THE PHYSIOLOGY AND MOLECULAR BIOLOGY OF MUSCLE GROWTH AND ITS REGULATION IN DIVERGENTLY SELECTED LINES

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SUMMARY

A detailed knowledge of the genetic control of growth is required firstly to make use of advances in transgenic biology now that genetic manipulation of pigs, sheep, cattle and poultry is possible, and secondly to develop more efficient breeding schemes. This paper briefly reviews the structure and development of muscle and its regulation by a number of growth factors, followed by our recent data on the somatotrophic axis, fibroblast growth factor and transforming growth factor-ß in lines of chickens divergently selected for growth. The results suggest that understanding the relationship between genotype and phenotype by investigation of the cellular and molecular biology of divergently selected animals is a good strategy to provide the required information.

INTRODUCTION

Inherited improvement in the rate of growth, in particular that of muscle, has been the aim of animal breeders for many years. Widespread application of quantitative genetic techniques has allowed considerable progress to be made although selective breeding requires large numbers of animals and is generally based on end-point parameters such as weight-for-age, muscle size and conformation and food conversion efficiency. This in part is a consequence of the fact that little is known about the biological basis of the genetic control of muscle growth or even total body growth. There is a lack of information on how the physiology and biochemistry of fast- and slow-growing animals is different and we are unable to indicate which genes are the most appropriate to manipulate in order to obtain the desired response. Numerous processes occur during embryonic and neonatal development which influence the number, type and size of muscle fibres and an alteration in the control of these could potentially result in beneficial changes in terms of animal production. Increased knowledge of the control of myogenesis is therefore of great significance if regulation muscle growth is to be achieved, possibly by incorporation of information into breeding schemes (Blair et al. 1990). More importantly, detailed knowledge of the genetic control of growth is required to make use advances in transgenic biology now that genetic manipulation of pigs, sheep, cattle and poultry is possible. This paper briefly reviews the current views on the control of muscle growth and some of our recent work on lines of chickens which have been selected for differences in growth rate. The first section outlines the structure and the complex development of muscle. The second indicates the regulatory factors thought to be important in muscle development. The final section describes some of our recent results using analysis of the cell and molecular biology of these factors in lines of chicken divergently selected for growth over many generations and illustrates the importance of this strategy in identification of 'trait-genes' and in determining the basic regulation of muscle development.

1. MUSCLE STRUCTURE AND DEVELOPMENT

Structure

Skeletal muscle has a unique and highly ordered structure in order to perform its specialised functions. Individual muscles are composed of a variable number of multinucleate fibres that are parallel to each other. Fibres can be anything from 10 to 100 μm in diameter and several centimetres in length. The characteristic contractile apparatus or myofibril consists of a contractile thread consisting of 12 - 14 proteins arranged in a highly organized structure. These proteins make up about 55% of the total muscle proteins with actin and myosin the most abundant. Several different isozymes of myosin are expressed in muscle fibres during different stages of development and the presence of a particular isoform tends to be characteristic for that stage of development and fibre type (Pierobon-Bormioli et al. 1981). The multinucleate nature of the muscle fibre is also a characteristic and it has been demonstrated that nuclei within the muscle fibre do not undergo division (see Goldspink, 1977).

Specific muscle fibre types have evolved to perform specific functions and have been characterised according to a number of different schemes which generally have three categories (see Peter et al. .1972). There are significant differences in morphology, biochemistry, histochemistry and size of different fibre types. In mice, different fibre type ratios exist between sexes and genetic lines (Vaughan et al. 1974) and fibre composition also changes during muscle development and during experimental manipulation such as compensatory hypertrophy (see Swatland, 1984). The biological basis of these changes is not understood but factors that affect fibre type and distribution within a muscle could alter ultimate muscle size or growth rate and should be considered.

