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Embryonic bone growth in lines of mice selected for large and small body size

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SUMMARY

Embryonic tibia growth was measured in large, small and control mouse embryos *in situ* and in organ culture. *In situ* tibias of the large line were longer than controls which were longer than tibias of the small line. Relative growth was approximately equal among lines. In culture tibias of the small line grew more than controls and as much as or more than tibias of the large line. Embryonic genotype appears to be more important in regulating tibia growth than uterine environment. Humoral differences among lines may influence tibia growth.

1. INTRODUCTION

This study is part of an effort to understand how genes at many loci control growth in lines of mice selected for high and low body weight at 6 weeks of age (Falconer, 1973). No differences have been observed between these large and small lines in embryonic size or cleavage rate prior to implantation (Bowman & McLaren, 1970), although differences in embryonic weight appear as organogenesis begins (I. Gauld, personal communication). These results suggested that the organ level of organization would be appropriate for the examination of growth differences between large and small mice.

The embryonic tibia was chosen as the organ for study because long bone growth should be an important component of overall growth differences between lines. Observations of tibia growth, measured as elongation, would, ideally, be made *in situ*; however, this entails killing the embryo and does not allow sequential measurements of growth. Further, embryos from large and small lines develop in different uterine environments, and it is possible that tibia growth could be influenced by uterine environmental differences as well as by gene differences which influence bone growth directly. Similarly, tibias from each line are formed and grow in different embryos, and differences in embryonic milieu may also influence tibia growth. In this study organ culture and embryo transfers were used to deal with these potential problems.

Experiments in this study were designed to try to answer the following questions:

(1) Has selection for differences in body weight altered embryonic tibia growth, and, if so, what aspects of growth have been affected?

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(2) To what extent is tibia growth influenced by differences in uterine environment and to what extent by differences in embryonic milieu?

(3) Is the control of tibia growth systemic or intrinsic to the bone itself?

2. MATERIALS AND METHODS

The mice used in this study were large, control, and small mice, replicates B and D, taken from generations 52–57 of Falconer's (1973) replicated selection lines for high and low body weight at 6 weeks.

Female mice, 6–10 weeks old, were randomly mated to males of the same line and examined daily for vaginal plugs. The day on which a plug was found was considered day 1 of pregnancy.

The uterus from each pregnant female was removed to a dish of warm, sterile phosphate-buffered saline. Embryos were rapidly removed and transferred to a dish of fresh saline. The pair of tibias from each embryo was dissected from the embryo, cleaned, and measured using a dissection microscope fitted with an ocular micrometer. Tibias from day 14 and day 15 embryos (Theiler's (1972) stages 22 and 23) were used for organ culture. They were cultured according to procedures modified from Aydelotte & Kochhar (1972) and Kochhar & Aydelotte (1974). Each bone of a pair was placed on a small piece of sterile ultrathin Millipore filter, and each pair of bones was placed on a sterile stainless steel organ culture grid (Falcon) in a sterile 35 mm petri dish (Falcon) containing approximately 1·5 mm BGJ_a medium (Biggers, Gwatkin & Heyner, 1961) and incubated at 37° C in an atmosphere of humidified 5% CO_2 in air. Preliminary experiments confirmed the observation of Biggers, Gwatkin & Heyner (1961) that most embryonic mouse tibia growth occurs within the first 4 days in culture. For this reason, tibias were incubated for 4 days (the day of explantation = day zero). Tibia lengths were measured on days zero, two, and four, and medium was replaced on day two.

BGJ_a medium was prepared from BGJ_b medium (Gibco Biocult) by adding 5 mg/100 ml adenosine (Sigma), 5 mg/100 ml glycine (Sigma), 35 mg/100 ml L-glutamine (Flow), 2·5 mg/100 ml streptomycin sulphate (Glaxo), and 5000 U/100 ml benzylpenicillin (Glaxo). The medium was supplemented with 15 mg/100 ml L-ascorbic acid (Fisons). Medium was made up in 50 ml quantities, sterilized by pressure filtration through a Swinnex filter (pore diameter 0·22 μm), refrigerated, and used within 3–4 days.

