BIOLOGY OF REPRODUCTION **64,** 918–926 (2001)

Culture of Preimplantation Mouse Embryos Affects Fetal Development and the Expression of Imprinted Genes[1]

Sanjeev Khosla,[3,5] Wendy Dean,[5] David Brown,[4,6] Wolf Reik,[5] and Robert Feil[2,5]

Laboratory of Developmental Genetics and Imprinting[5] and Laboratory of Computational Neuroscience,[6] The Babraham Institute, Babraham, Cambridge CB2 4AT, United Kingdom

ABSTRACT

Culture of preimplantation mammalian embryos and cells can influence their subsequent growth and differentiation. Previously, we reported that culture of mouse embryonic stem cells is associated with deregulation of genomic imprinting and affects the potential for these cells to develop into normal fetuses. The purpose of our current study was to determine whether culture of preimplantation mouse embryos in a chemically defined medium (M16) with or without fetal calf serum (FCS) can affect their subsequent development and imprinted gene expression. Only one third of the blastocysts that had been cultured from two-cell embryos in M16 medium complemented with FCS developed into viable Day 14 fetuses after transfer into recipients. These M16 + FCS fetuses were reduced in weight as compared with controls and M16 fetuses and had decreased expression of the imprinted *H19* and insulin-like growth factor 2 genes associated with a gain of DNA methylation at an imprinting control region upstream of *H19.* They also displayed increased expression of the imprinted gene *Grb10.* The growth factor receptor binding gene *Grb7,* in contrast, was strongly reduced in its expression in most of the M16 + FCS fetuses. No alterations were detected for the imprinted gene *Mest.* Preimplantation culture in the presence of serum can influence the regulation of multiple growth-related imprinted genes, thus leading to aberrant fetal growth and development.

developmental biology, gene regulation, IGF receptor, implantation/early development

INTRODUCTION

The oviduct provides the preimplantation mammalian embryo with a unique environment that nurtures the early embryo and thereby influences its further development. The use of specific media for in vitro culture of preimplantation embryos, mimicking the environment of the oviduct, has allowed examination of early development and manipulation of the mammalian genome [1]. However, there is increasing evidence that in vitro culture of preimplantation embryos can be associated with aberrant growth and specific phenotypic abnormalities during fetal and postnatal development [2–5]. In sheep and cattle, these aberrant phenotypes are collectively referred to as the large offspring syndrome. The main characteristic of this syndrome is increased fetal growth, and on average affected newborn animals are significantly heavier than controls [6]. The culture systems with which the large offspring syndrome has been associated are varied, but most involve the use of serum [5–7]. Little information has been obtained on growth effects in rodent species. However, mouse fetuses derived from in vitro cultured preimplantation embryos (from eight-cell stage to blastocysts) were lighter than those derived from control uterine blastocysts [8]. Pronuclear transfer in the mouse also has negative long-term implications for growth and development [9, 10].

Although the mechanisms responsible for the deregulation of development as a consequence of in vitro culture remain elusive, several hypotheses have been put forward to explain the diversity of abnormalities observed. Fragmentation of cytoplasm, documented for cultured bovine embryos [11], has been suggested as a possible reason for the deregulation, because interaction of cytoplasmic factors with the nucleus is essential for normal development [12–14]. Another proposal alleges that embryos do not receive the appropriate cues during in vitro culture and that this lack of signaling molecules could result in the deregulation of genes under their influence [5]. A third hypothesis is that in vitro culture of preimplantation embryos leads to aberrant epigenetic modifications in the genome. Such alterations would be maintained somatically and might affect gene expression at later stages of development [9, 15–17]. One heritable epigenetic feature that regulates gene expression in mammals is DNA methylation. During early embryonic development, extensive changes in genome-wide methylation take place [18], and therefore any perturbation caused in the methylation process could result in deregulation of development at later stages.

Imprinted genes are exceptional in that they are expressed in a parent-of-origin-specific manner. Genetic studies have shown that imprinting is evolutionarily conserved among eutherian mammals (e.g., mice, humans, sheep) and plays important roles in fetal growth and development [19–21]. In most imprinted gene loci, key regulatory regions have been identified that are methylated on one of the two parental chromosomes. For several of these imprinting control regions, the differential methylation originates from one of the germ lines and is maintained throughout pre- and postimplantation development [21–23]. This situation contrasts with that of nonimprinted genes, which acquire their methylation patterns after implantation [18, 24, 25]. Thus, imprinted genes may be particularly susceptible to methylation changes that occur during preimplantation development. Fetal abnormalities observed as a consequence of preimplantation in vitro culture have been proposed to result from aberrant changes in the methylation states of imprinted genes [9, 15, 16]. In addition, for several imprint-

[1]This work was supported by the Ministry of Agriculture, Fisheries and Food, the Biotechnology and Biological Sciences Research Council, and the Royal Society.

[2]Correspondence and current address: Robert Feil, Institute of Molecular Genetics, CNRS, 1919 Route du Mende, 34293 Montpellier cedex 5, France. FAX: 33 4 67040231; e-mail: feil@jones.igm.cnrs-mop.fr

[3]Current address: Wellcome/CRC Institute of Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QR, UK.

[4]Current address: Office for National Statistics, Ebury Gate, London SW1, UK.

Received: 21 July 2000.
First decision: 25 August 2000.
Accepted: 2 November 2000.

ISSN: 0006-3363. http://www.biolreprod.org

ed genes deregulation has significant effects on growth and development [19]. For instance, alterations in the expression of the insulin-like growth factor 2 gene (*Igf2*) have been shown to severely affect growth in mice [26–28]. In humans, deregulation of *IGF2* has been implicated in cases of the congenital overgrowth syndrome, Beckwith-Weidemann syndrome (BWS) [29, 30], and several of the phenotypes observed in BWS are similar to the ones observed in the large offspring syndrome [6]. Our recent observations on embryonic stem (ES) cells support the hypothesis that imprinted genes may become deregulated as a consequence of culture. Upon derivation and subsequent culture of ES cells in medium complemented with serum, we observed aberrant methylation changes in a number of growth-related imprinted genes. Significantly, these epigenetic alterations were not corrected during postimplantation development and were associated with aberrant imprinted gene expression and phenotypic abnormalities [15]. In the current study, we investigated whether in vitro culture of preimplantation mouse embryos can affect their subsequent development and can lead to a deregulation of genomic imprinting. Hence, we determined the effects of embryo culture media with or without serum on pre- and postimplantation viability and examined the regulation of different growth-related imprinted genes.