Development

The origin of striated skeletal muscles is from myoblast cells. These are derived from presumptive myoblasts (Boyd, 1960) of mesodermal origin which are difficult to distinguish morphologically from other stem cells but are derived from the somites and the limb buds (see Swatland, 1984; Dayton and Hathaway, 1989). These myogenic cells proliferate, differentiate and fuse to form multinucleate myotubes which synthesize muscle specific contractile proteins and ultimately are assembled into myofibrils. The nuclei which have been incorporated into a myotube are no longer capable of cell division and therefore the fusion of proliferating, mononucleated myoblasts to form non-proliferating multinucleate myotubes is the terminal step in muscle differentiation. Once this is completed during embryogenesis, the number of muscle fibres is fixed and the nuclei within the fibres are unable to divide. However, the DNA content of muscle fibres increases during postnatal growth by up to eight-fold (Winick and Noble, 1966) and is closely related to the rate and extent of muscle growth (Moss, 1968; Powell and Aberle, 1975; Harbison et al. 1976; Swatland, 1977;Trenkle et al. 1978). This DNA is derived from myogenic cells called satellite cells found between the plasmalemma and basal lamina of each muscle fibre (Mauro, 1961; Campion, 1984). These satellite cells proliferate and fuse with existing muscle fibres to provide nuclei for postnatal muscle growth (Moss and Leblond, 1970) but their origin and regulation of proliferation and fusion is poorly understood.

The mechanism and regulation of the transition from myoblast to myotube has been studied extensively and there have been three hypotheses put forward. The

mesodermal cells of the somites and limb buds undergo frequent mitosis with a peak of activity for example in the chick embryo at 5 days of incubation (Bloom and Buss, 1968). The cells are locked in the mitotic cycle (Bischoff, 1970) and the escape from this to a post mitotic myoblast is irreversible. The number of times that a clone of cells remain in this cycle is important in that one extra cycle by all the cells could potentially double the number of myoblasts and lead to extra fibres. Holtzer, (1978) suggested that differentiation occurs via a 'quantal mitosis' in which the daughter cells possess a more differentiated phenotype and that the final quantal mitosis results in production of myoblasts irreversibly withdrawn from the cell cycle and capable of fusing with others to produce a myotube. It has been suggested (Quinn et al. 1985) that there are a fixed number of cell divisions during the transition from the stem cell to the non-proliferating, fusion-capable myoblast. The second hypothesis (Bischoff, 1970; Buckley and Konigsberg, 1977) suggested that the differentiating muscle cells spend an increasingly longer time in the G_1 phase of the cell cycle where fusion occurs. The longer the time spent in this phase the greater the probability of fusion. The third model combines aspects of both the previous ones and suggests that early in the G_1 phase myoblasts either withdraw from the cell cycle and become committed to fusion, or remain in the cell cycle and proliferate. The process is dependent on the presence or absence of a number of growth factors (Nadal-Ginard, 1978).

Two morphologically distinct populations of fibres are formed. Initial fusion produces primary fibres which are still surrounded by myoblasts which subsequently fuse and give rise to another population; the secondary fibres. This continues until most myoblasts have been incorporated into myofibres but a significant proportion will remain as mononucleate cells and make up the satellite cell population. The origin of these fibres is from different myogenic cell lineages proliferating and differentiating at different times of development (Miller et al. 1985; Miller and Stockdale, 1986a, b; Stockdale et al. 1986; Narusawa et al. 1987). Two general myogenic cell types appear in these lineages corresponding to the formation of primary and secondary fibres and there is further division into subtypes based on the formation of distinct myotubes expressing a particular myosin heavy chain isozyme (Stockdale and Miller, 1987). A further myogenic lineage corresponding to the satellite cells is also possible since there are a number of features in which they differ from embryonic myoblasts (see Dayton and Hathaway, 1989).

The final stage is fusion to form multinucleate cells giving rise to muscle fibres. Fusion is preceded by a period of cell-cell recognition and adhesion and appears to be initiated at a single site between two myoblasts. A pore is formed to link adjacent cells which increases in size and leaves no trace of the intervening membranes accompanied by extensive remodelling of the cytoskeleton (Lipton and Konigsberg, 1972; Fulton et al. 1981). There is extensive literature on the fusion process which is reviewed by Wakelam, (1985). Further muscle growth is essentially the accumulation of muscle protein. This is a balance between the rate of protein synthesis and protein degradation. Again there is a substantial body of literature (see Goll et al. 1989).

Clearly, myogenesis is complex and is far from being fully understood. The factors controlling myoblast determination from the stem cell, development of myogenic cell lineage, myoblast and satellite cell proliferation, fusion, development of fibre number and type and also muscle protein accumulation are potentially candidates for manipulation since they ultimately regulate muscle mass, efficiency of muscle growth and even the organoleptic quality of the final meat product. The challenge to cell and molecular biologists is to clearly define the control of this process to allow the full potential of transgenic biology to make an impact in terms

of animal protein production. The route to this could also provide opportunities for improvements in present selection regimes (Blair et al. 1990).