In the first organ culture experiment wet and dry tibia weights were obtained after 2 and 4 days in culture as indicators of hydration. Tibias were removed from culture, gently blotted, weighed to the nearest 0·0001 mg on an electric balance, dried overnight at 100° C, and reweighed.

In order to determine effects of differences in uterine environments, day four embryos (morulae and blastocysts) from each of the three lines were transferred to the uteri of day three pseudopregnant females from each of the three lines (Aitken, Bowman & Gauld, 1977). In most cases four to five embryos were trans-

ferred to each uterine horn. Females were killed on day 15, and embryonic tibias were cultured as usual.

In order to examine the effects of differences in embryonic milieu on tibia growth, tibias from each of the three lines were incubated in the presence of embryo extracts from each of the three lines. Embryo extract from day 14 and day 15 embryos was prepared according to Paul (1965). All batches of extract were stored at $-20°$ C until needed and added to BGJ_a medium to a final concentration of 10%.

Hierarchical analysis of variance and computation of the components of variance due to replicates, to litters within replicates, to embryos within litters, and to tibias within embryos indicated that the embryo was the correct unit of observation for data analysis and that the error term was tibias within embryos. Therefore, data presented in this study are based on the mean score of the two tibias from each embryo. These data were subjected to least-squares analysis of variance in order to determine the main effects of treatments. Specific comparisons of least-squares means between lines were made using non-orthogonal linear contrasts. Tests of significance of differences between lines were made using Student's *t* test.

3. RESULTS

Data from the two replicates were pooled, as there were no significant differences between them.

(i) *Tibia lengths* in situ

Tibia lengths were measured in day 14 to day 18 embryos to determine size and growth relations among tibias from large, control, and small lines *in situ* (Table 1). At all times tibias from large embryos were longer than control tibias which were longer than tibias from small embryos. Increases in mean tibia lengths on successive days were used to estimate daily absolute and relative growth. Absolute growth is the increase in tibia length in millimetres. Relative growth is absolute growth expressed as a percentage of tibia length at the beginning of the interval. Except for the day 16–17 interval, the absolute growth of small tibias was less than that of controls while the growth of large tibias was greater. Relative growth was similar among lines at all times; during all but the day 15–16 interval tibia length increased by about 20%, and between days 15 and 16 this value doubled. The relative growth of small tibias was slightly less at this time than that of large or control tibias, but during the day 16–17 interval it was slightly greater, suggesting a small difference in the timing of growth among lines.

(ii) *Tibia growth in organ culture*

The results obtained in culture differed from those observed *in situ* (Table 2). After 2 days in culture large tibias were longer than control and small tibias, but there was no significant difference in length between control and small tibias. The relative growth of small tibias was substantially greater than that of controls. The

Table 1. *Tibia growth* in situ

(Least-squares means and standard errors of tibia lengths (mm) measured on day 14 to day 18 embryos. Absolute growth (mm) and relative growth (%) were computed for each daily interval. See text for explanation.)

Genotype of embryo	No. of embryos at each day	Tibia length (mm)				
		Day 14§	Day 15	Day 16	Day 17	Day 18
Small (S)	100	1·057	1·287	1·807	2·295	2·782
Control (C)	100	1·139	1·401	2·056	2·429	2·966
Large (L)	100	1·250	1·515	2·219	2·701	3·248
S.E.†		0·008	0·012	0·014	0·019	0·017
S–C‡		***	***	***	***	***
L–C		***	***	***	***	***
L–S		***	***	***	***	***

	Daily interval			
	Day 14–15	Day 15–16	Day 16–17	Day 17–18
Absolute growth (mm)				
Small	0·230	0·520	0·488	0·487
Control	0·262	0·655	0·373	0·537
Large	0·265	0·704	0·482	0·547
Relative growth (%)				
Small	21·7	40·4	27·0	21·2
Control	23·0	46·8	18·1	22·2
Large	21·2	46·5	21·7	20·3

† Least-squares standard error of each of the above means.
‡ Significance of differences between line means: *** indicates $P < 0{\cdot}001$.
§ Age of embryo.

absolute growth of small tibias was also greater than that of controls, but the difference was not significant among day 14 tibias. Neither absolute nor relative growth differed significantly between large and small tibias. The results after 4 days in culture were similar, except that small tibias were somewhat longer than controls, rather than slightly shorter, as they had been at 2 days.

