Genetics of mouse growth

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ABSTRACT   During development, mammalian organisms increase in size until a limit is reached that is mainly determined by the rate and duration of occurrence of cellular divisions increasing total cell number. This process is mostly regulated by an orchestration of the actions of genes participating in pathways that promote or inhibit growth through systemic or local effects. This view of growth control genes and of their effects on the cell cycle has begun emerging from the results of transgenic and gene knockout experiments, which have also re-emphasized the central involvement of some growth factors and hormones in growth signaling, although mechanistic relationships and details about the coordination of growth with patterning, differentiation and morphogenesis continue to remain largely elusive.

KEY WORDS: *growth, growth factors, hormones, gene knockout, mouse embryo*

"Through a glass, darkly"

Growth is a fundamental aspect of development and a vast topic. For the purpose of this review, which is a survey of genes that control growth and, therefore, determine body size, I had to narrow down the scope of the presentation to mammals, and more specifically to mice, with only infrequent references to other organisms. Even if the mouse is not a typical mammal, as some people tend to think, examining what we have learned (more or less recently) from transgenic and gene targeting research about its growth is worthwhile. This is exactly what I have attempted to do, although my account is not exhaustive.

Everybody knows that drawing conclusions about normal functions from the results of their perturbation defines the limitations of the attempt. Transgenes can tell us something about the effects of overexpression only if they simulate to some extent the performance of their endogenous counterparts, whereas knockouts reveal only *sine qua non* functions; some part of the picture often remains hidden because of opportunistic compensation ("redundancy" is not a kosher term; but this is another story). An additional difficulty is that genes are not solitary players, but members of pathways, and pathways do interact, with the potential of getting pleiotropic effects from knockouts. Still, we can get a glimpse of a generalization in the making, which, even if it does not surpass significantly the stage of a working hypothesis, suggests that body size brought about by growth is predominantly controlled genetically through general (whole-body) controls and local (tissue- or organ-specific) controls acting either positively (growth promoters) or negatively (growth inhibitors).

Nevertheless, the content of this review is only a blurred snapshot of the tip of the iceberg. When dealing, for example, with ligand/receptor interactions affecting growth, I will not go deeper than the cell surface. Little is known about intracellular signaling cascades in the context of the whole organism, and this topic will not be covered here in any detail. Also, I don't have the space to discuss extensively nutrition or the importance of the maternal genotype for the growth of the embryo. However, I will discuss briefly the role of the placenta.

Of mice and elephants: genes and body size

The smallest mammal (~2.5 g) is the Etruscan shrew *(Suncus etruscus)* and the largest is the blue whale (>100 tons). However, I know nothing about these organisms, and to make a point about the significance of body size, I will use as examples two other mammals.

We all know that an elephant is larger than a mouse, but we do not realize immediately that it is 167,000 times larger (divide 5 tons by 30 g). This difference affects all of the physiological parameters imaginable. In one minute, for example, mice breathe 150 times and elephants only 6, and an analogous difference holds for heartbeats (600 vs. 30). Mice live their lives much faster and die in 2-3 years. Elephants live 60 years. Differences in body size exist not only between, but also within species. An extreme example is the situation with dogs; various breeds differ by a factor as large as

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~100. Behind these differences are genes and cell numbers; a playground for evolution.

The insular size of elephants and rodents exemplifies a reaction of development to ecological change in a most interesting way (discussed by Raff and Kaufman, 1983; Bonner, 1988). The large European elephant, for example, gave rise to the eventually extinct elephant of Malta that was not larger than a pony. In contrast, the fossil record shows that the island forms of mice had increased sizes (up to two-fold, in comparison with the mainland forms). Whatever the selective pressures that bring about such phenotypic alterations may be (changes in the availability of food, predators, etc.), the general trend is that, in the isolated environment of islands, the size of smaller animals tends to increase, while larger animals are dwarfed (see Lomolino, 1985). Although it is inevitable that any change in size will be accompanied by some change in form, the allometric changes in these examples were not particularly drastic, and the dwarfism or giantism of these animals can still be considered as ateliotic (proportionate). Cases of disproportionate dwarfism are known – the mouse mutants *bm* (brachymorphic) and *cn* (achondroplasia) are such examples – but they will not be discussed.

Of mice and humans: growth and developmental timing

Growth is an increase in size. In a biological context (fortunately, I don't have to talk about the growth of the economy), this simple definition refers to a cell or to a cell population (a multicellular organism or a cell culture). Although it may sound trivial, it is useful to remember that, from the point of view of a single cell, growth and proliferation are opposite concepts. A cell first grows (doubles in size by duplicating its constituents) and then is split into two single-size daughter cells that go through the same steps. Asymmetric cell divisions do exist, of course, and cells can also grow without division (hypertrophy). From the point of view of the entire organism, on the other hand, growth is attained predominantly by cell proliferation, although secondary deposition of extracellular matrix and hypertrophy of particular tissues at particular developmental stages also make contributions. There is also cell proliferation without growth, as in the cleavage stages of early embryogenesis.

It is perhaps important to emphasize that very small increases or decreases in the timing of cell divisions have large effects on growth. The significance of the duration of the cell cycle becomes immediately obvious from a simple calculation. Assuming just a 10% difference in doubling time between two cultures of cells proliferating exponentially, we realize that, after only seven mitotic rounds, there will be a two-fold difference in cell numbers. In addition to rate, size is determined by the duration of growth (there are some thoughtful reviews on this topic by Snow, 1981,1986; Snow *et al.,* 1981; McCance and Widdowson, 1986). Thus, although the mouse embryo grows with a higher rate than the human embryo and reaches, for example, a weight of 1 g three times faster (17.5 vs. 55 days; Adolph, 1970), the human newborn is much larger because of a significantly longer period of intrauterine life (280 vs. 19 days). In addition to growth pattern, there are other differences between these two species: The order of appearance of equivalent stages of various embryonic structures is not in strict correspondence, and there is no simple proportionality in the pace of development. In fact, a comparison between mouse and human embryos shows that the ages of developmental equivalency can-

not be correlated with the percentage of elapsed gestation time (Otis and Brent, 1954). This is reflected in the degree of maturity that the embryo has attained at the time of birth. A comparison of several indices shows that, in relative terms, mice are born essentially in an "embryonic" state (lack of fur, low body lipid content, fused eyelids, etc.), whereas human newborns are relatively more mature. Of course, differences in growth and maturation patterns continue to exist postnatally.

Growth profile

Growth patterns are similar, but not identical between different strains of mice. However, for an overview of growth, a "generic" mouse will suffice. Embryos recovered from the oviduct can be followed until ~100 h postcoitum. By this time (just before implantation), seven slow cell divisions without growth have occurred (one every ~10 h), and the resulting blastocyst is composed of an average of 128 cells (Streffer *et al.,* 1980). The implanting blastocyst (~e4.5) weighs ~230 ng (Goedbloed, 1972; Hensleigh and Weitlauf, 1974) and its mass does not differ significantly from that of a fertilized egg (it is actually a bit smaller). The ensuing growth is a developmental continuum (birth is just a landmark), until a body size limit is reached (~30 g) sometime between 70 and 100 days of postnatal life. This is a 130 million-fold increase in size that progresses in a particular way, as described below.

The rate of growth by cell proliferation is slow between implantation and gastrulation, and then it becomes very high. When gastrulation starts, there are ~600 cells in the epiblast, increasing to ~15,000 a day later (Snow, 1977). This requires a doubling time of ~5 h (twice the previous rate). Importantly, growth controls are already in place, because there are regional differences within and between the differentiating embryonic regions. Thus, the proliferation rate is higher in the epiblast than in the primitive endoderm, and when mesodermal cells appear, they proliferate with an intermediate rate. In addition, there is a patch of epiblastic cells (~10%; proliferative zone) that exhibit cycle times of only 2-3 h (Snow, 1977).

Subsequent growth is usually followed by constructing growth curves: plots of weight vs. time. Although this approach is practically indispensable for the study of developmental kinetics, especially when the impact of a mutation on growth is examined, it has several shortcomings. First, growth occurs in time, but it is not a function of time. It would have been better to consider growth, as reflected by increase in weight, as the dependent variable of some intrinsic property of the embryo itself. However, the complexity of the system precludes the identification of a useful parameter, at least for now. Second, a growth curve provides nothing better than a coarse, average index of the growth process (tissues and organs grow at different rates). Third, even if experimental inaccuracies are ignored, there is always scattering of measured values because of developmental asynchrony of embryos, both within and between litters. Still, the range of weight values is relatively narrow for each embryonic age, in contrast to the considerable variability that is observed during postnatal life.

To derive conclusions about rates from a comparison of growth curves, some kind of regression analysis becomes necessary. And here is the rub. Any equation used for regression (there are several choices) is not a mathematical formulation deduced from growth principles, but only a tool applied empirically for approximate

analysis of growth curve data. Characteristic of the artificiality in this approach is the fact that some of these equations include constants that cannot be possibly assigned biological significance. Nevertheless, while being aware of the illusion, we are forced to use some mathematical treatment, and I will tell you what I have found to be useful. A simple method of regression analysis is based on the assumption that growth of the whole embryo is exponential (Goedbloed, 1972), according to the equation $W = W_0 \exp(kt)$, where t is the time, W_0 is the weight at $t = 0$, and k is the growth rate constant. However, a single rate constant cannot be used to describe the growth process during the entire embryonic period, because the rate of growth slows down with developmental time. Nevertheless, using the method as a simplification, it can be shown empirically that plots of logW vs. time do not deviate significantly from three straight lines with two breaks signifying points of retardation (three windows of time; e4.5-e10.5; e10.5-e14.0; e14.0-e18.5; Goedbloed, 1972). An alternative is to apply a Gompertz equation (Laird *et al.,* 1965) that usually fits the data in the form of a single curve quite well. In the Gompertz function $W = A \exp[-b \exp(-kt)]$, W and A are the weight at time t and the asymptotic weight, respectively, while b and k are constants. This mathematical treatment takes into consideration that the specific rate $(1/W) \times (dW/dt)$ of the exponential embryonic growth is not constant, but declines exponentially. Specific growth rate (γ) can be calculated using the equation $\gamma = -k \ln(W/A)$.

The decrease in growth rate continues postnatally until growth stops, but with a transient change in gear. Between 2 and 3 weeks after birth, the rate of growth declines considerably in comparison with the previous period, but is restored after weaning and then continues to decline more smoothly. Thus, for the time period of 70 postnatal days, growth is clearly triphasic. Postnatal growth curves are best fitted with a logistic equation $W = A/1 + \exp[-b (t-c)]$, where b and c are constants. Using a computer program, robust weight data can be fitted simultaneously by summation of three logistic functions with different sets of A, b and c values (Koops, 1986; Koops *et al.,* 1987).

Even if you don't take any of this seriously or you find it boring, it is worth remembering that, regardless of how exact our descriptions are, the whole body weight increases with decreasing rate. This probably reflects a competition between gene functions promoting and inhibiting growth. Although we can recognize some individual players, we don't know the rules of this game, which differs from a real tug-of-war: until a steady state is attained, the degree of growth at each developmental age is the cumulative outcome of continually shifting imbalances between the effects of positive and negative factors.

Embryonic vs. postnatal growth: growth factors and hormones

Some of the signaling molecules determining developmental pace are growth factors and hormones. Polypeptide growth factors got their name long ago, when they were recognized as serum components essential for the growth of cell cultures. Fibroblasts in G_0, for example, will stay quiescent, unless they become competent to enter the cell cycle by exposure to PDGF (reviewed by Pardee, 1989). Competent cells then progress to the S phase under the influence of other factors, like EGF and IGF-I, and pass through two control points in G_1, V and R (restriction). If not

provided with essential amino acids, the cells will arrest at the V point, whereas after R, they are committed to enter the S phase regardless of environmental signals. The only factor required for progression from V to R is IGF-I.

