Growth and the distal tip of mouse chromosome 7

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Summary

This review concerns the general problem of understanding growth control in the whole organism, starting with a saltatory change in size generated by a chromosome translocation or a mutation in a single gene. In particular, changes in insulin-like growth factor-II levels, by genetic and embryological manipulation, have major effects on wet weight size, but the intermediary events that link these levels to this measure of growth are uncertain. Thus it is currently impossible to eliminate any of the intermediary candidate processes that have been observed in model systems, including changed rates of apoptosis, cell multiplication, protein synthesis, capillary permeability and fluid transport.

1. Introduction

This review is a celebration of MRC Harwell's formidable analysis of chromosome differentiation determined by genitive sex. For brevity, it concentrates on the apparently simple observation that two paternal copies of mouse distal chromosome 7 are lethal before embryonic day 11·5 (E 11·5), while two copies of this region from the mother reduce the size of the placenta and fetus from E 13·5 onwards in development (reviewed by Cattanach, 1986; Searle & Beechey, 1990). The paternal duplication could be part rescued in chimeras, where it caused fetal overgrowth (Ferguson-Smith *et al.*, 1991). It is difficult to think of any other chromosome observations that have so clearly exposed the difficulty in translating a knowledge of genes into an understanding of the phenotype. Further, it exposes the general problem of fine-dissecting the functions of an extensive non-coding region of the genome that regulates gene imprinting.

(i) *The 'phenotype gap'*

It is frequently said that understanding of the phenotype has not kept pace with that of the genome.

This perception of a 'phenotype gap' is beguiling and misleading. A complete genome analysis of an organism would contain much more than the base sequence of the genetic material and a description of its protein products: it would also provide an encyclopaedia of all the interactions that can occur between the genes via their RNA and protein products binding back to the genetic material (e.g. transcription factor interactions with DNA and chromatin conformation) and their RNA and protein products regulating the production and export of mature mRNA from the nucleus. It would predict all the likely consequences of mutations, concentrating in the first instance on all the frequent polymorphisms that are maintained in wild populations of the species.

A complete phenotype analysis might start with a compendium of all the interactions that could occur between the protein products, such as the 18 million pair-wise interactions between the 6000 genes of yeast (Fields, 1997), and the extent to which these interactions are limited by subcellular site and developmental anatomy, such as the restriction of gene expression to particular cell types during development and the interplay of the products of these tissues that move from one place to another in the body (endocrinology). It is easy to see that gaps, potholes, and sloughs of despond are present at every level of current understanding of mammals and the above wish-list of knowledge is certainly not complete. For

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instance it does not include interplay with other individuals, other species, or the environment.

This review combines information from several biomedical disciplines to identify the big holes of ignorance that pit the road to understanding transformation of genetic information at the distal tip of mouse chromosome 7 into the size of the whole organism as registered by cell number. The first level is based on physical and chemical analysis of the chromosome and the alterations of phenotype following genetic change. The second level concerns cell biology, reproductive physiology, endocrinology and biochemistry of growth and metabolism, dipping into protein and coding nucleic acid sequence when this information can help. The population genetics of size in mammals is not discussed.

(ii) *Description of growth*

It is curious that most descriptions of mouse and human growth only record wet weight. These records are distanced from cell number, cell population kinetics and gene expression by the fluid content of the body, the material content of the alimentary canal, and the weight of extracellular material, such as external hair and internal teeth, bone and other extracellular matrices. For instance, 90 % of the wet weight of the conceptus bounded by the visceral yolk sac at E 16.5 is water ($n = 62$; unpublished study). Consequently any genetic analysis of wet weight growth may say little about cell population kinetics and a great deal more about body fluid content mediated by placenta and gut uptake, excretion by the placenta and kidneys, and thirst and suckling after birth.