MATERIALS AND METHODS

Embryo Culture and Blastocyst Transfer

To obtain one- or two-cell embryos, superovulation was induced in (C57BL/6J × CBA/Ca) F_1 females by i.p. injection with 7.5 IU of eCG (Intervet UK Ltd., Cambridge, UK) followed 44–48 h later by an injection of 7.5 IU of hCG (Intervet UK Ltd.). Females were mated with males of the same genotype. The one-cell embryos were recovered from oviducts on Day 1, 21–23 h post-hCG. The two-cell embryos were recovered by flushing oviducts 32–34 h post-hCG injection. M16 medium (Sigma Ltd., Poole, Dorset, UK; containing 4 mg/ml BSA) was used in our studies. To test the effect of serum, 5% or 10% fetal calf serum (FCS; a single batch from Sigma) was added to the M16 medium (after heat treatment of the serum at 55°C for 30 min). Culture dishes with embryo-containing microdrops were incubated under oil at 37°C in an incubator equilibrated with 5% CO_2 in air. Control blastocysts were obtained from females of the same genotype (by flushing the uterus on Day 4 postcoitum) and were transferred immediately into uteri of mature recipient (C57BL/6 × CBA) F_1 females. To avoid artifacts due to fluctuations in the maternal contribution, the control (10 blastocysts/horn) and experimental (11 blastocysts/horn) blastocysts that were compared were always transferred contralaterally to recipient females. For this study, we chose to transfer experimental blastocysts to the left uterine horn and control blastocysts to the right uterine horn. From each group, only blastocysts that had a morphology typical of embryonic Day 4 were transferred (expanded blastocysts).

Analysis of Gene Expression and DNA Methylation

Simultaneous isolation of total RNA and genomic DNA from E14 fetuses was carried out using an RNA/DNA isolation kit following the manufacturer's (Qiagen Ltd., Crawley, West Sussex, UK) instructions. For expression analysis, 20 μg of total RNA from each sample was electrophoresed through 1.5% formaldehyde/3-*N*-morpholino-propanesulfonic acid (MOPS) agarose gels in 1× MOPS (0.2 M MOPS, pH 7.0; 0.5 M NaAc; 0.01 M EDTA). RNA was then blotted onto Hybond N^+ membranes (Amersham-Pharmacia-Biotech Ltd., Little Chalfont, Buckinghamshire, UK), which were hybridized with radioactively labeled probes as previously described [20, 31]. The following probes were used: an intragenic 1-kilobase (kb) *Bam*HI fragment from the *H19* gene (H19-1) [32], a 0.9-kb *Kpn*I-*Bam*HI fragment from exon 6 of the *Igf2* gene [31], a 2.5-kb *Eco*RI fragment from the *Mest* gene [33], a 499-base pair (bp) fragment from the 3′ end of the *Grb10* gene (nucleotides 1532–2030 in GenBank sequence U18996), a 372-bp fragment amplified by the polymerase chain reaction technique from the 5′ untranslated region (UTR) of the *Grb7* gene (nucleotides 112–464 in GenBank sequence M94450), and a 250-bp *Hin*dIII-*Pst*I fragment from the 5′ end of the *Gapdh* gene. For analysis of methylation, 10 μg of restriction enzyme-digested DNA was electrophoresed through 1% agarose gels in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). DNA was transferred onto Hybond N^+ membranes in 0.4 N NaOH, and hybridization was carried out with a 1.1-kb *Sac*I-*Bst*XI fragment from the *H19* upstream region (H19-4) [32]. Following Northern and Southern hybridization, relative band intensities were determined by using the Quantity-One image software (BioRad Ltd., Hemel Hempstead, Herts, UK).

Statistical Analysis

The comparison between control and cultured groups was made using randomized block analysis of variance on the untransformed and logarithmically transformed data, where blocks were mouse litters. The results of this parametric analysis were confirmed using randomization tests. The logarithmic transformation was made to correct for any possible lack of homogeneity of variance within litter/treatment combinations. Applying the transformation before the analysis of variance did not affect the results. Correlation between fetal weight and gene expression was tested by performing multiple regression analyses on fetal weight and the *Igf2, H19, Grb10,* and *Grb7* expression levels for fetuses in the control and serum groups. These tests were performed using the Genstat software [34].

RESULTS

Serum Affects Preimplantation Development

To analyze the effect of serum on preimplantation viability, one- and two-cell embryos were cultured to the blastocyst stage in M16 medium in the absence or presence of 5% or 10% FCS. Of all the one-cell embryos (n = 112) that were cultured in M16 medium, 92% (n = 103) had developed into cavitating or hatching blastocysts after 4 days. In M16 + 5% FCS, in contrast, only 50% (56 of 111) of the embryos became blastocysts. An even greater reduction in viability was observed in M16 + 10% FCS, in which only 35% (36 of 103) of the embryos developed into blastocysts. Most of the embryos that did not develop to the blastocyst stage in the M16 + FCS media (both the 5% and 10% FCS groups) were between the one- and the eight-cell stage after the 4 days of culture. Therefore, the presence of serum inhibits preimplantation development from one-cell embryos to blastocysts. Development from two-cell embryos, in contrast, was not inhibited by the presence of serum in the culture medium. In M16 medium, 99% (108 of 109) of the two-cell embryos had developed into blas-

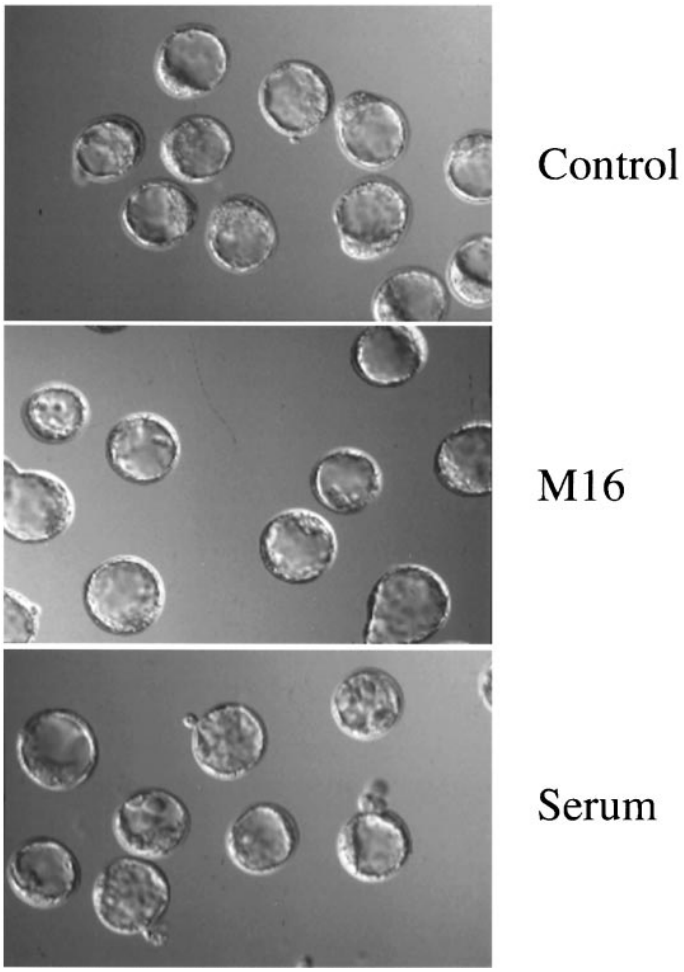

FIG. 1. Comparison of blastocyst morphology. Blastocysts were cultured from two-cell embryos (M16 and serum group) or obtained from donor females at Day 4 postcoitum (control group). Cavitating and hatching blastocysts had a similar size and morphology in all three groups. Original magnification ×100.