2. Regulation of muscle growth

A number of hormones and growth factors have been demonstrated to have major effects on the programme of events leading to muscle development. A series of papers have recently been published on the expression of a number of genes such as myd, MyoD and myogenin which are thought to play a central role in the determination of myogenic cells from the stem cell population (Pinney et al. 1988), although it may be that these gene products are not the primary switches and that their expression may be influenced by some of the growth factors discussed below (Vaidya et al. 1989). It is premature to review them here although this is likely to be a fruitful area in the near future. We will concentrate instead on recent evidence suggesting a major role in muscle growth and development for three families of growth factors; the insulin-like growth factors (IGF-I and IGF-II), fibroblast growth factor (FGF) and the transforming growth factor-ß family (TGF-ß).

Growth hormone

Discussion of the regulation of muscle growth is not complete without reference to growth hormone (GH). As indicated by Blair et al. (1990), the key role of the somatotrophic axis in regulating growth has been known for many years (see Kostyo and Reagan, 1976). Impaired growth resulting from hypophysectomy can be substantially reversed by treatment with GH. Administration of exogenous GH for example, to pigs markedly increases growth rate, improves feed efficiency, increases muscle growth and inhibits adipose tissue accretion (Etherton, 1989). Similar effects have been seen in some transgenic pigs which have GH genes inserted (Pursel et al. 1989) and in the classical experiments in transgenic mice (Palmiter et al. 1983). The mechanism by which GH has effects on muscle growth has not yet been established. Despite repeated attempts (see Florini, 1987) there has been only a single report of a well controlled experiment in which a direct effect of GH has been observed in muscle cells (Nixon and Green, 1984). It is likely that most effects of GH on muscle growth are mediated by the insulin-like growth factors although whether this is by stimulation of circulating IGFs or by stimulation of their action by an autocrine or paracrine mechanism is still a matter of debate.

Insulin-like growth factors

The IGFs are clearly involved in most aspects of muscle development. IGF-I and IGF-II stimulate proliferation (Ballard et al. 1986; Hill et al. 1986; Ewton et al. 1987), amino acid uptake (Hill et al. 1986; Ewton et al. 1987), differentiation (Ewton and Florini, 1981; Schmid et al. 1983; Ewton et al. 1987), protein synthesis (Ballard et al. 1986; Harper et al. 1987) and decrease protein degradation (Ewton et al. 1987) in a variety of cultured myogenic cells and myotubes. Although most cells have both a type I and type II IGF receptor it is likely that the effects of IGF peptides on muscle cells are via the type I receptor (Florini, 1987), despite the fact that muscle cells may contain up to 15-fold higher type II IGF receptors (Beguinot et al. 1985), particularly since the equivalent receptor in the chicken does not bind IGF-II (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989; Duclos and Goddard, 1990). Recently, the expression of IGF peptides, the type I IGF receptor and the secretion of an IGF-I binding protein, which are all components critical to IGF action, have been

demonstrated during fusion of a muscle cell line in a differentiation-dependent manner indicating an autocrine role for IGF peptides in muscle growth (Tollefson et al. 1989). This is supported by data from in situ hybridization and immunocytochemistry of developing muscle which show that the components of the IGF system are expressed and produced at the correct stage of development and are consistent with an autocrine or paracrine function (Han et al. 1987). Relatively little is known of the mechanism of action. The type I receptor is a ligand-activated tyrosine kinase likely to be involved in intracellular signalling. Ong et al. (1987) have reported that c-fos may be involved in the signal transduction mechanism and also IGF-I induced elevation of polyamines is essential to myoblast differentiation (Stoscheck et al. 1982; Ewton et al. 1984).