(iii) *Prenatal and postnatal growth in mice*

Prenatal genetic and environmental factors that influence the prenatal wet weight growth of mice have been carefully studied and reviewed (e.g. Snow *et al.*, 1981). A comprehensive survey sets out the changing kinetics from early development to adulthood (Goedbloed, 1972, 1974, 1975), but the integrated control of proportional growth summed up in these kinetics is not understood until mice are 3 weeks old. For instance, it is not known whether there is a systemically distributed hormone that regulates the local production of insulin-like growth factors (IGFs). After weaning, the absence of pituitary growth hormone (GH) slows down weight gain to such an extent that this hormone can be regarded as the master controller of cell number increase, in part exerting its influence by the intermediary, insulin-like growth factor-I (IGF-I; reviewed by Daughaday, 1992). It has therefore been rational to search for the anatomical source of an equivalent commanding hormone in the conceptus and newborn, but no site

has been found (reviewed by Snow *et al.*, 1981). This failure may indicate no more than that insufficiently subtle organ ablations and gene deletions have been conducted on the conceptus in the uterus. Certainly a mechanism for regulating fetal size must exist because aggregation chimeras are not abnormally large at birth despite their origin from several complete pre-implantation conceptuses. The placenta is a necessary condition for rapid prenatal growth but there is no simple relationship between placental and fetal wet weight. For instance, the placenta may be of normal weight and the fetus 60 % of normal, or the placenta may grow excessively large with no proportionate increase in fetal weight (McLaren, 1965; Liu *et al.*, 1993; Lau *et al.*, 1994).

A very important determinant of newborn size is the length of pregnancy. For instance, mothers of F1 progeny characteristically give birth nearly a day earlier than mothers of the same strain with inbred offspring (McLaren & Mitchie, 1963). The consequence is that F1 size heterosis is often underestimated. Another unexplained aspect of pregnancy effects emerged from embryo transfer experiments using mice selected for large and small body size. The two classes had similar cell numbers at the blastocyst stage but embryo weights diverged by 15 % at E 11·0 and E 16·5 and the difference became progressively greater, reaching 80–84 % by 6 weeks after birth. The difference at E 16 was maintained when the conceptuses were transferred to a random-bred strain uterus but the small conceptuses became larger than the large body weight line when they were transferred to a uterus that was 1 d behind in pseudopregnancy (Aitken *et al.*, 1977). The authors suggest that the effect is not simply due to extra cell divisions that might occur before implantation in the asynchronous transfers, but rather that the state of development or maturity of the implanting blastocysts gave them a head start. Apart from these interactions with the reproductive biology of the mother, the size of any one conceptus is dependent on the others. The following interactions with other offspring occur: total litter size (Gates, 1924), systemic effects between conceptuses in different horns (McLaren, 1965), and uterine position effects that are particularly apparent when there are eight or more conceptuses in a single horn (Healy *et al.*, 1960). Further, the growth phenotype of one genotype can be altered by that of another genotype in the same litter: inbred foetuses are smaller than usual if F1 foetuses are present in the same pregnancy (Musialek, 1965). This observation suggests that genetically small conceptuses may be further diminished by the presence of normal conceptuses in the same uterine horn.

The postnatal growth of the young is also altered by total litter size and the litter size also influences the effect of one pup genotype on another (e.g. Musialek, 1967; Fisher *et al.*, unpublished study). The physio-

logical basis of all these pre- and postnatal variables is uncertain but these studies warn that the expression of any genetic change in the conceptus depends on a tangle of interactions with many features of the uterine and lactational environment. Further, observations on very large numbers of litters are required to factor out all these variables and this degree of rigour has rarely been achieved.