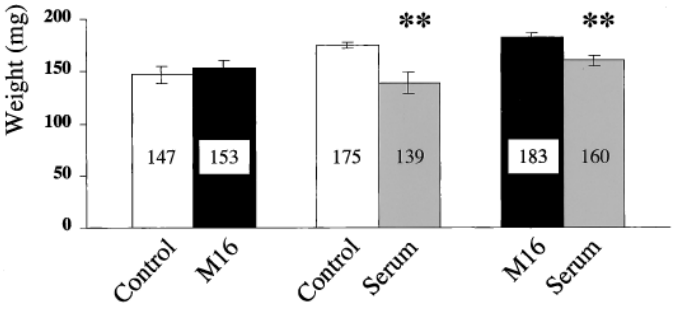

FIG. 2. The effect of serum on fetal weight. Wet weights (in milligrams) of different groups of E14 fetuses were compared. The comparison between control and M16 media shows data for 2 recipients (11 and 17 fetuses, respectively); data from 3 recipients were pooled for the comparison between control and serum group fetuses (25 and 20 fetuses, respectively). The difference in average weight between the two independent control groups reflects maternal effects and differences in the timing of blastocyst transfer and dissection of fetuses. For comparison between M16 and serum groups, data from 3 recipients were pooled (22 and 14 fetuses, respectively). Error bars represent SEM. $^{**}P < 0.001$.

tocysts after 3 days of culture. Of the two-cell embryos placed in M16 + 5% FCS, 85% (245 of 290) developed to the blastocyst stage. Similarly, 85% (68 out of 80) of the two-cell embryos that were transferred into M16 + 10% FCS became blastocysts. The cavitated and hatching blastocysts cultured in the presence of serum appeared morphologically identical to those cultured in the absence of serum and to control uterine blastocysts (Fig. 1). In two additional culture experiments, embryos cultured in M16 + 5% FCS traversed the second cell cycle more rapidly and remained for an extended period in the third cell cycle as compared with those cultured in M16 medium alone (data not shown). However, both groups formed cavitated blastocysts after 4 days of culture (Fig. 1).

Postimplantation Developmental Effects

To analyze whether serum during preimplantation culture affects postimplantation development, cultured blastocysts were transferred to the left uterine horn of recipient females. Control blastocysts flushed from a donor mother's uterus were transferred to the contralateral (right) horn of the same recipients. For these transfers, only fully expanded but not yet hatching blastocysts were selected. Because the percentage of blastocysts derived from one-cell embryos was greatly reduced in the presence of serum, all studies were carried out on blastocysts that had been grown in vitro from two-cell embryos. Fetuses derived from flushed control blastocysts (control group) were compared with fetuses derived from blastocysts that were cultured in vitro in the absence (M16 group) or presence (serum group) of 5% FCS. Fetuses were studied at embryonic Day 14 (E14) because rapid growth and organogenesis occurs at this stage of development and effects due to culture conditions would therefore be best distinguishable.

For computation of the viability of the groups of fetuses, each uterine horn was taken as a unit, and for each unit the ratio of viable fetuses to blastocysts transferred was calculated. Comparison of the averages of these ratios for each group showed that at E14 the percentage of viable fetuses derived from the serum group (33%, 66 of 202) was significantly lower ($P < 0.001$) than that derived from the control group (79%, 107 of 135). The percentage of viable fetuses developing from the M16 group of blastocysts (60%, 78 of 130), in contrast, did not differ significantly from that of the control group ($P = 0.184$). Therefore, the presence of serum during preimplantation development significantly decreased postimplantation viability. The finding that at E14 the dead conceptuses were partially resorbed cell masses (data not shown) suggested that loss occurred at an early postimplantation stage.

The average weights of the E14 fetuses were calculated for each recipient female. Comparisons were always made between fetuses of the two different groups dissected from the same recipient female. Also, to avoid minor effects that could have arisen from having reduced numbers of fetuses, comparison was made only when there were four or more fetuses present in each of the uterine horns. The results from the recipient females were then pooled, taking into account the differences that could have arisen between different recipients (see *Materials and Methods*). We found no difference in the average (±SEM) weights of fetuses from the control (147 ± 8 mg) and the M16 (153 ± 8 mg) groups (Fig. 2). However, fetuses of the serum group (139 ± 10 mg) were on average 20% lighter than the fetuses of the control group (175 ± 3 mg), and this difference was sig-

nificant ($P < 0.001$, Fig. 2). In the comparison of M16 and serum group fetuses, a significant effect of serum was observed; serum group fetuses were on 12% lighter than M16 fetuses (183 ± 3 mg versus 160 ± 3 mg; $P < 0.001$, Fig. 2).

To determine whether preimplantation culture in the presence of serum can be compatible at a low frequency with viable development until birth, we transferred 39 blastocysts of the M16 + 5% FCS group into three recipient females. This transfer gave rise to three (8%) viable newborn animals (two and one pup each in two of the recipients) that had no apparent developmental abnormalities and were killed at 2 wk of age. A similar experiment on 30 M16 group blastocysts yielded nine (30%) apparently normal newborn animals.

Aberrant Expression of Growth-Related Imprinted Genes

We studied gene expression in the M16 and the serum group of E14 fetuses in comparison with that in the control E14 fetuses to determine the extent to which preimplantation culture in the M16 and M16 + 5% FCS media had altered the expression of imprinted genes. For the evaluation of fetal weights, comparison of expression levels was always between control and cultured groups within the same recipient, and results were pooled after taking maternal effects into consideration. Several growth-related imprinted genes were analyzed, and relative expression levels were calculated as the ratio of the imprinted gene's expression to the *Gapdh* signal. To allow statistical analysis, average ratios were adjusted to 1 for the groups of control fetuses (Fig. 3). We first analyzed the expression of the neighboring imprinted *Igf2* and *H19* genes on mouse distal chromosome 7. In the M16 group of fetuses, the levels of expression of the *Igf2* (0.93 ± 0.07 relative to the control fetuses; $P = 0.24$) and *H19* (0.83 ± 0.11 relative to controls; $P = 0.07$) genes were not significantly different from those in the control fetuses (Fig. 3A). However, the comparison of the control and serum group fetuses showed significantly lower (−31%) average levels of *H19* expression in the serum group than in the control group (0.69 ± 0.05 relative to the controls; $P < 0.001$, Fig. 3B). The average level of *Igf2* expression was also significantly reduced (−15%) in the serum group as compared with the control fetuses (0.85 ± 0.05 relative to controls fetuses; $P < 0.001$, Fig. 3B).

The growth factor receptor binding 10 gene (*Grb10*) is another imprinted gene whose product affects the IGF/INS (insulin) axis [35]. It is located on proximal mouse chromosome 11 and expressed from the maternal chromosome exclusively [36]. Analysis of *Grb10* expression levels did not reveal differences between the M16 group of fetuses and the control fetuses (0.84 ± 0.1; $P = 0.09$). However, the serum group fetuses had a 27% higher *Grb10* expression than the control fetuses, and this difference was significant (1.27 ± 0.11 relative to control; $P < 0.01$). The *Grb10* probe used for Northern analysis hybridized with the 5.4-kb *Grb10* transcript and cross-hybridized with a related transcript of 2.3 kb (indicated by an arrow in Fig. 4A), and we generated a specific probe for this transcript. Two additional members of the GRB protein family, GRB7 and GRB14, have been reported in mice and are encoded by genes that are homologous to *Grb10* [37]. Using a *Grb7*-specific probe from the 5′-UTR of the gene we established that the 2.3-kb transcript corresponded to *Grb7* (Fig. 4A). Hybridization with the *Grb7* probe showed that in the serum group, the average *Grb7* expression was substantially reduced (−76%) as compared with the control group (0.24 ± 0.06 relative to control; $P < 0.001$). In 17 of the 20 serum group fetuses, *Grb7* expression was lower than in the control fetuses. The M16 group of fetuses, in contrast, did not show a significant difference in *Grb7* expression as compared with control fetuses (0.75 ± 0.17 relative to controls; $P = 0.17$).