Fibroblast growth factor

The fibroblast growth factor family also appear to play an important role in the control of muscle development. Stimulation of myoblast proliferation by FGF was initially reported by Godsparowicz et al. (1975) and confirmed by a number of authors (Olwin and Hauschka, 1986; Allen et al. 1985; Bischoff, 1986) although surprisingly, the L6 myoblast cell line which has been used extensively in muscle research does not respond to FGF (Ewton et al. 1987). In addition, FGF is a potent inhibitor of differentiation (Linkhart et al. 1980; Allen et al. 1985; Kardami et al. 1985a,b; Bischoff, 1986). Recent papers have demonstrated that the levels of FGF present in the developing chick limb bud are sufficient to inhibit differentiation of myoblasts and that FGF delays the onset of differentiation in precursor cells in limb buds (Seed et al. 1988; Seed and Hauschka, 1988). FGF is stored in fibre extracellular matrix in the rat and appears to regulate satellite cell function during work-induced muscle hypertrophy (Yamada et al. 1989). The action of FGF on differentiation is independent of its action on proliferation (Linkhart et al. 1982). Extensive studies have shown that inhibition of differentiation occurs at an early stage of the process between cessation of proliferation, fusion and expression of muscle-specific genes (Lathrop et al. 1985). FGF is not found in the circulation and it is likely to have its effects by an autocrine/paracrine action. The mechanism of action other than binding to a membrane receptor which has been identified in myoblasts (Olwin and Hauschka, 1986) is not known but may be related to changes in cellular oncogene expression in myogenic differentiation which has recently been reviewed (Schneider and Olson, 1988; Florini and Magri, 1989).

Transforming growth factor-ß

The third major regulator of muscle development is the transforming growth factor-ß family. Three laboratories reported that TGF-ß inhibited myogenic differentiation (Florini et al.1986; Massague et al. 1986; Olson et al. 1986). It inhibited all aspects of myogenic differentiation, fusion, elevation of creatine kinase activity, appearance of acetylcholine receptors, transition from ß- and γ- to α- actin, and the expression of other muscle specific proteins and their mRNAs. The effects are reversible and removal of TGF-ß results in differentiation by 72 h. Inhibition of differentiation by TGF-ß is similar to FGF but the critical time period for inhibition is slightly longer and although it inhibits commitment to myogenic differentiation, it has no effect on the expression of differentiated functions. TGF-ß is important physiologically in the regulation of muscle growth and development confirming the original observations in cells. In the embryo it may prevent fusion of myoblasts until sufficient muscle mass is attained. Raised local concentrations may prevent

satellite cell fusion with growing myotubes and then a fall in concentration may then allow differentiation of activated satellite cells in wound healing or postnatal muscle growth (Florini and Magri, 1989).

3. MUSCLE REGULATORY FACTORS IN DIVERGENTLY SELECTED LINES OF CHICKEN

Although these growth factors along with GH appear to be the most important regulators of normal muscle growth and development much information has been obtained from muscle cell lines. Little is still known of their effect in vivo, their inter-relationships, the regulation of their receptors or their signal transduction mechanisms, their role in muscle from embryos and postnatal, rapidly growing animals or their contribution to genetic differences in growth. We are currently attempting to answer some of these questions using a combined molecular and cellular approach in animals divergently selected for commercial traits and in transgenic animals. The rest of this paper will describe some of our current results on these major factors controlling muscle development. We have initially concentrated on the somatotrophic axis because of its importance in growth and the section reflects this, although we have included some recent work on FGF and TGF-ß near the end.

We have used chickens from three lines: a commercially available strain of broiler derived from a White Cornish heavy strain mixed population selected for more than 36 generations for an increase in body weight and breast muscle conformation, an offshoot of this line in which selection criteria were relaxed after 18 generations (Ross Breeders, Newbridge, Midlothian) and a closed population of White Leghorn layer chickens maintained at IAPGR, Edinburgh. The lines differ up to four-fold in body weight and up to eight-fold in muscle weight at 7 weeks of age (Bulfield et al. 1988). We have also shown that the differences in body weight are apparent in embryos as early as 8 days of incubation (R. Johnson, C. Goddard and G. Bulfield, unpublished observations) indicating that the response to selection is also due to events in embryogenesis.

Somatotrophic axis

a) GH

Research on GH in these lines has focussed on two aspects. We have demonstrated that plasma GH concentrations in all three lines increased during the rapid growth phase and then declined to basal levels. However, the slow-growing line had a considerably higher plasma GH than either of the broiler lines, and the relaxed selection line had a higher plasma GH than the selected broiler line showing that selection within a line for a higher growth rate resulted in lower mean plasma GH concentration (Goddard et al. 1988).