Regression analysis revealed no change in the pattern of tibia growth in culture when bones from all lines were standardized to the same initial length or when data were corrected for differences in litter size among lines.

The per cent dry weight of tibias did not differ significantly among lines, nor was there any consistent increase in water content from 2 to 4 days, which indicated that tibias were not becoming progressively hydrated in culture (Table 3).

(iii) *Tibia growth in culture following embryo transfer*

Embryo transfers were performed to determine whether observed differences in tibia growth were due to embryonic gene differences or to differences among

Table 2. *Tibia growth in organ culture*

(Least-squares means and standard errors of tibias, at explantation (L0), after 2 days in culture (L2), and after 4 days (L4), of absolute growth, and of relative growth for day 14 and day 15 embryos.)

Genotype of embryo	No. of embryos	Tibia length (mm)			Absolute growth (mm)		Relative growth (%)	
		L0	L2	L4	Day 0–2	Day 0–4	Day 0–2	Day 0–4
Day 14 embryos								
Small (S)	40	1·102	1·655	1·909	0·533	0·807	50·2	72·3
Control (C)	41	1·175	1·663	1·888	0·488	0·713	41·7	60·8
Large (L)	42	1·256	1·822	2·135	0·566	0·878	45·4	70·6
S.E.†		0·010	0·024	0·032	0·023	0·032	2·0	2·8
S–C‡		***	n.s.	n.s.	n.s.	*	**	**
L–C		***	***	***	*	**	n.s.	*
L–S		***	***	***	n.s.	n.s.	n.s.	n.s.
Day 15 embryos								
Small (S)	40	1·311	1·898	2·168	0·587	0·856	45·0	65·7
Control (C)	41	1·439	1·941	2·140	0·502	0·700	35·2	49·1
Large (L)	40	1·533	2·199	2·478	0·645	0·926	42·0	60·5
S.E.†		0·019	0·027	0·027	0·021	0·025	1·6	1·2
S–C‡		***	n.s.	n.s.	**	***	***	***
L–C		***	***	***	***	***	**	***
L–S		***	***	***	n.s.	n.s.	n.s.	n.s.

† Least-squares standard error of each of the above means.

‡ Significance of differences between line means: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$; n.s., not significant.

uterine environments. The effects of embryonic genotype and uterine genotype are considered separately (Table 4).

(*a*) *Effect of embryonic genotype.* The pattern of tibia growth produced by the three embryonic genotypes, regardless of uterine genotype, resembled that seen *in situ*. At explanation large tibias were longer than controls which were longer than small tibias. This result was consistent within each uterine genotype and within the surgical controls. Therefore embryo transfer does not seem to have affected tibia length *in situ*. In culture small tibias did not catch up with control tibias but remained significantly shorter, as *in situ*. The absolute growth of control tibias was greater than that of small tibias, although this difference was not significant at 2 days. Large tibias grew absolutely more than control and small tibias There were no significant differences in relative growth among lines.

(*b*) *Effect of uterine genotype.* The pattern of growth produced by the three uterine genotypes, regardless of embryonic genotype, resembled that seen in culture. Initially tibias from embryos which developed in large uteri were longer than those from embryos which developed in control uteri which were longer than those from embryos which developed in small uteri. After 2 days in culture tibias from embryos in large uteri were still longer than tibias from embryos in control

Table 3. *Least-squares means and standard errors of percentage dry weight of tibias from day 14 and day 15 embryos incubated for 2 or 4 days in culture*

Genotype of embryo	2 days		4 days	
	No. of embryos	% dry wt	No. of embryos	% dry wt
Day 14 embryos				
Small (S)	40	15·7	40	14·0
Control (C)	40	14·0	41	14·7
Large (L)	41	16·1	42	15·5
S.E.†		1·3		1·2
S–C‡		n.s.		n.s.
L–C		n.s.		n.s.
L–S		n.s.		n.s.
Day 15 embryos				
Small (S)	42	15·5	40	14·6
Control (C)	40	14·5	41	15·6
Large (L)	40	14·0	40	14·2
S.E.†		1·1		0·6
S–C‡		n.s.		n.s.
L–C		n.s.		n.s.
L–S		n.s.		n.s.