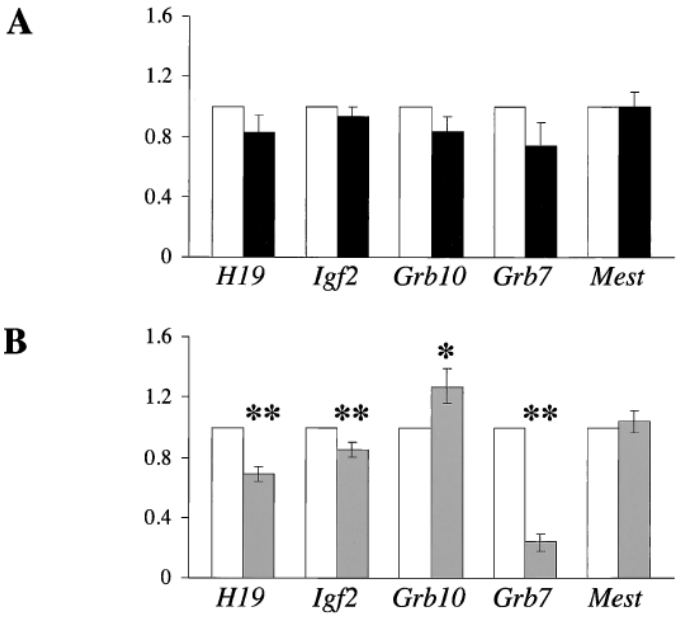

FIG. 3. Reduced expression of *Igf2, H19,* and *Grb7* and increased expression of *Grb10* in the serum group fetuses. Mean expression levels were compared between the control and the M16 group (**A**) and between the control and the serum group (**B**). Relative expression levels were calculated as the ratio between the specific-transcript's signal and the *Gapdh* signal. For *Igf2,* measurements were performed on its 1.8-kb transcript (see Fig. 4A). The expression ratios for the various genes are represented relative to the control group's mean expression ratios (which were adjusted to 1). Open bars represent control group fetuses, solid bars represent M16 group fetuses, and shaded bars represent serum group fetuses. Error bars represent SEM. *$P < 0.01$; **$P < 0.001$.

Because the imprinting status of this gene had not been reported, we carried out an allele-specific analysis of *Grb7* expression. Like *Grb10*, *Grb7* is located on mouse chromosome 11, and we therefore analyzed mice that were either maternally (Matdi11) or paternally (Patdi11) disomic for chromosome 11 to ascertain its imprinting status. We found *Grb7* expressed at equally high levels in kidneys dissected from adult Matdi11 and Patdi11 mice (Fig. 5), but the disomy-11 system did not allow us to ascertain its imprinting status in the embryo. We also analyzed the expression status of the growth-related imprinted gene *Mest* [33,38] but did not detect significant differences between control and M16 or serum group fetuses (Figs. 3 and 4A).

To test the correlation between fetal weight and gene expression, we performed regression analyses on fetal weight and the *Igf2, H19, Grb10,* and *Grb7* expression levels for fetuses in the control and serum groups (as depicted in Fig. 4B for one recipient). These calculations showed that the effect of serum on weight could not be accounted for by the combined action of the four genes ($P = 0.04$) or by the combined effects of specific combinations of two or three of the genes. Thus, these genes are noncoordinate in their deregulated expression in individual fetuses. When we considered *Grb7* on its own, however, its expression

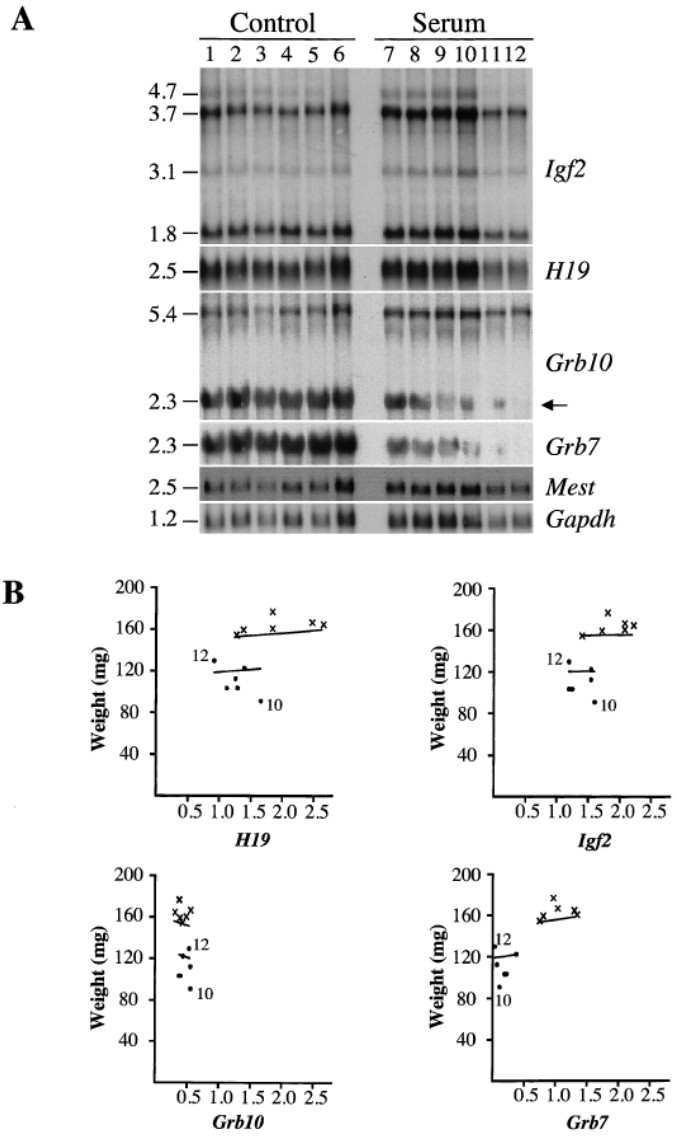

FIG. 4. Serum-induced stochastic deregulation of *Igf2, H19, Grb10,* and *Grb7*. **A**) Northern hybridization analysis of control and serum group fetuses. *H19, Igf2, Grb10, Grb7, Mest,* and *Gapdh* transcripts were analyzed using gene-specific probes (see *Materials and Methods*). Transcript sizes are indicated in kilobases. All fetuses shown (1–12) originated from a single recipient female. **B**) For the same recipient female, wet weights and relative levels of *H19, Igf2, Grb10,* and *Grb7* expression are shown for all of its fetuses. Crosses indicate data points for control fetuses, and dots are data points for serum group fetuses. Regression analysis between expression and weight was performed (lines).