Although extrapolations from cell culture experiments would have predicted otherwise, growth factors do not necessarily promote the growth of the whole mammalian organism (but they have kept their name; what's in a name?). Thus far, among the classic growth factors, only the IGFs have turned out to be a major growth signaling system for the entire mouse embryo, while one of the FGF receptors (FGFR3) has emerged as a component of a local growth-inhibitory pathway (see below). The growth role of other important factors, like PDGF and EGF, is less clear-cut. Lack of signaling through the EGF receptor appears to affect negatively, but modestly, embryonic growth by compromising the size of the placenta, depending on genetic background (see below), whereas the predominant role of the PDGF system is in patterning.

The monomeric chains of PDGF ligand (A and B) and PDGF receptor (αR and βR) are products of distinct genes, all of which have been knocked out (Levéen *et al.,* 1994; Soriano, 1994,1997; Boström *et al.,* 1996; see also reviews by Betsholtz, 1995; Betsholtz and Raines, 1997). Binding of ligand, which is either a homodimer or a heterodimer, induces receptor dimerization. βR can bind only the ligand B chain, whereas αR can bind either ligand. Thus, it is thought that the following combinations exist: $\beta\beta$BB, $\alpha\alpha$BB(or AA or AB) and $\alpha\beta$BB(or AB). The phenotypes resulting from knockout of either βR or B are strikingly similar. The mutants die perinatally, and exhibit hematological defects, bleeding and kidney abnormalities, but no obvious growth retardation. Knockout of αR results in defects in neural crest development and somite patterning. The severity of the phenotype at different developmental ages is variable, but the great majority of embryos die by embryonic day 16.0 (e16). Some embryos are smaller than wild-type to various degrees, but this could be due to increased apoptosis detected at e10, whereas proliferation assayed by BrdU labeling is normal, at least up to e14.0 (the most advanced age tested). Growth retardation (or a condition simulating growth retardation) is observed only with the A chain knockout that exhibits a phenotype distinct from that of the other family members. About 50% of the embryos die before e10.5, whereas the remaining are brought to term, and either die within a few days after birth (30%) or survive, but no more than 6 weeks (20%). Mice of the last category exhibit lung emphysema due to failure of alveolar septation. The weight at birth is ~70% of normal (hereafter referred to as %N) and becomes ~50%N at 4 weeks. While study of potential genetic background effects and combinations of the mutations are still in the works and may reveal unsuspected ligand/receptor relationships, it is uncertain whether the small body size of the PDGF-A nullizygotes reflects true hypoproliferation, rather than increased apoptosis or some other defect. When αR is missing, only the $\beta\beta$BB ligand receptor interaction remains intact, but there is no apparent proliferation defect. This interaction is preserved in the less severe PDGF-A knockout (in addition to the $\alpha\alpha$BB and $\alpha\beta$BB interactions). Does the PDGF system that apparently controls patterning also control growth? The jury is still out.

While growth factors are produced by many tissues and exert local (autocrine/paracrine) controls, hormones are long-range signals acting away from the sites of their production. This distinction, however, seems to be artificial to some extent, since growth

factors are also present in the circulation and can play roles analogous to those of classical hormones. Nevertheless, there is an interesting difference between embryonic and postnatal growth in the degree of involvement of these two types of signals. Postnatal growth is controlled by both hormones and growth factors, and it is pointless to make any attempt to assess which is more important. On the other hand, growth factors are certainly the predominant players during embryonic growth. For example, absence of IGFs compromises embryonic growth both in humans and mice (see below). In contrast, absence of growth hormone action in mutant animals (see below) or experimental ablation of the pituitary does not impair normal prenatal growth. The same holds true for humans (an anencephalic fetus lacking a pituitary gland has normal birthweight). There are differences, however, between humans and mice in other hormonal influences, and the case of insulin provides a dramatic example. Insulin inaction (that is, either absence of insulin or lack of a functional insulin receptor) during human embryonic development results in severe growth deficiency that is not observed in mice (discussed by Louvi *et al.,* 1997).

Growth control

Growth control is brought about mainly by genes of the developing organism itself. But what is a growth control gene? Suppose you knock-out a gene and you observe growth retardation. Does this mean that this gene is controlling growth? Not necessarily. It seems to me reasonable that, to assign a growth-promoting regulatory function to a gene, two criteria should be met: elimination of the gene function should result in growth retardation, whereas overexpression of the gene should result in overgrowth; and vice versa, for growth-control genes with an inhibitory function. In the latter case, however, even if results of overexpression are unavailable, it is quite safe to place a gene in the category of growth inhibitors, if its elimination results in overgrowth. The rationale is that it is difficult to sketch a scenario in which loss-of-function would result in overgrowth in a non-specific way. An additional criterion to recognize authentic growth-control genes is that they should be affecting the duration of the cell cycle, although they need not be cell-cycle control genes in the strict sense. If genes controlling the cell cycle directly (like those encoding cyclin-dependent kinases or their inhibitors, for example) fulfill the other criteria and their mutations (alone or in combination) have phenotypic manifestations on growth, they will constitute a subset of growth control genes. The complete set, however, will also include genes at any hierarchical level of growth-control pathways, which will affect the cell cycle only indirectly. For example, the genes *Prop1*, *Pit1*, *Ghrhr*, *Ghrh*, *Gh* and *Ghr*, which are members of the growth hormone (GH) pathway (see below), should all be considered as *bona fide* growth control genes. Additional known and unknown genes that meet at least some of the assigned criteria and promote or inhibit growth either generally (affecting many tissues or the whole body) or locally (affecting particular tissues) are listed in Table 1.

Knockouts of genes that are not involved in growth control, but result indirectly in growth retardation, are listed in Table 2 and discussed only selectively. This group includes mutations that deprive the embryo of nutrients and/or oxygen due to a variety of defects (for example, in the visceral endoderm, the yolk sac, the placenta, etc.; Table 2A-D). Nutrition might also be impaired by potential behavioral defects (the mice don't want to eat, for example; Table 2E). In other cases, nutrients are available, but the gene knockouts affect, one way or another, metabolic processes (Table 2F). Finally, growth retardation may be the result of a mitotic block (which I consider as being outside of the realm of physiological growth control), if known or presumed DNA-repair mechanisms are affected (Table 2G). Clearly, all of these genes are necessary for the maintenance of normalcy, but are not involved in the growth process itself. Their function is simply a precondition for normal growth, and when perturbed, growth is impaired in a non-specific way.

A gray area exists. Several knockouts cannot be classified easily, because the potential mechanisms involved in the manifestation of growth retardation are unclear (Table 2H). I don't exclude the possibility that some of these genes might be eventually promoted from Table 2 to Table 1. For example, a clue in this direction is the observation that cultures of embryonic fibroblasts from *Cbx2* nullizygotes grow very poorly (Coré *et al.,* 1997). An example of a possible growth control gene now in limbo (not listed) is *Zfx*. It is not certain yet whether knockout of this X-linked gene encoding a zinc-finger transcription factor is a true null mutation (Luoh *et al.,* 1997). Except for a diminished number of primordial germ cells in animals of both sexes, some incidence of perinatal lethality and fertility problems, histopathological findings are absent and developmental delays are not observed. However, growth retardation occurring earlier than e12.5 results in smaller animals. The unique feature of this phenotype is that, from e12.5 to postnatal day 40 (p40), the mice are ~80%N, as if an abrupt event in early development stunts their growth transiently and then the growth rate becomes normal. Finally, I confess that I have no clue about the role in growth control of the genes *Rbl1* and *Rbl2* encoding the retinoblastoma-like proteins p107 and p130, respectively. These gene products are inhibitors of cell cycle control proteins, and, therefore, the expectation would have been that their absence would result in overgrowth. Instead, the opposite occurs with particular combinations of mutations (Cobrinik *et al.,* 1996; Lee *et al.,* 1996). *Rbl1* or *Rbl2* nullizygotes do not manifest a phenotype, apparently due to compensatory mechanisms, whereas double nullizygotes die at birth without apparent growth retardation. However, double mutants that are nullizygous for *Rbl1* and heterozygous for *Rbl2* exhibit mild growth retardation (~80%N at p45), whereas the mice carrying the reciprocal combination of these mutations [*Rbl1(+/-)/Rbl2(-/-)*] are normal in size. Absence of p107 with reduced retinoblastoma gene dosage in *Rbl1(-/-)/Rb(+/-)* double mutants results in even more severe growth retardation (~50%N at p45). Apparently, therefore, the key player is p107, but how growth of the entire body is compromised in these cases is an enigma.

The GH and thyroid hormone growth pathways

Six classic dwarfing mutations have been described in mice, but very little is known about two of them: *mn* (miniature) and *dm* (diminutive). Miniature homozygotes (Bennett, 1961) are smaller than their normal siblings at birth and become 25-30%N at p75. Their dwarfism is proportionate and they lack anatomical abnormalities, but 96% of them die by the age of 2 months. Diminutive homozygotes (Stevens and Mackensen, 1958) also exhibit low

TABLE 1

GROWTH CONTROL GENES

Gene symbol (Chromosome)	Gene name (name of mutant)	Phenotype
A. General growth-promoting function (lack of function: growth retardation)		
Prop1 (11)	prophet of Pit 1 (Ames dwarf, *df*)	BW: N; GR ~p15; ~50%N p60
Pit1 (16)	pituitary transcription factor 1	
	(Snell dwarf, *dw*; Jackson dwarf, *dw^j*)	BW: N; GR ~p15; 25-30%N p40
Gsh1 (5)	genomic screened homeobox 1	BW: N; GR p10; 25%N p60; 30% die p2, 45% p60, ~all by p120
Ghrh (2)	growth hormone releasing hormone	
Ghrhr (6)	growth hormone releasing hormone receptor	
	(little, *lit*)	BW: N; GR ~p15; ~50%N p40
Gh (11)	growth hormone	
Ghr (15)	growth hormone receptor	BW: N; GR ~p15; ~50%N p40
Thra (11)	thyroid hormone receptor α	BW: N; GR ~p15; die by p35 (~20%N)
Cga (4)	glycoprotein hormones α polypeptide	BW: N; GR ~p15; ~60%N p50.
Igf1 (10)	insulin-like growth factor I	GR e13.5 (90%N); BW: 60%N; 30%N p60
Igf2 (7)	insulin-like growth factor II	GR e11.0 (73%N); BW: 60%N; 60%N throughout life
Igf1r (7)	type 1 IGF receptor	GR e11.0 (73%N); BW: 45%N; neonatal lethality
Insr (8)	insulin receptor	GR e18.5 (90%N); death by p5
Irs1	insulin receptor substrate 1	GR e15.5; BW: 60-80%N; 60%N p100
Irs2	insulin receptor substrate 2	BW: 90%N
Hmgi-c (10)	high-mobility protein group I/Y (pygmy, *pg*)	GR e15.5 (~80%N); nullizygotes 40%N p100
B. General growth-inhibiting function (lack of function: overgrowth)		
Igf2r (17)	cation-independent mannose 6-phosphate receptor/	
	type 2 IGF receptor	OG e13.5 (125%N); BW: 140%N; perinatal death
? (11)	unnamed imprinted gene	
	(candidate: maternally expressed *Grb10*)	PatDi: OG e12.5 (~120%N); BW: 140%N
Cdkn1b	cyclin-dependent kinase inhibitor 1B (p27Kip1)	BW: N; OG ~p20; nullizygotes 130%N p40
Hg (10)	high growth (not cloned)	OG e15.5; BW: 120%N; No OG until p20; ~140N p60
C. Local growth-promoting function		
Hfhbf1 (12)	HNF3/forkhead homolog, brain factor 1	small cerebral hemispheres
D. Local growth-inhibiting function		
Mstn	myostatin	skeletal muscle OG
Fgfr3 (5)	fibroblast growth factor receptor 3	bone OG

BW=birthweight; GR=growth retardation; OG=overgrowth; N=normal. For references, see text.

viability, but 25% of them survive to maturity (they have macrocytic anemia and skeletal defects). Their weight at birth is ~57%N, but it becomes ~68%N at p60.