† Least-squares standard error of each of the above means.
‡ Significance of differences between line means: n.s. indicates no significant difference.

and small uteri, which were not significantly different from one another. There were no significant differences in absolute or relative growth among tibias from large, control, or small uteri. Results after 4 days in culture were similar.

(iv) *Tibia growth in embryo extract*

Tibias were cultured in the presence of embryo extract to try to determine the effects of line differences in embryonic milieu on tibia growth. Again, the effects of tibia genotype and extract genotype are considered separately (Table 5).

(*a*) *Effect of tibia genotype.* Initially large tibias were longer than control tibias which were longer than small tibias. After incubation large tibias remained longer than control and small tibias, but control and small tibias did not differ significantly from one another. Small tibias from day 14 embryos grew more than large tibias, but there was no significant difference in growth between large and small tibias from day 15 embryos.

(*b*) *Effect of embryo extract.* There was a consistent tendency for tibias incubated in large embryo extract to grow more than tibias incubated in small embryo extract, but this difference was not significant.

4. DISCUSSION

Two patterns of tibia lengths were observed during the course of these experiments. In the first pattern large tibias were longer than controls which were longer

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Table 4. *Least-squares means and standard errors of tibia lengths of day 15 embryos and growth in culture following embryo transfer*

	No. of embryos	Tibia length (mm)			Absolute growth (mm)		Relative growth (%)	
		L0	L2	L4	Day 0–2	Day 0–4	Day 0–2	Day 0–4
Genotype of embryo								
Small (S)	63	1·484	1·948	2·186	0·465	0·702	31·6	47·8
Control (C)	67	1·523	2·046	2·254	0·522	0·730	34·6	48·3
Large (L)	61	1·607	2·129	2·394	0·521	0·787	32·8	49·5
S.E.†		0·013	0·020	0·024	0·019	0·024	1·3	1·7
S–C‡		**	**	*	*	n.s.	n.s.	n.s.
L–C		***	**	***	n.s.	n.s.	n.s.	n.s.
L–S		**	***	***	*	*	n.s.	n.s.
Genotype of uterus								
Small (S)	62	1·489	1·980	2·220	0·491	0·731	33·3	49·4
Control (C)	65	1·537	2·024	2·232	0·487	0·696	31·9	45·5
Large (L)	64	1·588	2·118	2·382	0·529	0·794	33·9	56·6
S.E.†		0·013	0·020	0·024	0·019	0·024	1·3	1·7
S–C‡		*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L–C		**	***	***	n.s.	**	n.s.	n.s.
L–S		***	**	**	n.s.	n.s.	n.s.	n.s.

† Least-squares standard errors of each of the above means.

‡ Significance of differences between line means: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$; n.s., not significant.

than small tibias. In the second pattern large tibias were longer than control and small tibias which were not significantly different from one another.

In situ the first pattern was maintained throughout development because tibias from all three lines grew at approximately the same relative rate. Gauld (personal communication), working with the same lines, has found that the same is true for embryonic weight from day 11 until birth. Taken together these results suggest that growth is similarly regulated in all three lines.

In organ culture the second pattern resulted from the increased growth of small tibias relative to controls. This may reflect a capacity for growth in small tibias which is suppressed *in situ* or a genotype-by-environment interaction in which small tibias were better able to grow in culture conditions than control or large embryos. If growth *in situ* did not result from such a genotype-by-environment interaction, then the breakdown in the pattern of growth seen *in situ* indicates that a bone's inherent capacity for growth is independent of the regulation of its growth. This in turn suggests that control of tibia growth is systemic and not intrinsic to the bone itself. Aitken, Bowman & Gauld (1977) have found that the regulation of whole embryo growth can be disrupted. On day 17 asynchronously transferred small embryos weighed more than synchronously transferred or untransferred large embryos.