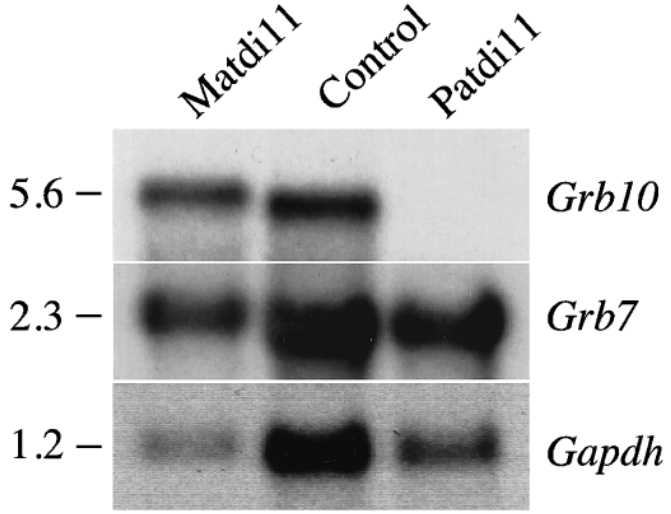

FIG. 5. Analysis of the imprinting status of *Grb7*. Mice with maternal (Matdi11) and paternal (Patdi11) uniparental disomy for chromosome 11 were generated by intercrossing heterozygotes for the Robersonian translocation Rb(11;13)Bnr [67]. Total RNA for the northern hybridization was extracted from kidney because it is one of the few tissues in which *Grb7* is highly expressed [48]. Transcript sizes are in kilobases.

was correlated significantly ($P < 0.001$) with fetal weight, and the same was observed for *H19* ($P < 0.01$) and *Grb10* ($P < 0.01$) when considered on their own. *Igf2*, when considered on its own, yielded a nonsignificant association with fetal weight ($P = 0.012$). Even though most of the weight-reduced fetuses from the serum group had reduced expression of *Igf2*, *H19*, and *Grb7*, not all fetuses showed this pattern. Similarly, only a proportion of the serum group fetuses had increased levels of *Grb10* expression. For the recipient shown in Figure 4B, for instance, fetus 10 was reduced in weight but showed normal levels of *Igf2* and *H19* expression. Although in this recipient *Grb7* expression was reduced in all fetuses of the serum group, there were a few serum fetuses (three) from other recipients that had normal levels of *Grb7* expression (data not shown). In addition, fetuses with lower *Grb7* levels did not necessarily show low levels of *H19* or *Igf2*. For example, weight-reduced fetus 10 showed low *Grb7* but normal *H19* and *Igf2* expression. Conversely, although fetus 12 had reduced levels of *Grb7, Igf2*, and *H19* expression, it was only slightly reduced in weight. Hence, in the serum group of fetuses, the *Grb7, Grb10, Igf2*, and *H19* genes are deregulated in their expression in a stochastic and noncoordinate fashion.

Because at E14 the fetuses of the serum group were on average 20% reduced in weight, there was a possibility that they were developmentally behind relative to the control fetuses. Hence, the reduction in *Igf2*, *H19*, and *Grb7* expression and the increase in *Grb10* expression could reflect their developmental stage rather than these genes being involved in the growth reduction. However, on external and internal examination of developmental stage-specific characteristics we did not find evidence that the growth-reduced fetuses were developmentally delayed (data not shown). Furthermore, we examined the levels of *Igf2*, *H19*, *Grb10*, and *Grb7* expression in control E13.5, E14, and E14.5 fetuses (four for each stage) that were of the same genotype as the experimental fetuses. This developmental analysis did not reveal detectable changes in expression levels during this 24-h period of fetal development (data not shown).

Gain of DNA Methylation at an Imprinting Control Element Upstream of H19

The allelic methylation status of a differentially methylated region (DMR) upstream of the *H19* gene [39] is essential for the imprinted expression of both *H19* and *Igf2* [40]. To test whether methylation levels in this DMR were correlated with the differences in *H19* expression levels observed between the control and the serum groups of fetuses, we assayed a methylation-sensitive *Aat*II restriction site located 3939 bp upstream from the *H19* transcription initiation site (Fig. 6A). In embryos, fetuses, and adult tissues, this *Aat*II site is fully methylated on the paternal allele and is unmethylated on the maternal allele [39, 41]. In our assay, methylation was determined as the ratio of the undigested (*Sac*I) fragment (methylated) to the *Aat*II-digested

(*Sac*I) fragment (unmethylated). To ascertain obtained methylation ratios, Southern hybridization experiments (enzymatic digestion of DNA, gel electrophoresis, transfer to filter, and hybridization) and the subsequent measurement of band intensities were performed in duplicate. No significant difference was detected in the levels of *H19* methylation between the control and M16 groups (Fig. 6B). However, in the serum group of fetuses, the methylation level was on average 19% higher than that in the control group of fetuses ($P < 0.001$). In some of the individual fetuses, the level of methylation was 25% higher than the control average value. Because only the paternal chromosome is normally methylated [39, 41], this finding suggests that the normally unmethylated maternal chromosome had become methylated at the *Aat*II restriction site in 50% of the cells in these fetuses.

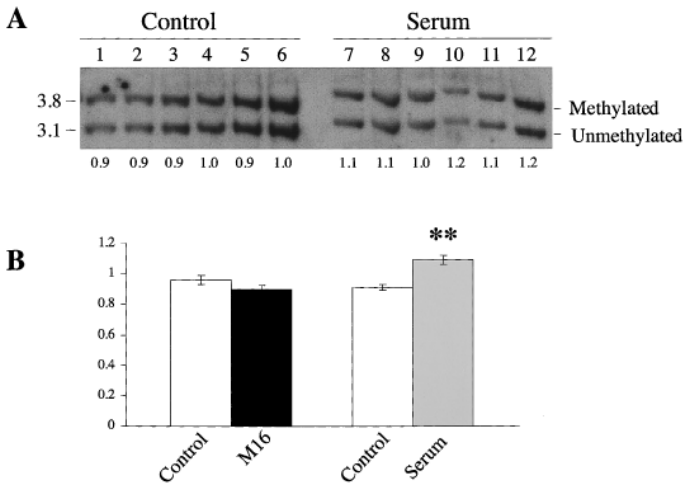

FIG. 6. **A**) Methylation at an *Aat*II site located upstream of *H19*. Genomic DNAs (1–12, corresponding to fetuses 1–12 in Fig. 4A) were digested with *Sac*I and *Aat*II and analyzed by Southern hybridization with probe H19-4. The upper band corresponds to the undigested (3.8-kb *Sac*I) fragment, and the lower band corresponds to the (3.1 kb) *Aat*II digestion product. The ratios between the intensities of these two bands (3.8-kb band:3.1-kb band) are indicated for each of the fetuses. **B**) Comparison of average methylation ratios (3.8-kb band:3.1-kb band) between control and the cultured groups of fetuses. Error bars represent SEM. $^{**}P < 0.001$.