These differences could be due to different growth hormone isomers, some of which could be biologically inactive in the slow-growing line. We have analysed pituitary-derived chicken GH and showed that it is found in a monomeric form together with small amounts of inter-chain disulphide-linked oligomers. Isoelectric focusing revealed ten distinct charge isomers. The predominant isomer was present in the pituitary gland of birds of both sexes and at all ages studied (3 -114 days). The more acidic forms were not apparent before 13 days of age (Houston and Goddard, 1988) and it is likely that the appearance of charge isomers with increasing age may reflect storage of GH as a consequence of a decline in serum concentrations which result in a decline in growth rate. Analysis of the major

isomer showed an amino acid sequence identical to that predicted by the sequence of a cGH cDNA and that it contained full biological activity (Houston et al. 1990). We could find no evidence for any differences between pituitary-derived GH from chickens of the different lines and current studies are focussing on the GH receptor. Initial results suggest that the chicken GH receptor gene consists of a number of exons and spans greater than 15 kb (A. Gray, unpublished observations).

b) IGF peptides

Serum IGF-I concentrations were measured in all three selected lines but we could find no differences between them although within each of the lines IGF-I concentration was correlated with body weight (Goddard et al. 1988). Using this information a cDNA library was constructed from the liver of a 5-week-old broiler in which the level of IGF-I mRNA was expected to be high. Clones were isolated from this library by homology to the human IGF-IA cDNA and then sequenced (Fawcett and Bulfield, 1990). Comparison with human IGF-I cDNA sequences demonstrated that these cDNA clones contained sequences homologous to human exons 1, 2, 3 and 5 and an additional exon (1A) spliced between exons 1 and 2 which is not found in human IGF-I cDNA. The deduced amino acid sequence predicted a 130 amino acid peptide, comprising putative mature chicken IGF-I (70 amino acids), the leader peptide (25 amino acids) and an E peptide (35 amino acids). There was 78% identity between chicken and human sequences with 8 substitutions, four of which are chemically similar amino acids. The physiological significance of the amino acid substitutions in the chicken is unknown although the substitution at position 26 is within the proposed receptor-binding domain and is a region which is unchanged in all the other IGF-I peptides analysed to date. Also of interest are the four substitutions within the C domain which is also important for high affinity binding of IGF-I to the type I receptor, but not to the type II receptor.

A chicken genomic library was screened with the human IGF-IA cDNA probe and ten clones were isolated. Two different cDNA clones were used to probe Southern blots and several bands were detected in both EcoRI and HindIII - cut DNA and indicated that the gene is at least 23 kb long. Messenger RNA isolated from the liver of 5 and 15 week-old chickens was analysed on Northern blots and multiple bands were detected which need further investigation. There is evidence for alternative splicing of the chicken IGF-I message as in other species giving at least two mRNAs differing in the presence or absence of exon 1A. The presence of this exon will not affect the mature peptide but it may give rise to prepeptides differing in the length and nature of their leader sequences.

The tissue specific and developmental stage specific expression of the chicken IGF-I gene is now in progress. A 224 base pair Sau3A fragment from exon 3 of the chicken IGF-I cDNA has been cloned into the bluescribe vector for use as a probe in Northern blot analysis and in situ hybridization. The use of this probe in animals from the different lines will soon be possible and a construct containing the human IGF-I cDNA driven by the 5' promoter region of the murine α-actin gene is being constructed and will be used to produce transgenic mice in which IGF-I expression is targetted to striated muscle.

We have recently found that when myoblasts from 13 day-old embryos or satellite cells from 5 day-old chicks from fast- and slow-growing lines are cultured at the same density their mitogenic response to growth factors contained in foetal calf serum is different. In all experiments so far, cells derived from broilers respond more quickly and to a greater extent than those derived from the layer strain (C. Goddard unpublished observations). The response to IGF peptides has been

characterized in the slow growing line and we have shown that IGF-I, IGF-II increased DNA synthesis three-fold in satellite cells. IGF-I and -II were almost equipotent. Combinations of maximum effective concentrations were not additive suggesting that their effect was mediated by the same receptor.