The remaining mutants are p*g* (pygmy; see below) and the GH-deficient Snell (dwarf, *dw*)/Jackson *(dw^j)*, Ames *(df)* and little *(lit)* dwarf mice. The allelic *dw* and *dw^j* mutations (Snell, 1929; Eicher and Beamer, 1980; reviewed by van Buul-Offers, 1983) affect the gene encoding the pituitary-specific transcription factor Pit1 (*dw* is a missense mutation of the Pit1 POU homeodomain, whereas *dw^j* is a rearrangement; Camper *et al.,* 1990; Li *et al.,* 1990; reviewed by Voss and Rosenfeld, 1992; Ryan and Rosenfeld, 1997). In the developing pituitary, expression of Pit1 is first detected at e13.5. Pit1 is involved in the differentiation of three cell types producing different hormones: somatotrophes (GH), lactotrophes (PRL) and thyrotrophes (TSH), which are absent from the hypoplastic pituitaries of Snell dwarfs. Pit1 activates transcription from the promoters of the *Gh*, *Prl* and *Tshb* genes and regulates its own gene via a late enhancer. Initial activation of *Pit1* does occur in Snell dwarfs, but then expression becomes extinct because of lack of autoregulation. An early enhancer of *Pit1* is apparently regulated by Prop1, the homeodomain protein product of an upstream gene in the pathway that is required for the establishment of the Pit1 cell lineages (reviewed by Ryan and Rosenfeld, 1997). Ames dwarfs (Schaible and Gowen, 1961) have a mutated *Prop1* gene (point mutation in helix 1 of the *Prop1* homeodomain; Sornson *et al.,* 1996). Although the lineages of cells are specified in Ames dwarfs, their populations do not expand (Gage *et al.,* 1996).

GH is released by the action of GHRH (growth hormone releasing hormone) binding to its cognate receptor (GHRHR). Circulating GH binds to its own receptor (GHR) and the ensuing signal transduction promotes cell proliferation in many tissues. A knockout of the homeobox gene *Gsh1* that acts in the hypothalamus and appears to be essential at least for the expression of the *Ghrh* gene results in severe growth retardation (~25%N weight at 8 weeks; Li *et al.,* 1996). The *lit* phenotype (Eicher and Beamer, 1976) is due to a missense mutation of the *Ghrhr* gene (Godfrey *et al.,* 1993; Lin *et al.,* 1993). Mutations in the genes encoding Pit1 (Pfäffle *et al.,* 1992; Radovick *et al.,* 1992) and GHRHR (Wajnrajch *et al.,* 1996; Baumann and Maheshwari, 1997), which result in severe GH deficiency and short stature, have also been described in humans.

In most mouse strains, the growth of Snell, Ames and *lit* mutants is indistinguishable from that of wild-type littermates for the first 2 weeks of postnatal life, at which time a reduction in body size begins to be manifested. Eventually, the Snell dwarfs reach a relative size of 25-30%N, whereas the Ames and *lit* mice become ~50%N in weight. Apparently, the Ames dwarfs are affected less severely than the Snell mutants because their pituitaries maintain a small fraction of somatotrophs (<1%; Andersen *et al.,* 1995; Gage *et al.,* 1996), and also a few tryrotrophs (and lactotrophes). The *lit* phenotype is also less severe because the mutation is hypomorphic (not a null mutation), and the mice maintain about 10% of the normal GH level (Cheng *et al.,* 1983). Snell dwarfs are lacking not only GH, but also prolactin and thyroid hormones. Mutants carrying *Prl* or *Prlr* gene knockouts (and, therefore, lacking prolactin or the prolactin receptor) do not exhibit growth retardation (Horseman *et al.,* 1997; Ormandy *et al.,* 1997). In contrast, thyroid hormones appear to be important for postnatal growth.

The nuclear receptors that mediate the action of the main thyroid hormone (T3) are encoded by two genes, *Thra* and *Thrb*. Mice with a targeted disruption of the *Thrb* gene exhibit impairment of auditory functions, but no growth defects or other abnormalities (Forrest *et al.,* 1996), whereas mutants lacking *Thra* die within 5 weeks after birth (Fraichard *et al.,* 1997). These mice appear to be normal for the first two postnatal weeks and then stop growing completely (their body weight never exceeds 5-6 g, and at the time of death they are ~20%N). They exhibit hypothyroidism, abnormalities in ossification and delays in the maturation of the small intestine. Injections of T3 (potentially acting through Thrb) extend the life-span and restore growth, but not completely (65%N at 5 weeks).

Growth retardation is also the consequence of a knockout of the *Cga* gene (Kendall *et al.,* 1995) encoding the common α subunit of the pituitary glycoprotein hormones TSH, LH and FSH, which possess individual β subunits. The *Cga* nullizygotes are hypogonadal and because of concomitant hypothyroidism they become growth retarded 2 weeks after birth (~60%N at 7 weeks). The phenotype of mice nullizygous for *Trh* (the gene encoding thyrotropin-releasing hormone that regulates TSH secretion) is mild and, despite hypothyroidism, growth retardation is observed only transiently (Yamada *et al.,* 1997). At 4 weeks, the mutants are ~70%N, but at 8 weeks they are indistinguishable in weight from control littermates.

Transgenic mice carrying a rat or human *Gh* gene driven by a metallothionein promoter express the transgene in the liver and other organs and exhibit 100-800 times higher than normal levels of serum GH. This results in overgrown animals, some of which reach nearly twice the weight of control siblings at 10 weeks of age (Palmiter *et al.,* 1982,1983). Accelerated growth begins between the second and third postnatal weeks, but cannot be correlated in a straightforward way with transgene copy number or serum GH levels. In examined lines, serum IGF-I concentration is elevated 2-3-fold, but these values are curiously inversely correlated with GH increases (Mathews *et al.,* 1988b).

When the human G*hrh* coding sequence is driven by a metallothionein promoter (Hammer *et al.,* 1985; Mayo *et al.,* 1988), overgrowth is again observed and the weight of mice becomes 125-150%N at 9 weeks depending on the transgenic line. The pituitaries of these animals are hyperplastic and the serum levels of GHRH and GH are up to ~20 and ~30 times above normal, respectively.

IGF story

The IGF signaling system (reviewed by Efstratiadis, 1994a; Jones and Clemmons, 1995; LeRoith and Bondy, 1996; Stewart and Rotwein, 1996) consists of two ligands (IGF-I and IGF-II), two receptors (type-1 and type-2; IGF1R and IGF2R) and several IGF binding proteins (IGFBPs).

The IGFs are structurally similar to proinsulin. They are small single-chain mitogenic polypeptides that are produced by many tissues and function in an autocrine/paracrine fashion (D'Ercole and Underwood, 1980; D'Ercole *et al.,* 1980,1984). They should also act as classical hormones (see Guler *et al.,* 1988), since they circulate in the plasma associated with IGFBPs.

The two IGF receptors are structurally and functionally unrelated. The signaling of both IGF ligands is mediated by IGF1R, which resembles the insulin receptor (InsR) and is a disulfide-linked heterotetrameric ($\alpha_2\beta_2$) transmembrane glycoprotein with extracellular ligand-binding and intracellular tyrosine kinase domains (reviewed by LeRoith *et al.,* 1995; Rubin and Baserga, 1995). IGF1R binds IGF-I with up to 20-fold higher affinity than IGF-II, depending on the assay system (see Germain-Lee *et al.,* 1992).

IGF2R is a single-chain protein consisting of a large extracellular domain (15 contiguous repeats) and a small cytoplasmic tail that lacks kinase activity (reviewed by Kornfeld, 1992; Ludwig *et al.,* 1995). It binds IGF-II avidly, but barely, if at all, recognizes IGF-I. In mammals, but not in other vertebrates, IGF2R is bifunctional. Thus, in chickens and frogs it serves exclusively as the cation-independent mannose 6-phosphate receptor (CI-MPR) that is involved in the trafficking of lysosomal enzymes carrying a mannose 6-phosphate recognition tag. The mammalian CI-MPR/IGF2R also serves the turnover of IGF-II by receptor-mediated endocytosis, but does not appear to participate in a signaling pathway.

There are six IGFBPs that bind IGFs with practically absolute specificity (reviewed by Clemmons, 1997), and apparently at least four additional IGFBPs of low affinity (see Kim *et al.,* 1997). The exact role of the IGFBPs remains unknown. They may serve as transport serum proteins; as IGF presentation and/or modulation molecules to the receptors; as protective molecules prolonging the IGF half-life; or as a means for tissue-specific IGF localization. The story of the *Igfbp* gene knockouts is still unfolding. What is known for the moment is that mice lacking IGFBP-2, -3 or -6 do not exhibit any obvious phenotype, whereas absence of IGFBP-4 results in mild growth retardation (~85%N birthweight) that is not aggravated further in double IGFBP-4/-2 nullizygotes (Wood *et al.,* 1993; J. Pintar, personal communication; and T. Ludwig and A.E., unpublished results).

Two of the genes in the IGF system, *Igf2* and *Igf2r*, are subject reciprocally to parental imprinting (monoallelic expression depending on parental legacy; reviewed by Solter, 1988; Efstratiadis, 1994b; John and Surani, 1996; Bartolomei and Tilghman, 1997). Only the paternal I*gf2* allele is expressed in most tissues (DeChiara *et al.,* 1991), while the transcriptionally active *Igf2r* allele is the maternal one (Barlow *et al.,* 1991). Interestingly, the *H19* gene, which is located about 90 kb downstream from *Igf2* in the same transcriptional orientation and encodes a non-translatable RNA of unknown function, has an imprinting mode opposite to that of *Igf2* (Bartolomei *et al.,* 1991; Zemel *et al.,* 1992).

The *in vivo* relationships in IGF ligand/receptor interactions (see Fig. 1) and the role of IGF signaling in embryonic and postnatal