DISCUSSION

Our results establish that serum in culture medium inhibits the in vitro development of preimplantation mouse embryos and affects their postimplantation developmental potential. The serum group embryos that were viable to E14 were reduced in weight and showed decreased expression of the imprinted *Igf2* and *H19* genes and increased expression of the imprinted gene *Grb10*. Another growth factor receptor binding gene, *Grb7*, was expressed at substantially reduced levels in almost all fetuses of the serum group.

Serum inhibited the in vitro growth of one-cell embryos; most of the embryos that had been cultured in M16 + FCS medium died or had development arrested between the one- and eight-cell stages. In agreement with reports on other defined media [42, 43], we did not observe a significant effect of M16 + FCS on development from two-cell stage embryos to blastocysts. The blastocysts that had been cultured from two-cell embryos in the presence of FCS appeared morphologically normal. However, 67% of these blastocysts did not survive to E14 after transfer into recipient females, and this result confirms findings by others on media complemented with serum [43, 44]. Thus, serum appears to have a direct or indirect adverse effect on gene(s) that are responsible for postimplantation development. FCS had a negative effect on the growth of many of the fetuses that survived to E14. However, at a strongly reduced frequency, preimplantation culture in the presence of serum can give rise to apparently normal development to term. M16 medium on its own seemed not to affect development to E14 in our study. This finding does not, however, exclude the possibility that serum-free M16 medium would not have effects at later fetal stages. For instance, a small reduction in fetal size at E17 of development has been noted in embryos that had been cultured from eight cells to blastocysts in medium without serum [8].

In the serum group of fetuses, the expression of the paternally expressed *Igf2* gene was lower than that in control fetuses, whereas the maternally expressed *Grb10* gene was increased in its expression. Given the growth-promoting roles of IGF2 via its activation of the IGF1 receptor, the observed reduction in *Igf2* expression is likely to be a determinant in the reduced weight of the serum group fetuses. GRB10, however, is thought to have a negative effect on cellular proliferation because it binds the IGF1 receptor and the insulin receptor via its *src* homology 2 (SH2) domain and could thereby inhibit their intracellular signalling [35, 45]. The increased *Grb10* expressed in the serum group fetuses, therefore, is also likely to contribute to the fetal growth reduction. Unexpectedly, expression of the *Grb10*-related gene *Grb7* was strongly reduced in the serum group of fetuses. Like GRB10, GRB7 belongs to a family of growth receptor binding adapter proteins that link specific receptor tyrosine kinases and other tyrosine phosphorylated proteins to downstream effectors [37, 46, 47]. Although its precise role is unknown, GRB7 has been shown to interact with different receptor molecules, including HER2 (erbB2), the epidermal growth factor receptor, and the insulin receptor [46, 47]. Its receptor specificity and its tissue-specific pattern of expression [48] suggest that GRB7 plays a different role in development than does GRB10. *GRB7* is overexpressed in a variety of human cancers and cancer cell lines, which suggests that it is involved in cellular proliferation [49, 50]. Although *Grb7* is not imprinted in the adult mouse, this does not exclude the possibility of imprinted expression at embryonic or fetal stages. It also remains to be determined whether the observed reduction in *Grb7* expression was caused by a direct or an indirect and possibly imprinted gene-mediated mechanism.

Statistical analysis indicated that the aberrant expression of the *Igf2*, *Grb10*, and *Grb7* genes could not account entirely for the observed reduction in weight in the serum group of fetuses. For the aberrant *Igf2*, *Grb10*, and *Grb7* expression to be the sole determinants of the growth phenotype, the association with fetal weight should have been apparent in all individual fetuses. Although we did observe a substantial (−76%) reduction of *Grb7* expression, the association between reduced fetal weight and aberrant levels of *Igf2*, *Grb10*, and *Grb7* expression was not apparent in all the serum group fetuses. Gene deregulation by culture in the presence of FCS may be a stochastic mechanism that affects the expression of multiple growth-related genes, including *Igf2*, *Grb10*, and *Grb7*. Such a stochastic effect would agree with our previous work on mouse ES cells, in which we demonstrated changes in imprinted gene expression as a consequence of in vitro culture (in the presence of FCS). In this ES cell study, alterations arose independently at different imprinted loci, and the alterations that

were observed differed among cell lines [15]. Because ES cells are representative of the inner cell mass of blastocysts, culture can influence the epigenetic regulation of gene expression at this preimplantation stage.

Given the aberrant *H19* and *Igf2* expression in the serum cultured group, we analyzed a differentially methylated region upstream of *H19* [39] that has been shown to be essential for the imprinting of *H19* and *Igf2* [40]. We found that the serum group of fetuses had significantly higher levels of methylation than the control fetuses. This increase in methylation was inversely correlated with decreased levels of *H19* expression. In some of the fetuses, the levels of *H19* methylation clearly indicated that in a considerable portion of cells they had acquired methylation on the normally unmethylated maternal chromosome, and this gain of maternal methylation is likely to be causally involved in the reduced *H19* expression. Our data on fetuses extend the finding by Sasaki and coworkers [51] that culture of preimplantation mouse embryos can lead to deregulation of *H19* imprinting in extraembryonic tissues. In these tissues, however, culture led to derepression of the normally silent *H19* allele, and it is therefore unclear whether a common causal mechanism is involved. However, it may be that the directionality of the methylation changes at *H19* is influenced by the culture medium used and by the presence of serum. In a recent study on the preimplantation stage of mouse development, Doherty and coworkers reported that culture of two-cell embryos to blastocysts in Whitten's medium (to which no serum was added) can lead to a loss of (paternal) methylation (and gain of expression) at the *H19* gene [52]. In agreement with our studies on the serum group fetuses, we previously detected gain (rather than loss) of maternal *H19* methylation upon culture of ES cells in FCS-supplemented medium [15, 32]. Using different ES cell lines, we established that methylation changes in *H19* (and other imprinted genes) are not corrected during postimplantation development and are associated with aberrant imprinted gene expression [15]. Relative to the developmental maintenance of altered imprints, it should be interesting to determine whether the increased *H19* methylation in the serum group of fetuses arose during the culture of preimplantation embryos, as has been shown for loss of *H19* methylation in Whitten's medium [52]. The serum-induced gain of methylation at the *H19* upstream region was not associated with a gain in expression of the flanking *Igf2* gene, as might have been expected from the chromatin boundary function of this imprinting control center [40]. Rather, decreased levels of *Igf2* expression were observed in the serum group fetuses. Whether *Igf2* had become deregulated via an independent epigenetic mechanism is unclear, but apparently independent deregulation of *IGF2* and *H19* has also been observed in sporadic cases of BWS in humans [29, 30].