2. Genome analysis

It is fortunate that chromosome abnormalities and mutations at distal chromosome 7 have saltatory effects on mouse size. Genome analysis of the distal tip of mouse chromosome 7 has revealed a sequence stretch decorated by genes that are more abundantly expressed from either the maternal or the paternal chromosome (reviewed by Bartolomei & Tilghman, 1997). Inactivating mutations of several of these genes has shown that they normally have major effects on growth. The first analysed mutation was in the insulin-like growth factor-II gene (IGF-II gene, *Igf2*), found to be expressed only from the paternal chromosome in most tissues and reducing birth weight by about 40 % (DeChiara *et al.*, 1990). The paternal activity of this gene in liver and gut was in turn regulated by the nearby *H19* enhancer and paternal deletion of the enhancer caused a 30 % reduction in weight (Leighton *et al.*, 1995*b*). A simple interpretation is that the enhancer's action on *Igf2* is responsible for 75 % of IGF-II's promotion of wet weight growth. On the maternal chromosome, *Igf2* transcription was increased by removing transcribed *H19* and adjoining sequences (Leighton *et al.*, 1995*a*) or by moving the enhancer closer to *Igf2* (Webber *et al.*, 1998): in the former case mouse size was increased by about 15 %. All these observations were consistent with a model in which either *Igf2* or *H19* had exclusive use of this enhancer on a particular chromosome with an undiscovered feature providing the enhancer's bias for *H19* on the maternal chromosome: prominent candidate regions are believed to exist in 5′ regulatory sequences that show differential methylation.

Still at the genome level, there are interactions of general and more limited transcription factors with the *Igf2* promoters, particularly by the WT1 protein (e.g. Rauscher *et al.*, 1990; Drummond *et al.*, 1992; Ward *et al.*, 1995). In addition there is regulation at the level of translation, and only a proportion of *Igf2* mRNA is translated in cell culture and during mouse development (Newell *et al.*, 1994; Nielsen *et al.*, 1990, 1995; Sussenbach *et al.*, 1991). The phenotype of the mice lacking IGF-II (DeChiara *et al.*, 1990) could be simply explained if there were no regulatory steps between *Igf2* transcription and growth. In reality, there are a hierarchy of systems for controlling the ultimate products of these transcripts at both the genotype and phenotype level.

3. Bio-availability of IGF-II protein

(i) *Growth promotion by IGF-II*

The wet weight phenotype of mouse mutants lacking IGF-II is remarkable: their growth rate suddenly diverges from wild-type. At E 10·5 the embryos have a normal weight, which abruptly changes to 70 % of normal at E 11 followed by a slight further decline to 60 % at E 18. The combined fetal and maternal components of the placenta change later, with the first reduction to 80 % at E 13·5. There is no obvious change in cell packing in histological sections and the sudden reduction in cell number suggested by these observations remains mysterious (DeChiara *et al.*, 1991; Baker *et al.*, 1993).

(ii) *Systemic or local effects?*

In adults, it has proved difficult to demonstrate any major effect of excess circulating IGF-II either on total body wet weight after birth or on the DNA content of organs that do not express extra IGF-II (Ren-Qui *et al.*, 1993 transgenic mice: DaCosta *et al.*, 1994; Rogler *et al.*, 1994; van Buul-Offers *et al.*, 1995; infusion to hypophysectomized rats: Glasscock *et al.*, 1992; but see Shaar *et al.*, 1989). Low levels of circulating IGF-II are found in adult mice (DaCosta *et al.*, 1994; van Buul-Offers *et al.*, 1995). These levels may fulfil any unidentified systemic requirements for this hormone. The absence of major systemic effects contrasts with the short-range actions of extra IGF-II: local production of IGF-II: leads to more cells in many places where it is expressed at high levels, such as the skin, alimentary canal and uterus (Ward *et al.*, 1994) and thymus (van Buul-Offers *et al.*, 1995).

It is therefore unclear whether systemic or local IGF-II levels alter the growth rate of the *Igf2* mutants in the second half of pregnancy. IGF-II mRNA and protein are so widely distributed in the fetus that the hormone may simply leak into the circulation. However, several observations support the view that circulating IGF-II influences growth in the uterus. First, when embryonic cells with the capacity to over-express IGF-II in later development are introduced into normal mouse blastocysts then the chimeras become excessively large fetuses (Ferguson-Smith *et al.*, 1991; Sun *et al.*, 1997); in the enlarged conceptuses, the growth of normal cells keeps up with those over-expressing IGF-II and the excess IGF-II must be acting at a distance or via intermediaries. Secondly, extracellular IGF-II excess can slightly increase wet weight when mini-pumped around fetal paws transplanted to the kidney capsule of adult rats (Liu *et al.*, 1989). If systemic IGF-II levels influence the growth