TABLE 2

KNOCKOUTS RESULTING INDIRECTLY IN GROWTH RETARDATION

Gene (Chr)	Name	Phenotype
A. Parietal endoderm and/or trophoblast defects?		
Thbd (2)	thombomodulin	slight GR e8.5; pronounced GR e9.5; death by e10.5
B. Visceral endoderm defects		
Tcf14 (2)	transcription factor 14 (HNF4)	GR, embryo disorganization e7.5 onward; death by e10.5
Hdh (5)	Huntington's disease gene homolog	GR, embryo disorganization e7.5 onward; death by e10.5
C. Placental defects		
Egfr	epidermal growth factor receptor	depends on genetic background; see text
Hgf (5)	hepatocyte growth factor	GR e14.5 onward; death e13.0-e16.5
Met (6)	proto-oncogene Met (HGF receptor)	GR e14.5 onward; death e13.0-e16.5
Wnt2	wingless-related MMTV integration site 2	GR e17.5 (87%N) onward; 50% die p1
D. Cardiac, vascular or hemopoietic defects		
Atrc1 (5)	amino acid transporter, cationic 1	BW: 77%N; death on p1
Arnt (3)	aryl hydrocarbon receptor nuclear translocator	GR e9.5; death by ~e11.0
Flk1 (5)	fetal liver kinase 1	slight GR e9.5; death e10.5
Flt1 (5)	FMS-like tyrosine kinase 1	GR e8.5; death ~e9.5
Lmo2 (2)	Lim only 2 (Rbtn2; rhombotin 2)	GR by e9.75; death by e11.5
Nkx2-5 (17)	Drosophila NK2 transcription factor related locus 5	GR e9.0; death ~e11.0
Tal1 (4)	T cell acute lymphocytic leukemia 1	GR e8.75; death by e11.5
E. Feeding defects?		
Drd1a (13)	dopamine receptor 1A	BW: N; GR p20; ~70%N p40; fare better with hydrated food
Mf3 (9)	mesoderm/mesenchyme fork head 3	some die by e14.5; others, BW: N; GR p2; 50%N p20, then catch up
F. Metabolic defects		
Gpi1-s (7)	glucose phosphate isomerase	GR, embryo disorganization; death by e10.5
Casr (16)	calcium-sensing receptor	BW: N; GR p2; ~30%N p13; death p3-p30
Cbs (17)	cystathionin β-synthetase	BW: N; GR p7; ~80%N p14; ~67%N p21;
Ctsd (7)	cathepsin D	BW: N; GR p20; ~60%N p25; death by p27; intestinal atrophy
Ggtb (4)	glycoprotein galactosyltransferase β-1,4	90% die by p20; ~53%N p30; catch-up growth (~80%N p50)
Ggtp	γ-glutamyl transpeptidase	BW: N; GR p10; stop growing p40, males 45%N, females 57%N
Gyk (X)	glycerol kinase	BW: N; GR p2; 67%N p3; die p3-p4
Pcmt1 (10)	protein L-isoaspartate(D-asp) O-methyltransferase 1	75%N p30; die p22-p60
Vdr (5)	vitamin D receptor	BW: N; GR p25; ~90%N p70
G. DNA-repair defects		
Atm (9)	ataxia telegiectasia gene mutated in human beings	GR noticeable at birth; 76%N p35
Brca1 (11)	breast cancer 1	GR, embryo disorganization e5.5; death between e5.5 and e9.5
Brca2 (5)	breast cancer 2	GR, embryo disorganization e6.5; death between e8.5 and e11.5
Rad51a (2)	RAD51A homolog	growth arrest ~e6.0
H. Miscellaneous		
Bcl2 (1)	B-cell leukemia/lymphoma 2	BW: N; GR p7; 64%N p50 (range 30-90%N)
Bmi1 (2)	B lymphoma Mo-MLV insertion region 1	BW: N; 50% die by p3; GR p7-p15; all die p20-p140
Cbx2 (11)	chromobox homolog 2 (M33)	BW: N; GR ~p7; 27%N p29; die by p40
Dbh (2)	dopamine β-hydroxylase	die in utero; 12% born; BW: N; GR p7; ~50%N p21, but 80%N p70
Edr (6)	early development regulator (Rae28)	BW: 93%N; die by p35 (80% neonatally)
Fst	follistatin	88%N e18.5; neonatal death
Il10 (1)	interleukin 10	BW: N; GR p20; ~70%N p50, but highly variable
Mll (9)	myeloid/lymphoid or mixed lineage leukemia	nullizygotes die by e11.0; heterozygotes: BW: 88%N; 54%N p20
Nfkbia	nuclear factor of κ light chain enhancer, inhibitor a	BW: N; GR p4; ~30% p7; death by p8
Plg (17)	plasminogen	BW: N; GR p60; ~50%N p180; rescue in fibrinogen null background
Prkg2	protein kinase, cGMP-dependent, type 2	BW: N; GR p20; 85%N p60; micromelia, abnormal growth plates
Rarg (15)	retinoic receptor γ	BW: N; 40-80%N p5; most die by p60; survivors ~N in weight
Sp4 (12)	trans-acting transcription factor 4	BW: N; GR p7, 30% survive; ~60%N p35, but ~80%N p70
Tcfe2a	transcription factor E2A	BW: N; GR p2; ~30% p15; death before weaning
Th (7)	tyrosine hydroxylase	die in utero; 2.5% born; BW: N; GR p7; 39%N p15; death by p30
Titf1 (12)	tryroid transcription factor 1 (T/EBP)	~85%N e19.5; die at birth; lack thyroid, pituitary; abn. brain, lungs
Zfp144 (10)	zinc finger protein 144 (Mel18)	BW: N; GR p7; 30%N p15; die p20-p40; intestinal obstruction

Unless cited in the text, the mutations listed are not referenced due to space constaints (I apologize to the authors).
References can be found by gene symbol or name at two Web sites: http://www.informatics.jax.org/locus.html and http://biomednet.com/cgi-bin/mko/mkosrch.pl

development were revealed by gene knockouts and analysis of phenotypes manifested by the mutations, alone or in combination (DeChiara *et al.,* 1990,1991; Baker *et al.,* 1993,1996; Liu *et al.,* 1993; Ludwig *et al.,* 1996; Eggenschwiler *et al.,* 1997; Louvi *et al.,* 1997).

I*gf1* nullizygotes exhibit some delay in the progress of ossification and have a birthweight that is ~60%N. Some of these neonates die within 6 hours after birth for unknown reasons, but others survive to adulthood at frequencies depending on genetic background. The growth rate of these mutants continues to be reduced postnatally and they become ~30%N in size at the age of two months. Bone development is delayed, and both sexes lack libido and are infertile. The females do not ovulate, while the males have low serum testosterone and reduced sperm counts, although sperm is capable of fertilizing wild-type ova *in vitro*.

IGF-I is also important for human fetal growth, as documented by the description of a 15-year old patient carrying a homozygous partial deletion (exons 4 and 5) of the *IGF1* gene (Woods *et al.,* 1996). This mutation was manifested with severe intrauterine growth retardation and postnatal growth failure, including delayed bone development. Although the growth deficiency of the *IGF1* null patient (~40%N birthweight) was relatively more severe than that of mice lacking IGF-I (60%), the overall phenotypic features were strikingly similar, except that the development of the reproductive system was apparently not affected (Woods *et al.,* 1996), in contrast to the mutant mice (Baker *et al.,* 1996).

The heterozygous mice carrying a paternally derived mutated *Igf2* gene [*Igf2(+/p)* mutants] and the *Igf2(-/-)* nullizygotes are phenotypically indistinguishable; they are viable dwarfs with ~60%N birthweight and, except for a slight delay in ossification, they do not exhibit developmental abnormalities. In contrast, when the disrupted *Igf2* allele is transmitted maternally, the offspring are phenotypically normal, since the maternal allele is normally silent due to imprinting.

Igf1r nullizygotes are severely growth-retarded and their weight at birth is ~45%N. Regardless of genetic background, these mutants are unable to breath and die immediately after birth. The primary cause of this respiratory failure is unknown (histological abnormalities of the lungs or absence of surfactant apoprotein were not observed). The mutant embryos of this class exhibit generalized organ hypoplasia, including the respiratory muscles, significant delays in ossification, and thin skin.

Double mutants lacking both IGF-I and IGF1R do not differ in phenotype from *Igf1r(-/-)* single mutants. This demonstrates that IGF-I interacts exclusively with IGF1R *in vivo*. In contrast, the birthweight of *Igf2(+/p-)/Igf1r(-/-)* double mutants is ~30%N (that is, the growth retardation of the double mutants is more severe than that manifested in either class of the single mutants). This was the first genetic evidence indicating that IGF-II interacts with IGF1R and also with an additional receptor, which, as it turns out, is the insulin receptor (see below). The phenotypes of double mutants lacking both ligands (IGF-I and IGF-II) or lacking both IGF-II and IGF1R are indistinguishable (30%N). Therefore, within the framework of the observed mutational effects, all ligand/receptor interactions have been accounted for (see Fig. 1).

When whole-embryo weight is used as a phenotypic criterion to study developmental growth kinetics during embryogenesis, it is observed that, in comparison with wild-type controls, growth retardation becomes evident for the first time at e11.0 in mutant

embryos lacking IGF-II, IGF1R or both (all 73%N in size), and at e13.5 in embryos lacking IGF-I (90%N). Between e11 and e12.5, the manifestation of growth deficiency has the same severity in mutants lacking IGF-II, IGF1R or both, indicating that during this window of developmental time IGF1R interacts exclusively with IGF-II. At e13.5, however, these three classes of mutants become distinct in size: *Igf2* > *Igf1r* > *Igf2/Igf1r* mutants. Thus, sometime between e12.5 and 13.5, IGF1R, which initially binds only IGF-II, begins an additional interaction with IGF-I, while IGF-II starts to interact also with InsR. Subsequently, the mutants exhibit their characteristic, lower-than-normal birthweights as a result of decreased growth rates.

Consistent with the information that *Igf2r* is imprinted, the phenotype of heterozygotes inheriting maternally the mutated gene [*Igf2r(m-/+)*] is indistinguishable from the phenotype of nullizygous mutants (Lau *et al.,* 1994; Wang *et al.,* 1994; Ludwig *et al.,* 1996). Mice lacking IGF2R have increased tissue and serum IGF-II levels and exhibit overgrowth (140%N birthweight) and generalized organomegaly, kinky tails and extra toes, heart abnormalities and edema, and usually die perinatally.

As demonstrated by genetic analysis, the lethality in the absence of IGF2R-mediated IGF-II turnover is caused by excess of this ligand overstimulating IGF1R. This is evidenced by the fact that double mutants lacking both IGF2R and IGF-II are fully viable dwarfs. In addition, the involvement of IGF1R overstimulation in the lethality of *Igf2r* mutants is demonstrated by the rescue of double mutants lacking both IGF1R and IGF2R, although each individual mutation is lethal. These double IGF receptor mutants have normal birthweight and are indistinguishable from wild-type siblings for the first two postnatal weeks. Their growth rate is then reduced up to p30, but afterwards they exhibit catch-up growth and sometimes attain normal adult weight. They are also fertile, in contrast to the *Igf1* nullizygotes. The surprising catch-up growth and fertility of the double IGF receptor mutants remain unexplained. Since both *Igf2r(m-/+)* and *Igf2r(-/-)* mutants are rescued in *Igf2* or *Igf1r* null background, it is clear that viability is not due to imprint relaxation (that is, transcriptional activation of the silent paternal *Igf2r* allele does not occur).

The normal embryonic development of mutants lacking both IGF receptors is sustained by excess of IGF-II acting through InsR, according to the following evidence. First, the significance of IGF-II for rescue is demonstrated by the phenotype of triple mutants lacking IGF1R, IGF2R and IGF-II. Second, the role of InsR mediating in part IGF-II action is demonstrated by the phenotype of double mutants lacking both IGF1R and InsR. In both of these cases, the mutant progeny are non-viable dwarfs with 30%N size, indistinguishable from other 30%N mutants (lacking IGF-I and IGF-II or lacking IGF1R and IGF-II), in which all of the ligand/receptor interactions are interrupted (see Fig. 1). Complementary evidence for an IGF-II/InsR interaction supporting the genetic data was provided from experiments with cultured cells (Morrione *et al.,* 1997b). The analyses revealing this particular ligand/receptor relationship were prompted by the known cross-interaction properties of the IGF and insulin systems. Whereas the affinity of insulin-binding to IGF1R is 500-1000 times lower than that of the IGFs, IGF-II binds to InsR with an affinity that is only 10 times lower than that of insulin (relative to insulin, the affinity of IGF-I for InsR is at least 50-100 times lower; see De Meyts *et al.,* 1994). Although InsR cannot compensate for an *Igf1r* knockout, the opposite apparently