Our study raises the question as to how embryo and cell culture can lead to improper maintenance of allelic methylation at imprinted gene loci, in particular where serum complements the medium. There was a difference in the kinetics of growth in the embryos grown in M16 + 5% FCS as compared with embryos grown in M16 medium. In particular, the embryos cultured in the presence of serum seemed to traverse the second cell cycle more rapidly and remained for an extended period in the third cell cycle; such a biphasic effect of serum also has been reported for bovine embryos [53]. Relative to the cell cycle, epigenetic regulation at imprinting control regions is highly complex. It involves DNA methylation on one of the two parental chromosomes, specialized features of chromatin, and for some regions nonhistone protein binding to the unmethylated allele. Recent data suggest that an intricate interplay between DNA methylation and such chromatin features is involved in the somatic maintenance of imprinting during the cell cycle [32, 54]. Given this considerable complexity, (serum-induced) alterations in the first cell cycles of mammalian development can result in the improper maintenance of methylation and chromatin imprints. Although the precise causal mechanisms have not been determined, methyltransferases and several chromatin regulatory complexes are cell cycle regulated and show dramatic alterations in their expression during preimplantation development [18, 55–57].

The findings in this study may be pertinent to other early embryonic interventions in the mouse that involve the use of culture media and to similar situations in other mammalian species. For instance, freezing of mouse embryos may affect fetal and postnatal development [58], and pronuclear transfer can lead to a decrease in growth of about 15% [10]. Different types of stress during early mouse development seem to have certain factors in common that can lead to a reduction in fetal growth. Such a directed acquisition of a phenotypic character as a consequence of environmental stress would agree with proposed theories on canalization and genetic assimilation [59, 60]. These theories apply in particular in instances where the newly arisen character is transmitted to the next generation(s), as for pronuclear transfer-induced growth retardation in mice [10]. In our current study on culture of mouse embryos, we could not address the heritability of the serum-induced growth reduction, but we did detect major directed shifts in the expression levels of *Igf2, Grb10,* and *Grb7,* genes that are involved in developmental signaling pathways. Support for a certain specificity in the deregulated gene expression is provided by our observation that *Mest,* another imprinted gene with a major impact on embryonic and fetal growth [38], was not altered in its expression by culture in M16 or M16 + FCS medium. In humans and ruminants, culture and manipulation-induced growth effects have also been reported. Human in vitro fertilization technologies, for instance, involve embryo culture and may give rise to reduced weight at birth [61, 62]. In cattle and sheep, intervention by manipulation and culture tends to lead to enhanced fetal growth [5, 6, 63]. In these ruminant species, other pathways may become activated or destabilized as a consequence of serum-induced environmental stress. However, in ruminant species the physiology of embryonic and extraembryonic development is dissimilar to that in multi-offspring rodent species, in which the relative size of individual offspring is regulated to a greater extent by maternal effects [64–66]. In addition, the sera used in studies of different species are frequently of diverse origin and are sometimes treated differently before use [2]. Therefore, in spite of apparent differences in the long-term phenotypic effects of serum during preimplantation culture, comparable molecular mechanisms could be involved in different mammalian species, and it would be relevant to test this hypothesis in nonrodent model systems.

ACKNOWLEDGMENTS

We thank G. Konfortova for excellent technical assistance and Dr. G. Kelsey for helpful discussion and for generating the disomy-11 mice from Rb(11;13)Bnr translocation and PCE strains (which were a kind gift from Dr. B. Cattanach).

REFERENCES

1. Lawitts JA, Biggers JD. Culture of preimplantation embryos. Methods Enzymol 1993; 225:153–164.
2. Bavister BD. Culture of preimplantation embryos: facts and artifacts. Hum Reprod Update 1995; 1:91–148.
3. Kruip TAM, den Daas JHG. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 1997; 47:43–52.
4. Walker SK, Heard TM, Seamark RF. In vitro culture of sheep embryos without coculture—successes and perspectives. Theriogenology 1992; 37:111–126.
5. Walker SK, Hartwich KM, Seamark RF. The production of unusually large offspring following embryo manipulation: concepts and challenges. Theriogenology 1996; 45:111–120.
6. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998; 3:155–163.
7. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod 1995; 53:1385–1391.
8. Bowman P, McLaren A. Viability and growth of mouse embryos after in vitro culture and fusion. J Embryol Exp Morphol 1970; 23:693–704.
9. Reik W, Römer I, Barton SC, Surani MA, Howlett SK, Klose J. Adult phenotype in the mouse can be affected by epigenetic events in the early embryo. Development 1993; 119:933–942.
10. Römer I, Reik W, Dean W, Klose J. Epigenetic inheritance in the mouse. Curr Biol 1997; 7:277–280.
11. Shamsuddin M. Effect of growth factors on bovine blastocyst development in a serum-free medium. Acta Vet Scand 1994; 35:141–147.
12. Evsikov SV, Morozova LM, Solomko AP. The role of the nucleocytoplasmic ratio in development regulation of the early mouse embryo. Development 1990; 109:323–328.
13. Latham KE, Solter D. Effect of egg composition on the developmental capacity of androgenetic mouse embryos. Development 1991; 113: 561–568.
14. Muggleton-Harris A, Whittingham DG, Wilson L. Cytoplasmic control of preimplantation development in vitro in the mouse. Nature 1982; 299:460–462.
15. Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. Development 1998; 125:2273–2282.
16. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 1993; 90:8424–8428.
17. Moore T, Reik W. Genetic conflict in early development: parental imprinting in normal and abnormal growth. Rev Reprod 1996; 1:73–77.
18. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 1997; 13:335–340.
19. Beechey CV, Cattanach BM, Selley RL. MRC Mammalian Genetics Unit, Harwell, Oxfordshire. Mouse imprinting data and references. Available at: http://www.mgu.har.mrc.ac.uk/imprinting/imptables.html.
20. Feil R, Khosla S, Cappai P, Loi P. Genomic imprinting in ruminants: allele-specific gene expression in parthenogenetic sheep. Mamm Genome 1998; 9:831–834.
21. Surani MA. Imprinting and the initiation of gene silencing in the germ line. Cell 1998; 93:309–312.
22. Constância M, Pickard B, Kelsey G, Reik W. Imprinting mechanisms. Genome Res 1998; 8:881–900.
23. Razin A, Cedar H. DNA methylation and genomic imprinting. Cell 1994; 77:473–476.
24. Kafri T, Ariel M, Brandeis M, Shemer R, Urven L, McCarrey J, Cedar H, Razin A. Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev 1992; 6:705–714.
25. Monk M, Boubelik M, Lehnert S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 1987; 99: 371–382.
26. DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor-II gene disrupted by targeting. Nature 1990; 345:78–80.
27. Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. Genes Dev 1997; 11:3128–3142.
28. Sun FL, Dean WL, Kelsey G, Allen ND, Reik W. Trans-activation of *Igf2* in a mouse model of Beckwith-Wiedemann syndrome. Nature 1997; 389:809–815.
29. Reik W, Maher ER. Imprinting in clusters: lessons from Beckwith-Wiedemann syndrome. Trends Genet 1997; 13:330–334.
30. Maher ER, Reik W. Beckwith-Wiedemann syndrome: imprinting in clusters revisited. J Clin Invest 2000; 105:247–252.
31. Feil R, Walter J, Allen ND, Reik W. Developmental control of allelic methylation in the imprinted mouse *Igf2* and *H19* genes. Development 1994; 120:2933–2943.
32. Khosla S, Aitchison A, Gregory R, Allen ND, Feil R. Parental allele-specific chromatin configuration in a boundary-imprinting-control element upstream of the mouse *H19* gene. Mol Cell Biol 1999; 19: 2556–2566.
33. Kaneko-Ishino T, Kuroiwa Y, Miyoshi N, Kohda T, Suzuki R, Yokoyama M, Viville S, Barton SC, Ishino F, Surani MA. *Peg1/Mest* imprinted gene on chromosome 6 identified by cDNA subtraction hybridisation. Nat Genet 1995; 11:52–59.
34. Payne RW, Lane PW, Digby PGN, Harding SA, Leech PK, Morgan GW, Todd AD, Thompson R, Tunnicliff, Wilson G, Welham SJ. Genstat^{TM}5, release 3: Reference Manual. Oxford, UK: Clarendon Press; 1993.
35. He W, Rose DW, Olefsky JM, Gustafson TA. Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains. J Biol Chem 1998; 273:6860–6867.
36. Miyoshi N, Kuroiwa Y, Kohda T, Shitara H, Yonekawa H, Kawabe T, Hasegawa H, Barton SC, Surani MA, Kaneko-Ishino T, Ishino F. Identification of the *Meg1/Grb10* imprinted gene on mouse proximal chromosome 11, a candidate for the Silver-Russell syndrome gene. Proc Natl Acad Sci U S A 1998; 95:1102–1107.
37. Daly, RJ. The Grb7 family of signalling proteins. Cell Signal 1998; 10:613–618.
38. Lefebvre LS, Viville S, Barton SC, Ishino F, Keverne EB, Surani MA. Abnormal behaviour and growth retardation associated with loss of the imprinted gene *Mest*. Nat Genet 1998; 20:163–169.
39. Tremblay KD, Duran KL, Bartolomei MS. A 5′ 2-kilobase-pair region of the imprinted mouse *H19* gene exhibits exclusive paternal methylation throughout development. Mol Cell Biol 1997; 17:4322–4329.
40. Thorvaldsen JL, Duran KL, Bartolomei MS. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of *H19* and *Igf2*. Genes Dev 1998; 12:3693–3702.
41. Olek A, Walter J. The pre-implantation ontogeny of the *H19* methylation imprint. Nat Genet 1997; 17:275–276.
42. Goddard MJ, Pratt HP. Control of events during early cleavage of the mouse embryo: an analysis of the '2-cell block'. J Embryol Exp Morphol 1983; 73:111–133.
43. Arny M, Nachtigall L, Quagliarello J. The effect of preimplantation culture conditions on murine embryo implantation and fetal development. Fertil Steril 1987; 48:861–865.
44. Caro CM, Trounson A. The effect of protein on preimplantation mouse embryo development in vitro. J In Vitro Fertil Embryo Transfer 1984; 1:183–187.
45. Wang J, Dai HP, Yousaf N, Moussaif M, Deng YP, Boufelliga A, Swamy OR, Leone ME, Riedel H. Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I-, and insulin-mediated mitogenesis. Mol Cell Biol 1999; 19:6217–6228.
46. Tanaka S, Mori M, Akiyoshi T, Tanaka Y, Mafune K, Wands JR, Sugimachi K. Coexpression of *Grb7* with epidermal growth factor receptor or Her2/erbB2 in human advanced esophageal carcinoma. Cancer Res 1997; 57:28–31.
47. Kasus-Jacobi A, Bereziat V, Perdereau D, Girard J, Burnol A. Evidence for an interaction between the insulin receptor and Grb7. A role of two of its binding domains, PIR and SH2. Oncogene 2000; 19: 2052–2059.
48. Leavey SF, Arend LJ, Dare H, Dressler GR, Briggs JP, Margolis BL. Expression of Grb7 growth factor receptor signalling protein in kidney development and in adult kidney. Am J Physiol 1998; 275:F770–F776.
49. Tanaka S, Mori M, Akiyoshi T, Tanaka Y, Mafune K, Wands JR, Sugimachi K. A novel variant of human Grb7 is associated with invasive esophageal carcinoma. J Clin Invest 1998; 102:821–827.

