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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/00, A01K 67/027, A61K 48/00, C07K 14/72, C12Q 1/68

A1

(11) International Publication Number: **WO 00/18902**

(43) International Publication Date: 6 April 2000 (06.04.00)

(21) International Application Number: PCT/GB99/03185

(22) International Filing Date: 23 September 1999 (23.09.99)

(30) Priority Data:
9820988.5 25 September 1998 (25.09.98) GB

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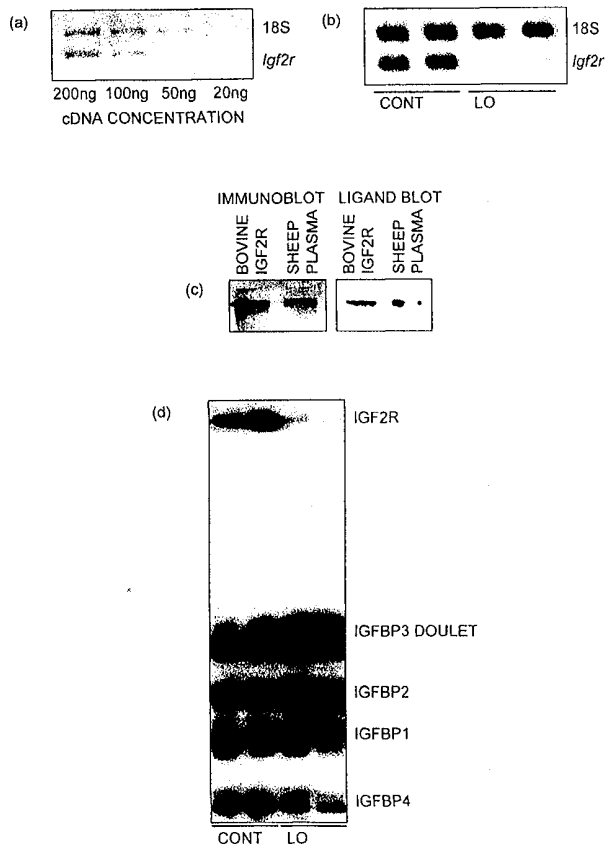
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF SCREENING FOR LARGE OFFSPRING SYNDROME

(57) Abstract

A method is provided for the diagnosis of Large Offspring Syndrome (LOS) in animals prepared by nuclear transfer or in animals prepared by methods of *in vitro* fertilisation or culture which comprises analysing Insulin–like growth factor–2 receptor *(Igf2r)* gene expression in the animal embryo or in a biological sample from the embryo.



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METHOD OF SCREENING FOR LARGE OFFSPRING SYNDROME

The present invention relates to a method of screening for abnormal development in an animal embryo or embryo-derived pluripotent cell-line and to methods for manipulating animal embryos or cells in *in vitro* culture which incorporate the screening method. The invention also relates to uses of the screening method in testing whether cell-culture environments or manipulation protocols induce abnormal development in an animal embryo.

Bovine and ovine embryos exposed to a variety of unusual environments prior to the blastocyst stage have resulted in the development of abnormally large offspring (Young *et al Reviews of Reproduction* - in press (1998)) which can also exhibit a number of organ defects, including skeletal and facial malformations (Walker *et al Theriogenology* **45** 111-120 (1996)), over-muscling and alterations in muscle fibre compositions (Maxfield *et al Am. J. Physiol.* **274** E1121-E1123 (1998)), cerebellar hypoplasia (Schmidt *et al Theriogenology* **46** 527-539 (1996)), liver and cardio-vascular defects (van Soom *et al Theriogenology* **41** 855-867 (1994); Wilmut *et al Nature* **385** 810-813 (1997); Campbell *et al Nature* **380** 383 (1996); Cibelli *et al Science* **280** 1256-1258 (1998)), urogenital tract defects (Campbell *et al Nature* **380** 383 (1996)), pulmonary defects (Cibelli *et al Science* **280** 1256-1258 (1998)), and placental defects including polyhydramnios, hydroallantois and enlarged placentomes (Cibelli *et al Science* **280** 1256-1258 (1998)). Enlargement of several organs has been reported (Farin, P. W. and Farin, C. E., *Biology of Reproduction* **52** 676-682 (1995); Cibelli *et al Science* **280** 1256-1258 (1998)) and associated features include lengthened gestation, dystocia (Kruip, T. A. M. and den Haas, J. H. G., *Theriogenology* **47** 43-52 (1997)) and physiological defects especially relating to thermoregulation, energy balance, respiration and metabolic pH regulation (Garry *et al Theriogenology* **45** 141-152 (1996)). Perinatal mortality is a

particular problem and prenatal losses are high (Walker *et al Theriogenology* **45** 111-120 (1996)). This group of symptoms is now generally described as Large Offspring Syndrome (LOS), although it has sometimes been referred to as Large Calf Syndrome (Willadsen *et al Theriogenology* **35** 161-170 (1991)). Not all features are observed in each case of LOS and in some cases, features are observed in the absence of obviously large size.