Table 5. *Least-squares means and standard errors of tibia length and growth in culture medium containing extract from small, control or large embryos*

	No. of embryos	Tibia length (mm)			Absolute growth (mm)		Relative growth (%)	
		L0	L2	L4	Day 0–2	Day 0–4	Day 0–2	Day 0–4
Day 14 embryos								
Genotype of embryo								
Small (S)	90	1·054	1·558	1·618	0·504	0·627	47·4	59·5
Control (C)	90	1·127	1·526	1·634	0·398	0·507	35·4	45·0
Large (L)	90	1·216	1·656	1·790	0·440	0·574	36·3	47·4
S.E.†		0·007	0·018	0·024	0·016	0·022	1·4	1·9
S–C‡		***	n.s.	n.s.	***	***	***	***
L–C		***	***	***	n.s.	*	n.s.	n.s.
L–S		***	***	**	**	n.s.	***	***
Genotype of extract								
Small (S)	90	1·135	1·567	1·672	0·432	0·537	38·3	47·6
Control (C)	90	1·127	1·580	1·707	0·453	0·581	40·6	52·0
Large (L)	90	1·135	1·592	1·726	0·457	0·591	40·5	52·3
S.E.†		0·007	0·018	0·024	0·016	0·022	1·4	1·9
S–C‡		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L–C		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L–S		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Day 15 embryos								
Genotype of embryo								
Small (S)	90	1·336	1·822	1·968	0·486	0·632	36·9	47·9
Control (C)	90	1·443	1·852	2·001	0·409	0·558	28·6	38·9
Large (L)	90	1·510	1·957	2·105	0·446	0·625	29·6	41·2
S.E.†		0·011	0·019	0·030	0·016	0·028	1·3	2·1
S–C‡		***	n.s.	n.s.	***	n.s.	***	***
L–C		***	***	**	n.s.	n.s.	n.s.	n.s.
L–S		***	***	***	n.s.	n.s.	***	*
Genotype of extract								
Small (S)	90	1·438	1·878	2·032	0·439	0·593	32·1	41·8
Control (C)	90	1·413	1·850	2·010	0·439	0·598	31·3	42·5
Large (L)	90	1·438	1·902	2·061	0·464	0·623	32·7	43·7
S.E.†		0·011	0·019	0·030	0·016	0·028	1·3	2·1
S–C‡		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L–C		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L–S		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

† Least-squares standard error of each of the above means.

‡ Significance of differences between line means: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$; n.s., not significant.

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Tibia growth appears to be controlled principally by the embryo rather than by the uterine environment. Aitken, Bowman & Gauld (1977) reached the same conclusion regarding embryonic weight. In the embryo transfer experiment the effect of embryonic genotype on growth in culture produced the same pattern of tibia lengths seen *in situ* whereas the effect of uterine genotype produced the same pattern previously seen in culture. Whether both uterine environment and the culture conditions inhibit the growth of control tibias relative to small tibias or whether they release small tibia growth from normal inhibition cannot be determined from these experiments.

The embryo extract experiment was an attempt to put tibias of given genotypes in different embryonic milieux. There was no significant effect of embryo extract at the concentration used (10%), and this concentration was not increased because tibias bent when this was done. Nonetheless, tibias incubated in large embryo extract always grew more than those incubated in small embryo extract, suggesting that humoral factors may have been responsible for the very small difference in growth.

Selection for differences in 6-week body weight has produced lines of embryos which differ substantially in tibia growth, but the reasons for this difference are not clear. Selection has not altered relative growth rate among lines and may not have diminished the capacity of small bones to grow. This selection programme has probably produced allelic differences among lines which have altered long bone organization. Differences among lines are established by day $11\frac{1}{2}$; forelimbs taken from embryos of this age, which have not yet begun to chondrify, show much the same pattern of growth in culture as older tibias (Blakley, unpublished results). The events which mediate the effects of gene differences on bone growth evidently occur before bones have formed.

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