of the conceptus, then other endocrinological side-effects must be considered. For instance, does the absence of circulating levels of IGF-II lead to compensatory changes in IGF-I levels? In mice, IGF-I plasma levels have not been altered by elevated IGF-II in many situations (Ren-Qui et al., 1993; DaCosta et al., 1994; Rogler et al., 1994; van Buul-Offers et al., 1995), but reciprocity between the levels of these two hormones has been described in rats, pigs and sheep (reviewed by Owens et al., 1990; Gluckman & Ambler, 1993).

(iii) *Actions on the cell cycle*

Currently there is no study of the cellular kinetics in mice lacking IGF-II ($Igf2^{+m/-p}$). However, when IGF-II levels are increased in the mouse conceptus by genetic manipulations then the 5′-bromo-2′-deoxy-uridine (BrdU) labelling index in the E 13·5 liver and heart increases (Eggenschwiler et al., 1998) and more cells are found in the heart, liver and intestinal epithelium (Ludwig et al., 1996). Cell culture studies identify additional cell cycle events that might be altered by raised IGF-II in the whole organism. For instance, IGF-II increases the rate of amino acid uptake in cultured human myoblasts and fibroblasts (Hill et al., 1986) and might therefore overcome any cell cycle arrest which is dependent on a supply of essential amino acids (reviewed by Pardee, 1989). Other possibilities include a decreased rate of cell death (e.g. mouse and human embryonal carcinoma cells: Biddle et al., 1988; Granerus et al., 1995; fibroblasts: Harrington et al., 1994; or increased bio-availability of IGF-I: Conover et al., 1994). Amongst these candidates only an increase in cell survival is supported by *in vivo* studies and these involve tumour formation in the developing pancreas (Christofori & Hanahan, 1994; Christofori et al., 1994).

(iv) *Endocrinology in the conceptus*

It is probable that IGF-II also has physiological growth-promoting actions in the whole organism and that an indicator of this physiological action is tissue fluid content. The association between high levels of both IGFs and unusually high wet:dry weight or wet weight:DNA content ratios has been observed in a variety of situations. During the latter part of pregnancy, the wet:dry weight ratio is greatly increased in the mouse embryo when IGF-II levels are elevated following mutations in the insulin-like growth factor-II/mannose 6-phosphate receptor gene (*Igf2r*; Ludwig et al., 1996; but see Lau et al., 1994; Wang et al., 1994). The embryo also has high tissue fluid and doubled exocoelomic plus amniotic fluid volume in mice with the T^{hp} deletion, which includes the *Igf2r* gene (Johnson, 1971; Babiariz et al., 1988). In adult

mice carrying *Igf2* transgenes, local IGF-II provokes excess tissue fluid with high ratios of wet weight:DNA content in the alimentary canal, skin and uterus where the transgene is expressed (Ward et al., 1994). These actions are not confined to IGF-II, because infusion of IGF-I into humans leads to a mild systemic oedema that is accompanied by increased movement of substances from the circulation to extravascular space in both the skin and the retina (Franzeck et al., 1995; Hussain et al., 1995).

These observations raise the possibility that increased tissue fluid is an indicator of increased transport of nutrients from the circulation to the rapidly growing conceptus. In such a situation, the effect of IGF-II on cell multiplication might be mediated by an action on vascular permeability with the consequent increase in amino acid and glucose supply. The consistent observation that individuals weigh less as the number of pups in the litter increases suggests that the supply of something limits their growth rate. Towards the end of pregnancy large mouse foetuses are surrounded by less fluid in the extravascular spaces bounded by the fetal membranes (McLaren et al., 1976). Perhaps extra fluid and nutrient supplies have increased fetal size earlier in development.

The conclusion from this section is that while mice with no IGF-II have low wet weight and those with excess IGF-II have high wet weight, there is no compelling reason to favour any one of this hormone's actions as the rate-limiting step for growth.