Manipulation of the pre-implantation embryo in nuclear transfer, *in vitro* culture of embryos, asynchronous embryo transfer or progesterone treatment of the mother soon after ovulation have all now been associated with such problems (reviewed by Walker *et al Theriogenology* **45** 111-120 (1996)), as has the administration of high urea diets to the mother (McEvoy *et al Animal Reproduction Science* **47** 71-90 (1997)). While alterations to the embryo environment *in vivo* have been of interest experimentally, the problems associated with LOS are of particular commercial importance in relation to *in vitro* treatments of the pre-implantation embryo, both in nuclear transfer and *in vitro* production. *In vitro* production can include any or all of the following three stages: (1) *in vitro* maturation (IVM) of the oocyte; (2) *in vitro* fertilisation (IVF) of the oocyte; and/or (3) *in vitro* culture (IVC) of the fertilised oocyte, generally to the blastocyst stage. Nuclear transfer can also include periods of *in vitro* culture, either of the oocyte prior to, during and subsequent to manipulation procedures or of the reconstructed embryo. Culture of the nuclear transfer- reconstructed embryo can either be *in vitro* or *in vivo* in a reproductive tract, usually ligated in a mammalian (usually sheep or rabbit) oviduct. Both provide unusual embryo environments and have been associated with the symptoms of LOS.

Fetal growth syndromes have also been described in humans and mice. However these are not associated with pre-implantation embryo manipulation but are,

respectively, the result of either chromosomal and other genetic abnormalities or experimental genetic manipulation. The absence of fetal overgrowth in humans and mice following embryo manipulation or culture may be due to intrinsic species differences from cattle and sheep, or, alternatively, to differences in the protocols used. For example, livestock embryos are usually transferred to recipients as blastocysts, whereas human embryos have historically been returned to the mother at around the four-cell stage and so it is possible that the human embryo is not exposed to the perturbing factor(s) at a critical period. Mouse embryos are cultured routinely to the blastocyst stage but all commonly-used media are serum free. Most cases of cattle and sheep LOS associated with *in vitro* embryo culture have involved exposure to co-cultured cells and/or serum (for example, see Walker *et al Theriogenology* **45** 111-120 (1996)), which presumably produce and/or contain the perturbing factors. Thus, it is possible that the same factors affecting bovine and ovine embryos could also affect embryos from other species in a similar manner if the culture conditions are significantly altered.

Perturbed fetal growth has important clinical significance in terms of the delivery and survival of offspring. Commercially, it is of considerable relevance in the production of bovine and ovine embryos by *in* vitro production (IVP), including *in vitro* maturation (IVM) or *in vitro* fertilisation (IVF). The technique may become increasingly relevant in the expanding use of this technology in other species, for example, in valuable individuals such as in horses (Hinrichs *Theriogenology* **49** 13-21 (1998)) and also in endangered species such as the panda (Saegusa *Nature* **394** 409 (1998)). LOS is also likely to assume greater importance in the production of transgenic animals using nuclear transfer technology. The phenomenon of LOS may well be relevant to human IVF given that the events of the pre-implantation period can affect the future prospects for offspring. For example, conventional human IVF can result in the production of babies with relatively low birth weight.

As this technology becomes increasingly invasive (e.g. intracytoplasmic sperm injection, cytoplasmic reduction, *in vitro* culture to the blastocyst stage, embryo biopsy, pre-implantation diagnosis), the health of the offspring is increasingly at risk (Walker *et al Theriogenology* **45** 111-120 (1996); Handyside *et al Trends in Genetics* **13** 270-275 (1997)).

Embryo-derived embryonal stem (ES) cells and embryonal germ (EG) cells in culture can also be used to produce transgenic or non-transgenic mice or other animals (Nagy *et al Proc. Nat'l. Acad. Sci. USA* **90** 8424-8428 (1993), Dean *et al Development* **125** 2273-2282 (1998)) or cells and tissues from such organisms. Embryonic stem (ES) cells are pluripotential cell lines isolated from the inner cell mass cells of blastocyst-stage embryos (Evans, M. J. and Kaufman, M. H., *Nature* **292** 154-156 (1981)), morulae (Eistetter, H. R. *Dev. Growth Differ.* **31** 275-282 (1989)) or isolated epiblast cells (Brook, F. A. and Gardner, R. L. *Proc. Nat'l Acad. Sci. USA* **94** 5709-5712 (1997)). The establishment of these lines and lines of pluripotential embryonal germ (EG) cells, derived from mouse primordial germ cells has enabled genetic modification of these cell lines in culture and the production of transgenic mice in which specific genes have been altered, introduced or replaced (McWhir, J., *Roslin Institute Annual Report* 1995-1996 80-83 (1996); L. M. Houdebine (ed) *Transgenic Animals: Generation and Use*, Overseas publishers Association, Amsterdam (1997)). While so far only ES and EG cells from mice have been able to contribute to the germ-line and thus produce a route to transgenic animals, there is currently much interest in the production of pluripotent cell lines from the inner cell mass or primordial germ cells of other species. Recently, there have been reports of chimeric pigs (Golueke *et al Theriogenology* **49** 238 (1998)) and cattle (Cibelli *et al Nature Biotechnology* **16** 642-646 (1998)) being produced using ES cell-like cells, derived from blastocyst inner cell mass cells. Since both *in vitro* culture (in the presence of both serum and co-cultured

