Short-term culture of ovine embryos modifies fetal myogenesis

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Maxfield, E. K., K. D. Sinclair, P. J. Broadbent, T. G. McEvoy, J. J. Robinson, and C. A. Maltin. Short-term culture of ovine embryos modifies fetal myogenesis. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E1121–E1123, 1998.—Certain reproductive techniques culture embryos in vitro; however, little is known about the impact of culture on fetal growth. Coculture of day 1 ovine zygotes on a bovine granulosa cell layer to blastocysts followed by transfer to synchronous recipients increased fetal weight by 11 and 40% at days 61 and 125, respectively, compared with the transfer of in vivo-produced blastocysts. Plantaris muscle weights were increased by 40% in cultured fetuses at day 125. Examination of myogenesis in plantaris muscle showed that primary fiber number was unchanged at day 61 by culture but that primary fiber area was increased significantly by 15 and 25% at days 61 and 125, respectively; secondary fiber area was increased by 40% at day 125 by culture, and the ratio of secondary to primary fiber numbers was 18–20% greater in the cultured groups compared with the controls at days 61 and 125. The results show that coculture of preimplantation embryos may alter myogenic programming. These changes may contribute to the abnormally large muscles observed in oversize fetuses. Early embryo manipulation that has impact on fetal muscle development or growth may also have impact on fetal and neonatal or postnatal size. Asynchronous embryo transfer (temporary 3-day transfer of an ovine day 3 embryo (day 0 = estrus) to an advanced (day 6) uterine environment) has already been shown to alter myogenesis and its regulation (9) but with little impact on fetal or muscle weight. In contrast, the coculture of ovine embryos with bovine granulosa cells has been shown to lead to an alteration in the rate of growth and coordination of fetal development and, hence, to fetal oversize (10). The aim of the present study was to assess the effect of embryo coculture on myogenesis in sheep. To provide a good assessment of primary and secondary myogenesis, muscle was derived from fetuses at day 61 and day 125 of gestation.

METHODS

Embryo manipulation. The methods for the transfer of embryos and creation of pregnancy have been described previously (10). All the animals, the donor and recipient ewes and the ram, were husbanded and treated according to the Home Office Animals (Scientific Procedures) Act 1986. Briefly, Scottish Blackface ewes were primed with progesterone for 12 days (30 mg, Chronogest, Intervet). After 10 days of progesterone priming, gonadotrophin treatment was begun using ovine follicle-stimulating hormone for 4 days (Ovagen, ICP), with a total of 9.0 mg administered in equal doses twice per day. Ewes were inseminated by laparoscopy 48 h after progesterone withdrawal by use of fresh semen from one Suffolk ram. Zygotes were recovered at day 1 (estrus being day 0). All recipient ewes were also given 12 days of progesterone priming followed by an intramuscular injection of 400 IU of pregnant mares' serum at progesterone withdrawal to induce estrus. Recovered day 1 zygotes were maintained in culture for a period of 5 days on a bovine granulosa cell layer. Embryos were then transferred to permanent recipient ewes. The control group was derived from embryos recovered at day 6 and transferred in a synchronous manner to day 6 permanent recipients. Fetuses were humanely euthanized at either day 61 or day 125 of gestation. Fetuses were weighed, and plantaris muscles and tissue-free humerus bones were removed and weighed or measured.

Muscle recovery and analysis. Plantaris muscles recovered from fetuses at both day 61 and day 125 were mounted on cork blocks and frozen in liquid nitrogen (8). Ten-micrometer transverse sections were cut of each whole muscle and stained to demonstrate Ca\(^{2+}\)-activated myofibrillar ATPase...
**RESULTS**

Fetal weights were significantly increased in cocultured fetuses compared with controls at both days 61 and 125 of gestation (Table 1). Plantaris muscle weights and humerus lengths from cocultured fetuses were also increased (P < 0.05) compared with controls at day 125 of gestation (Table 1). Coculture did not alter primary fiber number at day 61; however, the mean S/P was increased at both day 61 and day 125 in the coculture group (Table 2). In addition, primary and secondary fiber cross-sectional areas were increased in cocultured fetuses; primary fiber cross-sectional area was increased at both day 61 and day 125 (P < 0.05), whereas the increase in secondary fiber cross-sectional area was seen at day 125 (P < 0.001) (Table 2). Estimates of percent frequency indicated that there was a 10–15% reduction in primary frequency in the cocultured group at both day 61 and day 125, whereas there was a small increase in percent frequency for secondary fiber at these time points (Table 2). These differences were also evident in terms of percent area. Compared with control data, at day 61 there was a significant decrease in primary fiber percent area and a significant increase in secondary fiber percent area in the cocultured group (Table 2). These differences were also clear at day 125, at which time they were more pronounced (Table 2).

**DISCUSSION**

Fetal oversize due to the coculture of ovine zygotes has been documented (10) and has been shown to be associated with altered embryo development and metabolism (14) as well as perturbation of allometric growth relationships (10). The present study extends these observations to demonstrate that fetal oversize after coculture of zygotes with bovine granulosa cells may be associated with increased muscle weight and alterations of the developmental programming of muscle.

The present observations show that coculture of preimplantation blastocysts leads to changes in the development of plantaris muscles. Specifically, an increase in the S/P, an increase in secondary fiber frequency, and an increase in primary and secondary fiber cross-sectional area were observed in muscles from fetuses from in vitro cocultured embryos compared with in vivo controls. These results are interpreted in terms of alterations in the hyperplastic and hypertrophic programming of plantaris muscle.