4. Modifiers of IGF-II growth phenotype

The growth phenotype of mutants with too little or too much IGF-II depends on the activity of other genes. These phenotypic interactions involve at least four receptors and many binding proteins. The insulin and type 1 IGF receptor (alias IGF-I receptor) are dimeric tyrosine kinase receptors and the latter is required for many of the growth-promoting actions of IGF-II, insulin and IGF-I (mouse gene *Igf1r*). The monomeric type 2 IGF receptor regulates the plasma levels of IGF-II, amongst other things (aliases IGF-II/mannose-6-phosphate receptor, IGF-II/M6PR, IGF2R, mouse gene *Igf2r*). Glypican is a cell surface glycoprotein and human mutants have an overgrowth syndrome. Glypican also binds IGF-II and there is therefore a presumption that it normally down-regulates IGF-II levels.

The phenotypic interactions of these IGF-II modifiers are displayed when inactivating mutations in two or three players are combined in one mouse. Thus the foetal overgrowth phenotype of mice lacking the type 2 receptor and high IGF-II levels is prevented by combining the mutants ($Igf2r^{-m/+p}$) with mice lacking IGF-II ($Igf2^{+m/-p}$) or with mice that lack the receptor

required for IGF-II growth effects ($Igf1r^{-m/-p}$; Ludwig *et al.*, 1996). In the latter case, the weight of the fetus is normal and the most likely explanation is that fetal growth is maintained by interactions between the excess IGF-II and the insulin receptor (Louvi *et al.*, 1997).

Less information is available about the modification of the IGF-II phenotypes by the IGF binding proteins (IGFBPs) in life. There is extensive information about IGF binding protein distribution and changed levels in a variety of circumstances (reviewed by Rajaram *et al.*, 1997). In particular, IGFBP1 is 100–500 times more abundant in human amniotic fluid than serum. The problem of untangling their function is presented by the response of cell cultures: in some systems they inhibit IGF-driven proliferation while in others they promote it. The best evidence of one *in vivo* function comes from studies on the mammary gland. In this gland, transgenic over-expression of either IGF-I of IGF-II causes duct hypertrophy and a failure of normal involution of the mammary epithelium between pregnancies (Hadsell *et al.*, 1996; Neuenschwander *et al.*, 1996; Fisher *et al.*, unpublished observations). The transgenic expression of IGFBP3 at the same site reproduces this phenotype in an otherwise normal mouse and the suggestion is that local IGFBPs concentrate the action of circulating IGFs (Neuenschwander *et al.*, 1996).

The conclusion from this section is that there are many proteins that can potentially influence the IGF-II growth phenotype. Demonstrably during mouse development, IGF2R regulates IGF-II levels and the type 1 IGF receptor and the insulin receptor are required for IGF-II promotion of wet weight. The regular functions of the glypican receptor and the binding proteins *in vivo* remain to be established.

5. Towards an explanation of the reciprocal chromosome translocation

This is a salutary rehearsal of the events that intervene between the transcription of a single gene and the flowering of the growth phenotype. It can be said to start the analysis of a reciprocal translocation at the distal tip of mouse chromosome 7. In doing so, it has omitted any reference to other imprinted genes in the same stretch of the chromosome, namely $p57^{Kip2}$, *Ins2* and *Mash2*. Of these, *Mash2* is required for normal trophoblast development (Guillemot *et al.*, 1995); it is expressed from the maternal chromosome and its absence from paternal duplications might account for their lethal phenotype. Surprisingly the cyclin-dependent kinase inhibitor $p57^{Kip2}$ has no major effect on general body growth during development, which proceeds to term (Yan *et al.*, 1997; Zhang *et al.*, 1997). It is expressed from the maternal chromosome and its absence from paternal duplications of distal chro-

mosome 7 is unlikely to explain the translocation phenotype. At the genome level, the mechanistic interaction between this cluster of interacting genes is still uncertain (Leighton *et al.*, 1995*b*). At the phenotype level, the fascinating interactions between all the proteins that bind to the products of this region are sufficiently well analysed for us to know that they do not have trivial effects on the growth phenotype.

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