Fig. 1. Summary of IGF mutant phenotypes. *Each panel shows the interactions between IGF ligands (IGF-I=I; IGF-II=II; squares) and receptors (IGF1R=1R; IGF2R=2R; InsR=IR; ovals) in wild-type (WT) and mutant mice. The genotype of each single, double or triple mutant is indicated on top of each panel, together with the corresponding phenotype of embryos [E; % of normal weight at e18.5 (except for panel Q; e16.5-e17.5); rounded numbers]. The relative weight of the placenta (P), if determined, is also shown. Ligands and/or receptors that are absent from each of the mutants are indicated with black symbols. Signaling interactions are represented with arrows, whereas a «lollipop» symbol denotes the IGF2R-mediated turnover of IGF-II. In the absence of turnover (panels F, K, Q), the amount of IGF-II increases (II, larger letters). An IGF-II increase also occurs by imprint relaxation (ΔH19 =H deletion mutation; panel O). Expression of IGF-II only from the relaxed maternal allele is denoted by II in outline (panels P and R). In panels O and Q, the expression of IGF-II is biallelic.*
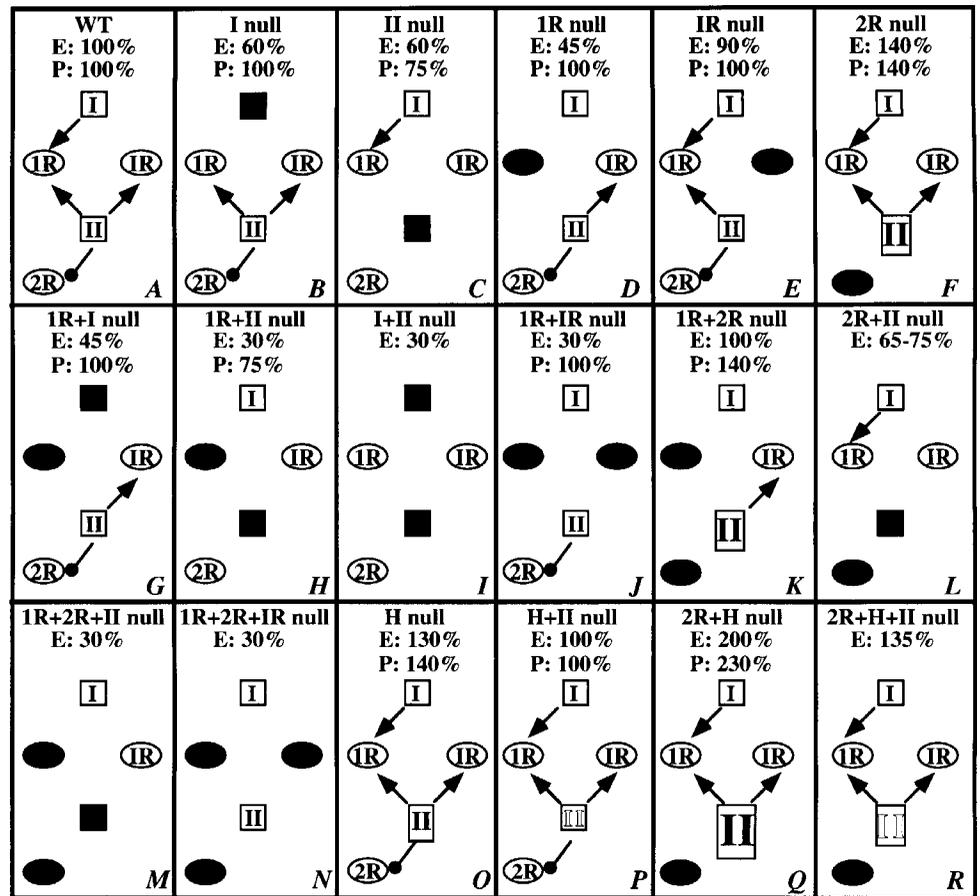
occurs, because mutants lacking *Insr* (Accili *et al.,* 1996; Joshi *et al.,* 1996) have a birthweight that is about 90%N (Louvi *et al.,* 1997). However, these mutants die of diabetic ketoacidosis within a few days after birth. The phenotype of *Insr* nullizygotes is identical to that of double mutants lacking both mouse insulins (I and II), whereas *Ins1* or *Ins2* single nullizygotes are viable and fertile (Duvillié *et al.,* 1997). In contrast to humans and most other vertebrate species, which possess a single gene encoding insulin II, the genome of rats and mice contains an additional nonallelic gene (a functional retroposon) encoding insulin I (Soares *et al.,* 1985). Further genetic evidence for the InsR role was provided from the 30%N phenotype of triple mutants lacking all three receptors (IGF1R, IGF2R and InsR). Thus, in these triple mutants, the excess of IGF-II in the absence of IGF2R and the lack of harmful overstimulation in the absence of IGF1R cannot sustain viability, since InsR is also missing.

In *Igf2r* mutants, overgrowth is due to increase of IGF-II by perturbation of a post-translational mechanism (absence of turnover). Overgrowth can also occur by *Igf2* imprint relaxation (perturbation at the transcriptional level). I have already mentioned the linkage of *Igf2* and *H19* genes. The paternal *Igf2* and maternal *H19* genes are expressed with similar tissue- and developmental-specificity using, at least in tissues of endodermal origin, the same enhancers located downstream from *H19* (Bartolomei *et al.,* 1993; Leighton *et al.,* 1995b). In mice carrying a maternally-derived 13 kb deletion mutation *(ΔH19),* which eliminates *H19* and 10 kb of

upstream sequence (Leighton *et al.,* 1995a), the normally silent maternal *Igf2* allele becomes transcriptionally active (imprint relaxation), and the increased amount of mRNA from both alleles yields a higher-than-normal level of translation product resulting in increased birthweight (130%N; "*H* phenotype"). Activation of the maternal *Igf2* allele, when the *ΔH19* mutation is in *cis,* is apparently caused by elimination of a negative imprinting signal, making possible an interaction between the intact 3' enhancers and the normally dormant *Igf2* promoters (Leighton *et al.,* 1995a). Interestingly, *Igf2/ΔH19* double mutants (with paternal and maternal mutant alleles, respectively) have normal size. In contrast to the mutants lacking IGF2R that exhibit abnormalities and die perinatally ("*R*" phenotype), the *ΔH19* mutants are fully viable. From the comparison of the *R* and *H* phenotypes, it was surmised that absence of IGF-II degradation in mutants lacking IGF2R permits accumulation of ligand at a level higher than that attained by biallelic transcription in *ΔH19* mutants, also shown to exhibit incomplete imprint relaxation in some tissues, including the liver that contributes the circulating form of IGF-II (Leighton *et al.,* 1995a). In double mutants (Eggenschwiler *et al.,* 1997) generated by combining the *ΔH19* mutation with a targeted *Igf2r* allele, both inherited maternally, the level of IGF-II becomes extremely high (7- and 11-fold higher than normal in tissues and serum, respectively) and causes a phenotype *("R/H")* with features of the Beckwith-Wiedemann syndrome (BWS; reviewed by Elliot and Maher, 1994; Weng *et al.,* 1995). The manifestations include somatic overgrowth,

visceromegaly, placentomegaly, omphalocele, and cardiac and adrenal defects. *R/H* embryos die by e17.5, at which time they are ~200%N (their placentas are ~230%N at e16.5). Analogous observations about the consequences of IGF-II overexpression were also made with a different experimental approach using chimeras (Sun *et al.,* 1997). Interestingly, the *R/H* mutants also exhibit skeletal defects and cleft palate that are frequent features of the Simpson-Golabi-Behmel syndrome (SGBS; reviewed by Hughes-Benzie *et al.,* 1996; Weksberg *et al.,* 1996). The BWS and SGBS overgrowth disorders overlap phenotypically, but they are distinct and variably manifested clinical entities of different etiology. SGBS is caused by mutations of the X-linked gene *GPC3* (Pilia *et al.,* 1996), which encodes a cell-surface proteoglycan (glypican-3). Preliminary biochemical data (Pilia *et al.,* 1996; not reproduced with the rat *GPC3* homolog; Song *et al.,* 1997) suggested that glypican-3 interacts with IGF-II and potentially sequesters or down-regulates this factor. It remains to be seen, however, whether the lack of *GPC3* resembles in mechanism the absence of IGF2R. In contrast, the evidence for an involvement of IGF-II overexpression in the pathogenesis of BWS is quite strong (reviewed by Weksberg and Squire, 1996; Reik and Maher, 1997), since this disorder is linked to the 11p15.5 chromosomal region, where the human *IGF2* gene resides. However, BWS is genetically heterogeneous and complex, and at least some of the cases can be explained by mutations of the gene encoding p57^{Kip2} (see Hatada *et al.,* 1996; Zhang *et al.,* 1997) also located in 11p15.5 (~700 kb upstream from *IGF2*).

Mice carrying an *Igf2* transgene driven by its own regulatory sequences did not exhibit overgrowth, but some of them had cleft palate, when the transgene was in the homozygous state (Wise and Pravtcheva, 1997). An *Igf2* transgene expressed under a keratin gene promoter (Ward *et al.,* 1994) did not affect birthweight, and postnatally caused overgrowth only in tissues expressing the transgene, while the transgenic animals, despite a 3-fold increase in circulating IGF-II, were up to 8% lighter than the controls due to lower fat content (Da Costa *et al.,* 1994). Similarly, whole-body weight was reduced by 5-17% in transgenic mice expressing (predominantly in the liver) *Igf2* under the control of the major urinary protein promoter, although serum IGF-II was increased up to 30-fold (Rogler *et al.,* 1994). Finally, in a line of mice carrying an *Igf2* transgene under the control of the *H2-Kb* gene promoter/ enhancer, whole-body weight was unaffected and overgrowth was observed only in the thymus, while the serum IGF-II content in homozygous transgenic animals reached a level that was up to 8-fold higher than in controls by 6 weeks of age (van Buul-Offers *et al.,* 1995).

Overexpression of IGF-I was achieved by using a human IGF1 cDNA transgene driven by a metallothionein promoter (Mathews *et al.,* 1988a; Quaife *et al.,* 1989). The level of IGF-I was increased in several tissues and was 1.5-fold above normal in serum, whereas the concentration of insulin was reduced, and GH was below detectable levels. An increase in body weight became apparent between 6 and 8 weeks of age, and was shown to be 123%N at 10 weeks. There was disproportionate overgrowth of some organs, but no increase in the length of long bones. *Igf1* mRNA expression in liver from the endogenous gene was reduced 3-fold. It seems, therefore, that moderate overgrowth was the net effect of transgenic expression with concomitant reduction of expression from the endogenous locus.

GH/IGF relationship

In rodents, *Igf2* expression ceases a few weeks after birth (except in the choroid plexus and the leptomeninges). Thus, in contrast to embryonic growth, the postnatal growth of mice is IGF-II-independent, and the mutants lacking IGF-II that are born with a 60%N weight maintain this relative size thereafter. Postnatal growth rate is also normal in *ΔH19* mutants overexpressing *Igf2*, which overgrow as embryos, but retain a 130%N size throughout postnatal life. In contrast to IGF-II, IGF-I is a ligand that has a continuous function throughout development. Thus, the postnatal rate of growth in surviving *Igf1* nullizygotes is lower than normal, as it was during embryogenesis, including the first 2 postnatal weeks. As a consequence, these mutants, which exhibit a 60%N weight at birth, become 30%N at 8 weeks and remain at this level thereafter.

GH comes into play at around p15. The original "somatomedin hypothesis" (Salmon and Daughaday, 1957; for a review, see Daughaday, 1989) was proposed on the basis of results demonstrating that GH (somatotropin) stimulated sulfate incorporation into cartilage indirectly through a serum factor (somatomedin; mediator of the effects of somatotropin). In its more general form, the hypothesis posited that GH stimulates the production of circulating somatomedin (IGF-I) by the liver, which then mediates the hormonal effects on all target tissues. Interestingly, the level of *Igf1* mRNA in liver and also in some other tissues is regulated by GH. Thus, it is decreased by hypophysectomy and restored by administration of GH (Roberts *et al.,* 1986; Hynes *et al.,* 1987).

Despite an apparent endocrine role of the plasma IGF-I (see Guler *et al.,* 1988), modified versions of the somatomedin hypothesis have been proposed (reviewed by Daughaday, 1989; Isaksson *et al.,* 1991; Scanes and Daughaday, 1995) to account for data indicating additional autocrine/paracrine actions of IGF-I produced locally under the influence of GH (see, for example, Nilsson *et al.,* 1986). Moreover, a "dual effector theory" exists, according to which GH stimulates the differentiation of progenitor cells making them responsive to IGF-I (see Green *et al.,* 1985). Finally, there are suggestions that GH exerts some direct effects on target tissues that are not mediated by IGF-I (see Green *et al.,* 1985; Lindahl *et al.,* 1987).