It appears that both fetal size and muscle size may be determined by the extent of myogenic hyperplasia and hypertrophy during fetogenesis. For example, low-birth-weight pigs, or “runts,” have fewer fibers per muscle than their normal-sized littermates (1, 2), whereas, in contrast, double-muscled cattle (7) appear to have increased numbers of fibers per muscle. Hence, in the present study in which both fetal and muscle weight were increased by coculture, it is, perhaps, not surprising that myogenesis is also altered.

Myogenesis proceeds in two phases: the first cohort of myoblasts fuses to form primary myotubes, and the second cohort of myoblasts forms on their surface and fuses to give rise to secondary myotubes. From studies on polytocous species, it has been suggested that the number of primary fibers is genetically determined (hence, the variation between litters), whereas the secondary fibers, which vary between littermates, appear to be determined by local and environmental signals such as nutrition (1, 2). The present results from day 61 fetuses may be interpreted as supporting the contention of a genotypic programming of primary muscle growth.

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### Table 2. Plantaris muscle fiber analysis of day 61 and day 125 ovine fetuses

<table>
<thead>
<tr>
<th>Day 61</th>
<th>Primary Fiber No.</th>
<th>S/P</th>
<th>Primary Size, µm²</th>
<th>Secondary Size, µm²</th>
<th>Primary %Frequency</th>
<th>Secondary %Frequency</th>
<th>Primary %Area</th>
<th>Secondary %Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>1.957 ± 534</td>
<td>1.89 ± 0.15</td>
<td>1.062 ± 134</td>
<td>394 ± 68</td>
<td>34.87 ± 1.72</td>
<td>65.13 ± 1.72</td>
<td>59.13 ± 3.13</td>
<td>40.87 ± 3.13</td>
</tr>
<tr>
<td>Coculture (n = 8)</td>
<td>1.908 ± 503</td>
<td>2.20 ± 0.19†</td>
<td>1.229 ± 137*</td>
<td>510 ± 147</td>
<td>31.25 ± 2.00†</td>
<td>68.75 ± 2.00†</td>
<td>52.89 ± 7.49*</td>
<td>47.11 ± 7.49*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 125</th>
<th>Primary Fiber No.</th>
<th>S/P</th>
<th>Primary Size, µm²</th>
<th>Secondary Size, µm²</th>
<th>Primary %Frequency</th>
<th>Secondary %Frequency</th>
<th>Primary %Area</th>
<th>Secondary %Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>7.78 ± 0.57</td>
<td>2.962 ± 637</td>
<td>2.366 ± 437</td>
<td>11.45 ± 0.70</td>
<td>88.55 ± 0.70</td>
<td>13.30 ± 1.64</td>
<td>86.7 ± 1.64</td>
<td></td>
</tr>
<tr>
<td>Coculture (n = 6)</td>
<td>9.36 ± 0.65</td>
<td>3.675 ± 403‡</td>
<td>3.376 ± 321‡</td>
<td>9.67 ± 0.56§</td>
<td>91.33 ± 0.56§</td>
<td>10.45 ± 1.10§</td>
<td>89.55 ± 1.10§</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD; n = no. of fetuses. S/P, ratio of secondary to primary fiber. Coculture at day 61 vs. control at day 61: *P < 0.05, †P < 0.01. Coculture at day 125 vs. control at day 125: ‡P < 0.05, §P < 0.01.
fiber number, since embryo culture did not significantly change primary fiber number; hence, the increase in S/P may be interpreted as indicating an increase in secondary fiber number. However, estimates of secondary fiber percent frequency were significantly increased in the coculture groups at both days 61 and 125, with a concomitant reduction in primary fiber percent frequency (Table 2). Thus it is possible that the overall increase in S/P derives from a combination of the small, nonsignificant reduction in primary fiber number and an increase in secondary fiber number. Hence, it may appear that the coculture procedure has in some way altered the hyperplastic programming of the tissue to alter the relative numbers of primary and secondary fibers.

In addition to a possible increase in secondary fiber numbers, the present observations indicate that the cross-sectional area of both primary and secondary fibers was increased, pointing to some alterations of hypertrophic programming.

It has been suggested that the surface area of the primary fibers, on which the secondary fibers develop, plays an important part in determining secondary fiber number (12). In the present study, muscle from cocultured animals showed an increase in cross-sectional area in both primary and secondary fibers, suggesting that the larger primary fibers provided a larger area for secondary myogenesis and hence gave rise to the increase in apparent secondary fiber frequency. Interestingly, the high-growth-phenotype mouse, which has an increased postnatal growth rate and body size, has an increase in both the number and size of the secondary fibers of soleus, gastrocnemius, and tibialis anterior muscles, with primary fiber cross-sectional area also being higher early in postnatal life (13). It is interesting to speculate whether, had fetuses from the coculture group been taken to term, the increase in secondary fiber percent frequency and cross-sectional area would have given rise to higher postnatal growth rates.

One of the striking features of this study is that the manipulations of the embryos were imposed very early in development, before implantation. The indications are that myogenic programming can be altered by events that occur even before apparent myogenic cell determination and commitment events are entrained. This has implications not only for the implementation of embryo transfer technologies but also for human reproductive technologies.

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REFERENCES


