Minitab). The contribution of being transgenic to the coefficient of variance was used to calculate the percent difference in the transgenic mice.

DISCUSSION

Local Actions of IGF-II. The organ overgrowths we observed were probably due to the locally produced transgene IGF-II. First, the only organs which grew excessively contained transgene transcripts (Table 1). Second, the organs which overgrew in this study did not respond to injected and presumably circulating IGF-II in Snell dwarf mice (14). Third, the enlarged organs in this study are not among those repeatedly shown to grow in response to the administration of recombinant IGF-I, such as the kidneys, adrenals, and spleen (e.g., refs. 32 and 33). This is a particularly strong reason for believing that local production drives the overgrowth, because most of the cellular multiplication responses to IGF-I and IGF-II are mediated through the same type 1 IGF receptor (refs. 5 and 10; reviewed in refs. 34 and 35). Normally, IGF-II promotes growth only during embryogenesis, whereas IGF-I acts both before and after birth (10, 36). Since type I IGF receptors are widely expressed during development (37) and persist at lower levels into adulthood (38), it seems likely that the growth response to excess IGF-II is mediated by this receptor and is autocrine or paracrine in nature. Type 1 IGF receptors are important for skin growth, since a striking hypoplasia of the epidermis of the skin was reported in mice lacking a functional type 1 IGF receptor gene (36). This is in contrast with the excess of epidermis in mice with BKVI promoter/*Igf-2* transgenes (Fig. 1 *B* and *C*).

Circulating IGF-II was increased ≈3-fold in transgenic animals of the Blast line, which might account for the global reduction in fat seen in these animals (a 17% reduction in the mass of lipid was observed at 2–4 months; ref. 30), particularly as a similar lean phenotype was recently reported in mice with elevated serum levels of IGF-II (39). Despite the high plasma IGF-II levels, whole body growth was only slightly increased in these transgenic mice (Table 2). The change in live weight was the balance between the weight gain which followed the excess growth of specific organs and the weight loss due to the reduction in carcass fat. These observations therefore support previous observations that systemic IGF-II is a poor promoter of whole body growth when compared with IGF-I in intact mice (6–8, 15).

There was no gross distortion of the enlarged uterus and colon, which grew in both length and diameter (Figs. 3 and 4). It follows that the tissues which make up these organs must have each contributed to the increase in cell number (Table 1). This observation does not establish that the cells of each tissue multiply in direct response to IGF-II, because there is ample evidence that tissue layers normally interact with each other to ensure the integrated growth of organs in normal development (40). In all the enlarged organs, except the skin, the wet weight increased more than the DNA content: this suggested either that cell mass was increased, or that there was more extracellular matrix, or that the organs contained more fluid.

IGF-II and Tumor Growth. There is extensive evidence that IGF-II is involved in human tumor growth (reviewed in ref. 41). The substantial multiplication response of both embryonic and tumor cells suggests that developmental stage can be critical to the response (e.g., refs. 42–46). This view is supported by high IGF-II mRNA expression and by alterations in the human IGF-II locus in some embryonal tumors (e.g., refs. 47–50).

Sixty Blast mice did not develop macroscopically obvious tumors over a 9-month period, and thus there is no evidence that IGF-II overproduction by itself causes tumor formation on this genetic background. However, a more extensive analysis of older animals might be required to confirm this

observation, particularly in those visceral organs which express the transgene, since there is a recent report of transgenic mice which both overexpress IGF-II as adults and develop diverse malignancies in later life (39). Reported IGF-I transgenes are similar to the IGF-II transgenes in the present paper, with a display of disproportionate growth and no increased tumor incidence (e.g., refs. 51–53).

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