Although the original "somatomedin hypothesis" is not tenable any longer, the important issues pertaining to the exact relationship between GH and IGF-I have not been resolved yet. In fact, in regard to bone development, the picture is still quite unclear, while more confusion exists when other potential IGF-I or GH target tissues are considered. There is a difference, for example, in murine reproductive functions. Whereas *Igf1* null mice are infertile, mouse reproduction is GH-independent, as demonstrated by the performance of nullizygotes carrying a *Ghr* gene knockout, which are fertile (except that litter sizes are small; Zhou *et al.,* 1997). These mutants become ~50%N in size at 8 weeks, and, therefore, their growth retardation is less severe than that of *Igf1* null mice (~30%N). With a different *Ghr* knockout, it was shown that *Ghr/Igf1* double nullizygotes practically stop growing at weaning and their relative weight does not exceed ~15%N at 3 months of age (F. Lupu and A.E., unpublished results). Mice lacking GHR function are potential models of Laron-type dwarfism, a growth-retardation disorder resulting from a variety of mutations in the human *GHR* gene (reviewed by Rosenfeld *et al.,* 1994).

Interestingly, a knockout of the mouse gene encoding HNF1α (hepatocyte nuclear factor 1α), a presumptive transactivator of the *Igf1* and insulin gene promoters, results in a dwarfing phenotype associated with diabetes and infertility (Lee *et al.,* 1998). Nullizygous mutants brought to term (less than 50% of the expected number) are only slightly smaller than normal, but grow with a reduced rate and become ~50%N at 5 weeks. As a consequence of low hepatic *Igf1* mRNA levels, the serum IGF-I is reduced in these mice (20-30% of normal), despite a GH increase to levels up to 100-fold higher than normal.

Irs1 and *Irs2*

The InsR and IGF1R action is coupled to various signaling effectors (PI-3 kinase, Grb2, Sos etc.) by tyrosine phosphorylation of at least four intermediary docking proteins (IRS, insulin receptor substrates; reviewed by Cheatham and Kahn, 1995; Jones and Clemmons, 1995; Yenush and White, 1997). The only phenotypic manifestation in mice lacking IRS1, other than mild insulin- and IGF-resistance and abnormal glucose tolerance, is growth retardation. However, the results from two independent *Irs1* gene knockouts are not in agreement. According to one report, the mutants are ~80%N at birth and have reduced growth rate postnatally becoming ~60%N at 15 weeks (Tamemoto *et al.,* 1994). In contrast, the reported growth deficiency at birth is more severe in a second knockout (40-60%N; Araki *et al.,* 1994), but the postnatal growth rate is apparently normal, since the mutants maintain the same relative weight (50-60%N) at least up to 4 months. Whether this significant difference can be attributed to genetic background is an open question. Nevertheless, considering what is now known from the *Insr* knockout (see above), we can surmise that IRS1 should mediate at least some of the IGF actions on growth. In contrast to the *Irs1* nullizygotes, mutants lacking IRS2 develop diabetes that becomes progressively severe (Withers *et al.,* 1998). However, the growth retardation of these mutants is minimal (their body size at birth and throughout postnatal life is ~90%N).

Pygmy story

Chance and tenacity revealed an unsuspected player in growth control in the case of *pg* mutants. Pygmy mice were first noticed as undersized animals in a strain selected for small size. The *pg* phenotype (King, 1950,1955) was found eventually to be identical to that of a transgenic insertional mutant (mini-mouse; Xiang *et al.,* 1990), and the two mutations were shown to be allelic. It was then demonstrated that the phenotype arises from inactivation of the *Hmgi-c* gene, a conclusion verified by knockout (Zhou *et al.,* 1995). Growth retardation is first noted at e15.5 (~80%N; Benson and Chada, 1994). The growth rate continues to be reduced postnatally, and homozygous mutants become ~40%N at 14 weeks (King, 1950; Benson and Chada, 1994). Interestingly, the heterozygotes, although indistinguishable from wild-type for two weeks after birth, also become smaller than normal (apparent haploinsufficiency; ~85%N at 14 weeks). Both sexes are sterile (King, 1950) and the mutants are unresponsive to GH treatment (Rimoin and Richmond, 1972). The levels of serum GH (Sinha *et al.,* 1979) and IGF-I (Sugisaki *et al.,* 1993) are normal.

Hmgi-c encodes a member of the high-mobility group (HMG) of DNA binding proteins. HMGI-C binds to AT-rich sequences in the minor groove of the DNA helix and is thought to be a chromatin architectural factor involved in the formation of higher-order nucleoprotein complexes. Expression studies (both RNA and protein) show that *Hmgi-c* is transcribed at least as early as e10.5. However, expression declines progressively, and is almost undetectable from e15.5 onward (Zhou *et al.,* 1996). The surprise then is that there is haploinsufficiency and postnatal growth retardation, whereas the gene is not expressed after e15.5 (except at exceedingly low levels in only two of the tissues examined postnatally). Therefore, considering the putative function of the protein, it is possible that this reflects aberrant chromatin templating affecting the cell cycle. One could argue that the *Hmgi-c* gene provides only a precondition for growth and is not a growth control gene *per se*. In fact, an open question is whether overgrowth will be observed upon *Hmgi-c* overexpression. Although this argument cannot be rejected conclusively at present, the absence of *Hmgi-c* does affect the cell cycle, and real, albeit indirect, control of growth may be brought about by some sort of templating action of the gene product in the cooperative assembly of enhanceosomes of other genes potentially participating in growth pathways.

Whole-body growth inhibitors

As already described, *Igf2r* plays a balancing, but in essence negative role on growth by removing the excess of IGF-II. Genetic analyses of the effects of transmission of malsegregated, rearranged chromosomes, used to study the phenomenon of imprinting, revealed that an unnamed growth control gene (potentially antagonizing IGF action) is located somewhere in the proximal third of mouse chromosome 11 (centromere to 11A/B border; Cattanach and Kirk, 1985; Cattanach, 1986; Cattanach *et al.,* 1996; Beechey and Cattanach, 1997). Maternal disomy for the proximal 11 region results in dwarfism, whereas paternal disomy results in overgrowth. The manifestations (in either direction), which are strikingly similar to those observed from lack or overexpression of *Igf2*, are evident as early as e12.5, and the respective birthweights are 60%N and 140%N. These relative sizes of mice change very little, if at all, postnatally. Except for their sizes, the mice of both sexes are normal and fertile; organ weights are proportional to body weights; and the level of circulating IGF-I is normal. The gene encoding Grb10 (growth factor receptor bound protein 10), a signaling effector inhibiting IGF1R-mediated cell proliferation (Morrione *et al.,* 1997a), has been proposed as a likely candidate for the imprinted gene on proximal 11 (Miyoshi *et al.,* 1998). The hypothesis is that, because the imprinted *Grb10* (chromosome 11; 8 cM) is expressed maternally, absence of its negative action on IGF-dependent growth promotion results in overgrowth in the case of paternal disomy of proximal 11. Reciprocally, overexpression due to maternal disomy leads to dwarfism.

Another general growth inhibitory function was revealed when three groups knocked-out independently, and with overall consistent results, the gene encoding the p27[Kip1] cyclin-dependent kinase inhibitor (Fero *et al.,* 1996; Kiyokawa *et al.,* 1996; Nakayama *et al.,* 1996). At birth, nullizygotes lacking p27[Kip1] are indistinguishable from normal littermates. Differences in weight become evident at ~3 weeks or later (and some heterogeneity in weights is observed between individuals). At 6 weeks, the nullizygotes are ~130%N and the heterozygotes ~115%N. Thus, there is clearly a (negative) gene-dosage effect. Some organs of the nullizygotes (like the liver

and the brain) are increased in size more or less proportionately to the body weight, whereas others (like the thymus and the spleen) are almost doubled in size. Organ overgrowth is due to hyperproliferation that is associated in examined tissues with smaller cell sizes (Fero *et al.,* 1996). Interestingly, the dramatic hyperplasia of the thymus has no impact on the normal differentiation of thymocytes. On the other hand, the development of ovarian follicles is abnormal and results in female infertility. Despite pituitary hyperplasia, the levels of GH and IGF-I in the circulation are normal.

A putative gene named *Hg* (high growth) that probably encodes still another general growth inhibitor was discovered in a line of mice (generation 25) that were selected for high weight gain at 3-6 weeks after birth (Bradford and Famula, 1984). Mice that are apparently homozygous for a mutated allele *(hg/hg)* exhibit an increase in embryonic growth from e15.5 onward, and their birthweight is about 120%N (Summers and Medrano, 1997). Surprisingly, however, overgrowth is not detected postnatally before p30 (~140%N weight at p60; Medrano *et al.,* 1991). The phenotype is affected by the genetic background. The concentration of GH in plasma is reduced, whereas the concentration of IGF-I is increased. The *Hg* gene is located on mouse chromosome 10, and the *hg* mutation co-segregates with a deletion of the microsatellite marker D10Mit69 (Horvat and Medrano, 1996). Although the exact length of the deleted segment is unknown, this information and the observation that the mutated allele is at least partially recessive suggest that the gene is involved, directly or indirectly, in negative growth regulation (even if the deletion involves only regulatory elements and not the body of the gene).

Local growth control

The gene *Hfhbf1* encoding brain factor 1, a winged helix transcription factor, provides an example of positive tissue-specific growth control. *Hfhbf1* is expressed in the region of the developing telencephalon that gives rise to the cerebral hemispheres. In *Hfhbf1* nullizygotes, proliferation of telencephalic neuroepithelial cells is severely reduced, and cell differentiation is premature, depleting the progenitor cell population. The mice die at birth with cerebral hemispheres drastically reduced in size (Xuan *et al.,* 1995). Two additional mutations in uncloned genes affect brain growth in the opposite direction (the mechanisms are unclear). One of them (*fog*, forebrain overgrowth; Harris *et al.,* 1997) is apparently related to abnormalities in neural tube closure from excessive growth due either to hyperproliferation or to failure in apoptosis. The other mutation (*mceph*, megencephaly; Donahue *et al.,* 1996) provides an interesting example of overall brain cell hypertrophy (not hyperplasia) that is curiously associated with whole body growth retardation. Thus, at 4 months, the weight of mutants is ~75%N, whereas the brain weight is ~124%N (ratio of mutant to normal brain normalized for body size: ~1.6).

The *Fgfr3* and *Mstn* genes are examples of local negative growth regulators. The fibroblast growth factor family is large and includes at least 15 ligands and 4 receptors, one of which is FGFR3. In mice, the *Fgfr3* gene is expressed in bone cartilage during endochondral ossification and its action apparently restricts osteogenesis, because *Fgfr3* nullizygotes show overgrowth of long bones and vertebrae and other skeletal (and inner ear) anomalies (Colvin *et al.,* 1996; Deng *et al.,* 1996). The increase in bone length

occurs postnatally and is attributable to cellular expansion of proliferating and hypertrophic chondrocytes in the growth plate. Despite bone overgrowth, there is variable growth retardation in one of two *Fgfr3* knockouts (Colvin *et al.,* 1996), potentially attributable to poor nursing. There is also a high incidence of unexplained lethality (48%) by p21. It is possible that missense mutations of the human *FGFR3* gene, which cause dominantly inherited skeletal dysplasias associated with dwarfism, correspond to gain-of-function mutations.

The *Mstn* gene encoding myostatin, a factor of the TGFβ superfamily of proteins, appears to function specifically as a negative regulator of skeletal muscle growth (McPherron *et al.,* 1997). *Mstn* is expressed exclusively in the myotome compartment of developing somites in early embryos and in many muscles throughout the body later in development. In *Mstn* nullizygotes, which are viable and fertile, there is a widespread increase in skeletal muscle mass (at least 2-fold higher than normal) that results predominantly from hyperplasia with some contribution from hypertrophy. The body weight of these mutants is increased (120-130%N from 2 months onward), but, because overgrowth is local, their body shape is abnormal.