Receptor binding studies on satellite cells demonstrated the presence of specific IGF receptors. Both IGF-I and IGF-II inhibited binding of ^{125}I-labelled IGF-I, with IGF-II slightly more potent than IGF-I. Affinity cross-linking of ^{125}I-labelled IGF-I and IGF-II, followed by SDS-polyacrylamide gel electrophoresis showed that they bound to a receptor with the structural characteristics of a type I IGF receptor and demonstrated the lack of a type II IGF receptor in these cells. The concentrations of IGF-I, -II required for biological action and to displace ^{125}I-labelled IGF-I binding were similar and support the hypothesis that their effects on proliferation were mediated exclusively through a type I IGF receptor. We have yet to examine the effects of these peptides in cells derived from the broiler (M. J. Duclos and C. Goddard, unpublished observations).

Other growth factors

We are currently working on the effects of FGF and TGF-ß, on proliferation of the same cells. FGF alone is a potent mitogen but its effects are inhibited in a dose-dependent manner by TGF-ß in satellite cells from both fast- and slow-growing lines. TGF-ß alone has no effect on proliferation in cells derived from the layers but preliminary experiments suggest that it may stimulate proliferation in the broiler cells (C. Goddard, unpublished observations). It must be stressed that these results need confirmation.

In order to analyse further the role of TGF-ß, the gene encoding TGF-ß2 has been cloned from a genomic DNA library prepared from the White Leghorn line, using a monkey TGF-ß2 cDNA as a heterologous probe. The use of chromosomal walking methods has allowed the isolation of most of the TGF-ß2 gene from overlapping genomic clones and the total cloned so far exceeds 60kb. DNA sequencing has revealed at least seven exons and six introns with the first two introns being very large. Further sets of overlapping clones are currently being characterized. If exon I and the promoter region are present in these, the TGF-ß2 gene will be at least 75kb in length. Comparison of avian and mammalian TGF-ß2 precursor sequences show an exceptionally high degree of homology at the amino acid level, implying that TGF-ß2 has a highly conserved function maintained by strong selection pressures. The mature peptide is probably released from a 414 amino acid precursor by proteolytic cleavage after 5 basic residues at positions 326-330 and is highly conserved with 108 amino acids identical in all species examined so far (D. Burt, unpublished observations).

Differences in the signal transduction mechanism for these factors may play a role in genetic differences in muscle growth. As indicated earlier, elevation of polyamines is essential to myoblast differentiation (Stoscheck et al. 1982; Ewton et al. 1984). Ornithine decarboxylase (ODCase) catalyses the first step in polyamine synthesis and is induced by a wide range of hormones and growth factors in a variety of target tissues including IGF-I in L6 myoblasts. We have previously demonstrated that the activity of ODCase is 20-fold higher in muscle from rapidly growing broiler chicks when compared to slow growing layers chicks at 7 days of age (Bulfield et al. 1988) and have recently extended this to include chick embryos between 5 - 10 days of incubation in similar strains (R. Johnson and G. Bulfield, unpublished observations). In order to investigate this at the molecular level, a number of ornithine decarboxylase cDNA clones have been isolated from a chicken

cDNA library. The longest of these has been sequenced and shows 78% homology to the mouse and human sequences which is equivalent to 90% at the predicted amino acid sequence. This clone has been used to probe Southern blots of genomic DNA prepared from both broiler and layer chicks and has shown the presence of a polymorphism in the 3' end of the ornithine decarboxylase gene in the broiler. Further analysis on Northern blots of muscle mRNA have demonstrated the expression of two transcripts of 1.7 and 2.0 kb. (R. Johnson and G. Bulfield, unpublished observations). The relationship of this to induction by growth factors in muscle cells prepared from fast and slow growing strains requires further analysis.

CONCLUSIONS

The biological basis of genetic selection for rapid growth, carcass composition or food intake in animals is not well understood and more information is needed before manipulation of individual genes can be exploited by gene transfer techniques or before direct and more efficient selection can be applied to physiological traits. We believe that a major contribution to the problem can be made by the cellular and molecular analysis of farm and laboratory animals divergently selected for growth rate, body composition or metabolic traits. The long-term aim is to identify the important metabolic processes, genes and gene products which control these commercially important characteristics and manipulate them genetically. This review has highlighted progress on a number of genes and their products involved in muscle growth and development and suggests that understanding the relationship between genotype and phenotype is likely to be a fruitful area in the next decade.

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