50. Stein D, Wu J, Fuqua SAW, Roonprapunt C, Yajnik V, Deustachio P, Moskow JJ, Buchberg AM, Osborne CK, Margolis B. The Sh2 domain protein Grb7 is co-amplified, overexpressed and in a tight complex with Her2 in breast cancer. EMBO J 1994; 13:1331–1340.
51. Sasaki H, Ferguson-Smith AC, Shum ASW, Barton SC, Surani MA. Temporal and spatial regulation of *H19* imprinting in normal and uniparental mouse embryos. Development 1995; 121:4195–4202.
52. Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted *H19* expression in the preimplantation embryo. Biol Reprod 2000; 62:1526–1535.
53. Pinyopummintr T, Bavister BD. In vitro-matured/in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined protein-free culture medium. Biol Reprod 1991; 45:736–742.
54. Feil R, Khosla S. Genomic imprinting in mammals: an interplay between chromatin and DNA methylation? Trends Genet 1999; 15:431–435.
55. LeGouy E, Thompson EM, Muchardt C, Renard JP. Differential preimplantation regulation of two mouse homologues of the yeast SWI2 protein. Dev Dyn 1998; 212:38–48.
56. Robertson KD, Keyomarsi K, Gonzales FA, Velicescu M, Jones PA. Differential mRNA expression of the human DNA methyltransferase (DNMTs) 1, 3A and 3B during G(0)/G1 phase transition in normal and tumor cells. Nucleic Acids Res 2000; 28:2108–2113.
57. Voncken JW, Schweizer D, Aagaard L, Sattler L, Jantsch M, van Lohuizen M. Chromatin association of the polycomb group protein BMI1 is cell cycle-regulated and correlates with its phosphorylation status. J Cell Sci 1999; 112:4627–4639.
58. Dulioust E, Toyama K, Busnel MC, Moutier R, Carlier M, Marchaland C, Ducot B, Roubertoux P, Auroux M. Long-term effects of embryo freezing in mice. Proc Natl Acad Sci U S A 1995; 92:589–593.
59. McLaren A. Too late for the midwife toad: stress, variability and Hsp90. Trends Genet 1999; 15:169–171.
60. Waddington CH. Canalization of development and inheritance of acquired characters. Nature 1942; 150:563–565.
61. Doyle P, Beral V, Maconochie N. Preterm delivery, low birthweight and small-for-gestational-age in liveborn singleton babies resulting from in-vitro fertilisation. Hum Reprod 1992; 7:425–428.
62. Tan SL, Doyle P, Campbell S, Beral V, Rizk B, Brinsden P, Mason B, Edwards RG. Obstetric outcome of in vitro fertilisation pregnancies compared with normally conceived pregnancies. Am J Obstet Gynecol 1992; 167:778–784.
63. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 1997; 278:2130–2133.
64. McLaren A. Genetic and environmental effects on foetal and placental growth in mice. J Reprod Fertil 1965; 9:79–98.
65. McLaren A, Mitchie D. Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. I. Factors affecting the implantation and survival of native and transferred eggs. J Exp Biol 1956; 33:394–416.
66. Papaioannou VE, Mkandawire J, Biggers JD. Development and phenotypic variability of genetically identical half mouse embryos. Development 1989; 106:817–827.
67. Cattanach BM, Kirk M. Chromosome-11 parental source effect upon fetal growth in mice. Genet Res 1985; 45:220–221.