Embryonic growth and the placenta

The imprinted *Mash2* gene encoding a transcription factor is highly expressed in cells of the trophoblast lineage exclusively from the maternal allele after e7.5 (Guillemot *et al.,* 1994,1995). A M*ash2* knockout results in failure of the placenta (lacking spongiotrophoblast layer) and death of nullizygotes or *Mash2(m-/+)* heterozygotes at ~e10 (Guillemot *et al.,* 1994). In nullizygotes with a targeted disruption of the *Vcam1* gene, encoding an adhesion molecule, there is failure of fusion of the allantois with the chorion that results in placental abnormalities and embryonic death by e11.5 (Gurtner *et al.,* 1995; Kwee *et al.,* 1995). In the cases of the *Mash2* and *Vcam1* mutations, lethality is apparently acute, and there is no time for the manifestation of growth retardation (mutant embryos are indistinguishable in size from their normal littermates at the time of their death). In *Hgf* or *Met* mutants (lacking hepatocyte growth factor or its receptor, respectively) some degree of growth retardation is evident, but in addition to impairment of the labyrinthine trophoblast layer of the placenta, there is extensive loss of parenchymal cells in the embryonic liver (probably by apoptosis) and abnormalities in myogenesis (Bladt *et al.,* 1995; Schmidt *et al.,* 1995; Uehara *et al.,* 1995). These mutants die between e13.0 and e16.5, but a small reduction in size is first observed at e14.5, becoming more pronounced at e15.5 and e16.5. Growth retardation due to placental defects is manifested in *Wnt2* nullizygotes from e17.5 (87%N) onward (Monkley *et al.,* 1996). The placentas of these mutants are thin, disorganized and edematous, probably as a result of defective vascularization. About 50% of these mutants die during the first postnatal day.

Three independent knockouts of the *Egfr* gene encoding the epidermal growth factor receptor have provided an outstanding example of the action of gene modifiers. At the same time, the importance of the placenta for embryonic growth was re-emphasized. Targeting (in 129/Sv background) involved the regions around promoter/exon 1 (referred to here as "mutation A"; Sibilia and Wagner, 1995) or around exon 2. In the latter case, there was either a partial deletion (*Egfr^{m1Cwr}* allele; mutation B; Threadgill *et*

al., 1995) or an insertion of a cassette (mutation C; Miettinen *et al.,* 1995). The effects of two random-bred colony stocks (CF-1 and CD-1) were examined with mutation B. 129/SvxCF-1 hybrids die at or near implantation due to inner cell mass degeneration, whereas 129/SvxCD-1 mutants are brought to term and live for up to three weeks, exhibiting skin, kidney, brain, liver, and gastrointestinal tract abnormalities. Their birthweight is normal, but the postnatal growth rate is reduced (~30%N at p18). In inbred 129/Sv mutants (mutation A or B) and in 129/SvxC57BL6 hybrids (mutation A), the severity of the effects is intermediate (between the two extremes described above): death of homozygous mutants occurs by e12.5 due to placental defects (reduction of the spongiotrophoblast placental layer and disorganization of the labyrinth). The size of the placenta is reduced to ~50%, while the birthweight of a few 129/Sv x C57BL6 hybrids that come to term is ~80%N (growth retardation is detectable from e14.5 onward). Mice carrying mutation C in a 129/SvxSwiss Webster Black background are brought to term, but die by p8. Their birthweight ranges from normal down to ~30%N. However, even the normal-size pups lose weight rapidly, apparently because of dehydration and malnutrition.

Although a conclusion that a functional placenta is required for normal embryonic growth can be reached (and it's not startling), a question that has no clear-cut answer remains: what degree of reduced placental growth will affect the growth of the embryo? Limited data on this point imply that there is a significant margin in the ability of the mouse placenta to meet nutritional demands. Consistent with the known negative correlation between litter size and embryonic weight (McLaren, 1965), superovulation in mice resulted in growth retardation (Evans *et al.,* 1981). Thus, the average relative weights of embryos and placentas in the superovulated group were 77% and 64% of the control values, respectively. Despite a disproportionately smaller placenta, the glucose concentration in embryonic serum was normal (Evans *et al.,* 1981). Analogous observations have been made in rats (Karlsson *et al.,* 1982; Ahokas *et al.,* 1983).

In the case of the IGFs, the size of the placenta is normal in mutants lacking IGF-I or IGF1R, but is compromised in mutants lacking IGF-II (alone or in combination with other mutations; see Fig. 1). Nevertheless, histopathological analysis of *Igf2* null placentas did not reveal any major abnormalities (only the number of glycogen cells was significantly reduced; Lopez *et al.,* 1996). IGF-II overexpression, on the other hand, increases the size of the placenta, as is the case with *R*, *H* and *R/H* phenotypes. Particularly interesting for the question of the embryo/placenta relationship are two double mutants. When both IGF1R and IGF2R are absent, the embryo has normal size, whereas the placenta is large (140%N). This indicates that placental overgrowth does not boost the growth of the embryo beyond normal levels. When both IGF1R and InsR are absent, the placenta is normal, but it cannot rescue from growth retardation the mutant embryo (30%N) that is lacking all of the ligand/receptor signaling interactions. Together these observations also provide genetic evidence that an unknown, placenta-specific receptor (XRp), which is distinct from IGF1R and InsR, mediates the IGF-II growth-promoting role in this organ.

Growth and the cell cycle

The dwarfism of IGF mutants is apparently a consequence of a decrease in tissue cell number (hypoplasia) and not in cell size,

since measurements of cell dimensions in several tissues of *Igf1r* nullizygotes did not reveal differences from wild-type. Thus, a likely mechanistic explanation accounting for the growth deficiency in any of these mutations is an elongation of cell cycle time. This is consistent with the observation that the doubling time in post-crisis cell lines of *Igf1r* null embryonic fibroblasts grown in 10% serum is 2.5-fold longer than in wild-type fibroblasts (109 vs. 43.6 h; Sell *et al.,* 1994). Surprisingly, not only G_1, but all phases of the cell cycle were elongated in the mutant cells, against expectation from earlier cell culture results (see Pardee, 1989). Therefore, a reasonable interpretation is that fewer than normal proliferative events occurring in the mutants during the same gestation period as in wild-type embryos generate fewer cells and result in dwarfism. From this point of view, the IGFs are indispensable efficiency factors maintaining the rate of growth at a normal level.

Measurements of cell sizes in *Igf2r* mutants with increased levels of IGF-II again showed that overgrowth was due to hyperplasia and not hypertrophy (Ludwig *et al.,* 1996). Although the length of the cell cycle was not determined in these mutants or in mutants with *H* or *R/H* phenotypes overexpressing IGF-II (see above), hyperproliferation assessed by BrdU labeling was observed in the liver and heart, but not in the kidney of e13.5 embryos (Eggenschwiler *et al.,* 1997).

Tissue culture experiments also showed that the proliferation of *Hmgi-c* null embryonic fibroblasts is 4-fold lower than wild-type after 4 days in culture, whereas increase in cell death was not noted (Zhou *et al.,* 1995). Surprisingly, embryonic fibroblasts from nullizygotes lacking cyclin D1 did not show any differences from controls in proliferation rates or cell cycle kinetics (Fantl *et al.,* 1995). These mutants exhibit retinopathy, poor acinar cell development in mammary glands during pregnancy and some other abnormalities, but they are viable and fertile in their majority (Fantl *et al.,* 1995; Sicinski *et al.,* 1995). However, the detected hypoproliferation in the retina and in mammary glands leaves unexplained the overall growth retardation of the mutants, which are normal at birth and begin to grow slowly after the tenth postnatal day.

The relationship between growth and the length of the cell cycle is dramatically illustrated by the phenomenon of "size regulation" of experimentally manipulated early mouse embryos. Double embryos are formed by aggregation of two uncompacted 8-cell embryos (morulae), and maintain twice the normal number of cells until ~e5.5 (Buehr and McLaren, 1974; Lewis and Rossant, 1982). However, regulation occurs sometime between e5.5 and e6.5 and the cell number is reduced before gastrulation to that of normal, single-size embryos, apparently by a general (in all tissues) elongation of the cell cycle time (Lewis and Rossant, 1982). Quadruple aggregates (4 morulae) also regulate downward and become single-sized embryos by e6.5. However, in contrast to the double embryos that show signs of regulation after implantation, the ratio of mean sizes of quadruple to control embryos begins dropping from the blastocyst stage (Rands, 1986a). Upward regulation of half embryos produced by destruction of one blastomere at the 2-cell stage occurs between e7.5 and e10.5 (Rands, 1986b) or no earlier than e10.5 according to another report (Tarkowski, 1959). Half embryos develop to term at relatively high frequencies and their postnatal growth does not differ from that of controls (intact embryos transferred to foster mothers; Papaioannou *et al.,* 1989).

Use of a cytotoxic agent (mitomycin C; MMC) provided an example of compensatory growth in the mouse embryo (Snow

and Tam, 1979; see also Snow *et al.,* 1981; Snow, 1986). Pregnant mice at 6.5 or 7 days postcoitum received a single intraperitoneal injection of MMC, an alkylating agent that inhibits DNA synthesis, and embryos were examined at various times thereafter. It was observed that the extraembryonic portion of the conceptus was not affected significantly at e7.5, whereas the size of the embryo proper was dramatically reduced due to extensive random cell death that eliminated ~86% of the normal cell number. Nevertheless, about 90% of the treated embryos were able to develop, despite some asynchrony and delays in organogenesis, reaching an almost normal weight after e13.5 because of a compensatory increase in growth rate. Survivors were brought to term and lacked major anatomical abnormalities (a reduction in the germ cell population, minor skeletal defects, and some other occasional malformations were noted). Postnatally, however, mortality, runting and neurological disorders were observed in a significant fraction of the progeny.

Apparently there is a mechanism sensing whole embryo size, which, if not normal, is regulated. However, the embryo has no means to count cell numbers or cell sizes, because developing tetraploid embryos produced by electrofusion of 2-cell stage embryos (initially a 1-cell "fused" tetraploid) possess half of the normal number of cells that are twice the normal size, but upward or downward regulation does not occur (Kaufman and Webb, 1990; Henery *et al.,* 1992). The mechanism that the embryo uses to adjust its size back to normal after experimental manipulation eludes us, but nobody will be surprised if growth control factors acting systemically are involved.

Growth and other developmental processes

During development, growth by cell proliferation is temporally linked with other processes. While cells are increasing in number, they form ordered spatial arrangements (pattern formation); become specialized by differential expression of characteristic sets of proteins (differentiation); move to generate form and structure of tissues and organs (morphogenesis); and some of them die by apoptosis (programmed cell death). The question then is whether growth is also mechanistically linked with these other developmental processes (collectively referred to here as ODPs, for brevity; I wish a proper term were available). Or, to phrase it differently: is there some cross-talk between the pathways of growth and ODPs for coordination of the developmental program? This is a difficult question that probably does not have a unique answer.

Before I go into any details, let me get rid of a trivial point. In regard to the issue under discussion, phenotypes manifested during the very early steps in embryogenesis are excluded from consideration as uninformative. This includes cases of preimplantation lethality due to defects of the trophectodermal cell lineage resulting, for example, from knockouts of the genes *vav* (Zmuidzinas *et al.,* 1995), *Cdx2* (Chawengsaksophak *et al.,* 1997), *Cdh1* (E-cadherin; Larue *et al.,* 1994; Riethmacher *et al.,* 1995) and *Catna1* (α-E-catenin; Torres *et al.,* 1997). Similarly, the question of cross-talk between pathways cannot be addressed if a mutation is known or presumed to cause a complete block in proliferation immediately after implantation, rather than reduction in the rate of cell divisions, because all ODPs will be canceled by default and the embryo will die. This happens, for example, in nullizygous *Os* mutants (oligosyndactylism; see Pravtecheva and

Wise, 1996, and other references therein) and in knockouts of the genes *Mdm2* (Jones *et al.,* 1995; Montes de Oca Luna *et al.,* 1995), *Ccna2* (cyclin A2; Murphy *et al.,* 1997), and *Fgf4* (Feldman *et al.,* 1995).

Also, I would like to place aside indirect effects. An outstanding example of local hypoproliferation in one region of the embryo affecting morphogenesis in another region for mechanical reasons has been provided by the phenotype of *ct* (curly tail) mutants (Copp *et al.,* 1988a,b). In 60% of ct/*ct* homozygous mice (incomplete penetrance) there is some form of neural tube defect (NTD), most often a curled, coiled or kinked tail, or an open lumbosacral spina bifida, and infrequently exencephaly. The phenotype is a result of delayed neurulation at the posterior neuropore, but the primary defect appears to be a reduced rate of proliferation of gut endoderm and notochord cells, while the growth of the neuroepithelium itself is unaffected. It is thought that enhanced ventral curvature of the embryonic caudal region because of the hindgut hypoproliferation delays the closure of the posterior neuropore by opposing dorsal neurulation movements. Consistent with this model of imbalance in growth rates, food deprivation of pregnant females reduced the incidence of NTD and prevented the occurrence of open spina bifida almost completely. These results were interpreted as indicating that the slowly growing notochord and gut endoderm were affected less than the rapidly growing neuroepithelium in the growth-retarded embryos, thus, restoring balance in growth.

Turning now to growth control genes to examine the question of pathway cross-talk, we find that in the cases of (practically) ateliotic dwarfism resulting from lack of IGF-II or HMGI-C or presumptive excess of Grb10, growth and ODPs appear to be completely dissociable. Hypoplasia has no detrimental effects on ODPs and a small, but otherwise normal organism is brought to term. We can safely hypothesize that the dwarfism of insular elephants was brought about by selectively advantageous mutations in growth control genes of this class (dissociability allowing heterochronic changes has been a pet idea of evolutionists for quite some time; see Gould, 1977). Reciprocally, there are numerous cases of gene knockouts resulting in abnormal patterning of the embryo, but not in growth retardation (I cannot reference them all, and I am at a loss which example to pick). But how general is this picture? As it turns out, it is not.

Embryos nullizygous for *Fgfr1* (Deng *et al.,* 1994; Yamaguchi *et al.,* 1994) are growth retarded, and although they do gastrulate and form allantois, amnion and yolk sac mesoderm, they exhibit abnormal patterning of the primitive streak, truncation or disorganization of posterior embryonic regions and never form somites. As shown by chimeric analysis, signaling through *Fgfr1* is most probably not involved in mitogenesis. Instead, the mutants are defective in the ability of epiblast cells to migrate through the primitive streak (Ciruna *et al.,* 1997), and this apparently impairs proper proliferative events at the sites where the cells should have been deployed. These observations are very much in line with the information (see Growth profile, above) that the cell cycle is modulated early in development to acquire features characteristic of particular cell lineages. The implication is that there is engagement of growth and ODPs in this case, which is likely to be affected by the position of cells responding to cell-cell communication signals and diffusable factors provided by the cells themselves or by their neighbors. In addition to such short-range signaling circuitry, long-range (systemic) controls should also exist, as exemplified by the phenom-

enon of size regulation (see above). A glimpse of engagement can also be gained by considering terminally differentiated cells, like neurons and skeletal muscle cells, which exit the cell cycle when they reach this state. I don't know how this could be happening without some kind of communication between the pathways of growth and differentiation.

The conclusion that growth and ODPs are sometimes engaged and some other times disengaged is vexing. In fact, additional difficulties arise by considering, for example, that the disengagement of processes in the case of complete absence of IGF-II is not phenotypically reciprocal to the consequences of its overexpression, because excess of cells produced by hyperproliferation results in heart dysmorphogenesis. However, even if the assumption is made that the coordination of growth and morphogenesis is perturbed when IGF-II is in excess, there is no clear-cut view that these two processes are mechanistically linked.

Unfortunately, crucial information is still missing, and we cannot figure out what exactly is going on, when it happens, and under which conditions. Despite allegations based on embryo culture experiments about the importance of growth factors for early embryos (see e.g., Harvey *et al.,* 1995), there is no example yet of a knockout of a gene encoding a true mitogen that results in early manifestation of growth retardation (on this score, we cannot extract any useful information from the *Fgf4* and *Egfr* knockouts). We need, however, such an intrinsic impairment in the program of proliferation of embryos shortly after gastrulation (but before the commencement of organogenesis), to see whether the ODPs will be perturbed. If they are perturbed (early engagement), the next question will be whether disengagement can occur later in development. This can be answered with a conditional mutation of the same gene induced late in gestation, by examining whether or not it simulates the absence of IGF-II, for example. Size regulation of half embryos and compensatory growth of embryos treated with MMC do not address the question because the small size is the result of experimental manipulation, not growth retardation (the developmental program remains intact).

Lacking anything better, I have to resort to information from knockouts, which through known or presumed mechanisms, result in growth retardation indirectly. This extrapolation is not exactly legitimate, because there is no reason to assume that growth control itself has been affected in these cases. Instead, the question really becomes: does curtailed proliferation by deprivation of nutrients and/or oxygen also affect ODPs, and if so, when? The criterion I use is whether or not growth retardation is associated with disorganization of the embryo, a term used here loosely to indicate perturbations (in any of the ODPs) that result in histologically dysmorphic embryos. Such cases I consider as clearly distinct from simple delays in reaching a particular developmental milestone, if extensive histopathological findings are absent. As it turns out (at least on the basis of the evidence currently available), if the embryo passes the e8.5 time point mark (early organogenesis) before a growth-retarding mutation has a chance to exert a significant effect, growth and ODPs become dissociable. In contrast, any mutation with an earlier impact results both in growth retardation and disorganization. If not an oversimplification, this generalization is tentative at best. Nevertheless, I present below what some knockouts are saying about timing.

During early embryogenesis and before the establishment of the placenta, the visceral endoderm and later the yolk sac provide nutrition to the embryo that is developing from the epiblast. Disruptions of the genes that encode the transcription factor HNF4 (*Tcf14*; Chen *et al.,* 1994) and huntingtin (*Hdh*; the murine Huntington's disease homolog; Duyao *et al.,* 1995; Nasir *et al.,* 1995; Zeitlin *et al.,* 1995) result in strikingly similar lethal phenotypes characterized by growth retardation and disorganization associated with a high incidence of apoptotic cell death in the epiblast (Table 2B). In both cases, abnormalities are seen at e7.5, but not earlier, and all embryos die by e10.5. The results of chimeric analyses with the *Tcf14* (Duncan *et al.,* 1997) and *Hdh* (Dragatsis *et al.,* 1998) mutations are consistent with the view that the nutritive functions of the visceral endoderm are impaired in both cases, although the pathogenetic mechanisms differ. HNF4 is expressed exclusively in the visceral endoderm, where it becomes involved in the transcription of several genes encoding proteins with transporting functions. Absence of these proteins is the apparent cause of death in mutants lacking HNF4. On the other hand, transporters are available in mutants lacking huntingtin, but nutrients and co-factors are not supplied to the embryo presumably because of defective transporting mechanism(s). Analogous growth retardation and disorganization with similar timing of events characterizes embryos lacking glucose phosphate isomerase (*Gpi-s* mutants; West *et al.,* 1990; Kelly and West, 1996). In this case, nutrients are available, but there is a failure in energy supply, since the glycolytic pathway is inoperative. Overall, early postimplantation lethality is always manifested with growth retardation and disorganization of the embryo (reviewed by Copp, 1995), but this cannot help this discussion because either the genes and/or the mechanisms involved are unknown.

The impact of the knockout of the gene *Thbd* encoding thrombomodulin (Healy *et al.,* 1995; Weiler-Guettler *et al.,* 1996) becomes detectable exactly at the e8.5 time point, when the embryos are only slightly smaller than normal. The mutants are clearly growth-retarded at e9.5 and die by e10.5, but specific pathological findings are not seen. The lethality is probably the consequence of defects in trophectoderm and/or parietal endoderm, and this is consistent with the observation that the mutants are able to reach *in vitro* a more advanced developmental stage than *in vivo*.

The knockouts of the genes *Nkx2-5*, *Tal1*, and *Lmo2* result in remarkable phenotypes of embryos that pass the e8.5 time point unharmed and then become much smaller than their normal littermates without being disorganized or dysmorphic. Embryos lacking the function of the homeobox gene *Nkx2-5* die at ~e11.0 because of defects in heart looping morphogenesis that results in circulatory insufficiency (Lyons *et al.,* 1995). At the time of death, these mutants are tiny embryos (something like 10%N in size) that have stopped developing at the stage of 20 somites. However, they are not malformed. The *Tal1* gene encodes a transcription factor that is essential for primitive erythropoiesis in the blood islands of the yolk sac. *Tal1* nullizygotes (Robb *et al.,* 1995; Shivdasani *et al.,* 1995) are bloodless and die of this anemia by e11.0. Growth retardation is first noticeable at e8.75, but, except for pallor and dilated pericardial sac, the miniature mutants examined at e9.5-e10.0 do not exhibit any dramatic malformations and are developmentally progressed. *Lmo2* nullizygotes also die by e11.5 because of failure of yolk sac erythropoiesis (Warren *et al.,* 1994). At e9.75, they are about half the normal size, but neurulation, turning and development of up to 20 somites have proceeded normally.

When the maturation of the vascular network of the yolk sac (angiogenesis) is impaired, as in *Arnt* nullizygotes (lacking the transcription factor arylhydrocarbon-receptor nuclear translocator; Maltepe *et al.,* 1997), the embryos are not viable past e10.5, whereas they are normal at e8.5. At e9.5 they are smaller than wild-type and developmentally delayed, but individual organs are morphologically normal. Analogous conclusions can be drawn from the phenotypes of the *Flk1* knockout (lack of yolk sac blood islands and vessels; Shalaby *et al.,* 1995) and the *Flt1* knockout (disorganization of vascular endothelium; Fong *et al.,* 1995). Absence of vascular endothelial growth factor (a ligand of the Flk1 and Flt1 receptors) in *Vegf* mutants results in a severe phenotype that provides an outstanding example of haploinsufficiency (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). Angiogenesis and blood-island formation are impaired in heterozygous mutants resulting in several developmental abnormalities and delays (death occurs between e11.0 and e12.0). However, growth retardation is relatively mild and can be explained by the significant increase in apoptosis that is observed. When the integrity of vascular endothelium is affected, as in *Tie1* and *Tek* knockout mutants (Dumont *et al.,* 1994; Sato *et al.,* 1995; Partanen *et al.,* 1996), embryos die because of hemorrhage, but they are neither malformed nor growth-retarded, although some organs (the heart, for example) are smaller than normal. Similarly, in contrast to primitive hematopoiesis, anemia from defects in definitive hematopoiesis in the fetal liver results in lethality, but not in growth retardation or malformations. This is exemplified by nullizygotes for *c-myb* (death at ~15.5; Mucenski *et al.,* 1991). A similar phenotype is observed when the definitive hematopoiesis is affected more severely than the yolk-sac hematopoiesis, as in the mutants lacking *GATA2* (death by e12.0; Tsai *et al.,* 1994), erythropoietin or erythopoietin receptor (death between e13.0 and e15.0; Wu *et al.,* 1995; Lin *et al.,* 1996). If the number of erythrocytes is reduced by half, as is the case with the knockout of the *Atrc1* gene (encoding an amino acid transporter; Perkins *et al.,* 1997), all mutants die on p1 and they are 77%N in size, but this moderate growth retardation is not accompanied by other overt abnormalities.

Punch lines

1. Growth is a developmental continuum from implantation to some time point in adult life, when a determinate body size characteristic for the particular mammalian organism is attained.
2. Body size is the outcome of the rate and duration of growth and is correlated with physiological parameters.
3. From gastrulation onward, the organism increases in size towards a limit with practically continual decrease in growth rate.
4. Growth is attained predominantly by cell proliferation that is controlled by genes of the organism itself.
5. Growth control genes are members of pathways that are either promoting or inhibiting growth by systemic or local action.
6. Growth control genes affect, directly or indirectly, the duration of the cell cycle.
7. Among the growth control genes are those participating in some growth factor or hormonal signaling pathways. In contrast to embryonic growth, when hormones participate less, if at all, post-natal growth is effected by both classes of signaling systems.
8. Before organogenesis, the pathways of growth and other developmental processes (patterning, differentiation and morphogenesis) appear to interact, but the details and principles of this putative cross-talk are unknown. After the commencement of organogenesis, there are some examples indicating that a complete disengagement of the pathway of growth from the pathways of other developmental process is possible.

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