cells) and nuclear transfer techniques were used in the production of the cattle ES cell-like cells, there is a high risk that such techniques may result in the abnormalities characteristic of Large Offspring syndrome. This also applies to any future isolation of EG cells from primordial germ cells, also embryo derived. In mice, changes in ES cell-lines during the derivation or culture phases may be sufficient to induce abnormalities with some similarities to LOS (Nagy *et al Proc. Nat'l. Acad. Sci. USA* **90** 8424-8428 (1993), Dean *et al Development* **125** 2273-2282 (1998)), without *in vitro* culture of the resulting embryo (since the embryo culture techniques used in these and other mice studies have not been reported to induce a phenotype similar to LOS). Since it has recently been suggested that genetically-modified bovine ES cells may provide a therapeutic source of tissue for xenotransplantation to treat human disease (Cibelli *et al Nature Biotechnology* **16** 642-646 (1998)), screening methods to ensure normal development may be of considerable value. Pluripotent human EG cells and ES cells are being actively sought and it has been suggested that these could provide an "unlimited source of *in-vitro*-derived differentiated cells to treat specific diseases by transplantation" (First, N.L. and Thomson, J., *Nature Biotechnology* **16** 620-621 (1998)). Furthermore, the possibility that a human or other oocyte may be able to reprogramme a nucleus from a differentiated human cell in a similar manner to that demonstrated recently in cattle (Cibelli *et al Science* **280** 1256-1258), sheep (Campbell *et al Nature* **380** 64-66 (1996); Wilmut *et al Nature* **385** 810-813 (1997); and Schnieke *et al Science* **278** 2130-2133 (1997)) and mice (Wakayama *et al Nature* **394** 369-373 (1998)), has also been suggested as having important therapeutic applications (First, N.L. and Thomson, J., *Nature Biotechnology* **16** 620-621 (1998)).

Other types of stem cells have been suggested which may be used to generate fetuses, cells or tissues, which may or may not be transgenic. These include

embryonic and fetal-derived stem cells as well as those which persist in the adult animal. Examples include gonadal stem cells, haematopoietic stem cells, muscle stem cells, epidermal stem cells and neuronal stem cells (WO 94/24274). Since use of these stem cells in production of fetuses, cells or tissues, by methods such as embryo aggregation, blastocyst injection or nuclear transfer, all involve periods of *in vitro* embryo and/ or cell culture, these cells may also potentially be perturbed to induce phenotypes similar to LOS.

Fetal overgrowth in humans and mice has resulted from alterations in the expression of several genes. The fetal overgrowth observed in Beckwith-Weidmann syndrome has been attributed to the *H19* and/or *Igf2* genes (Reik *et al Human Molecular Genetics* **4** 2379-2385 (1995); Eggenschwiler *et al Genes and Development* **11** 3128-3142 (1997); Sun *et al Nature* **389** 809-815 (1997)) and to the *GPC3* gene in Simpson-Golabi-Behmel syndrome (Pilia *et al Nature Genetics* **12** 241-247 (1996)). In addition, mice that are null mutants for *H19*, *Igf2* and *Igf2r* all have marked growth phenotypes. For example, *H19* null mutants are 27% heavier (Leighton *et al Nature* **375** 34-39 (1995)) and *Igf2* null mutants are 40% lighter (De Chiara *et al Cell* **64** 849-859 (1991)) than their respective wild type counterparts. Experimental deletion of the maternal *Igf2r* allele in mice also leads to a 30% increase in mean birthweight and elevated plasma concentration of the fetal mitogen, insulin-like growth factor II (IGF-II) (Lau *et al Genes and Development* **8** 2953-2963 (1994). With the exception of *GPC3*, all of these genes have been shown to be imprinted, at least in the mouse. Parental (genomic) imprinting is the process by which the differential expression of maternal and paternal alleles occurs at certain genetic loci in mammalian embryos and cells (Moore, T. and Reik, W. *Rev. Reprod.* **1** 73-77 (1996)). Aberrant expression of imprinted genes has been implicated in a range of embryonic and fetal abnormalities. Although the proximate functions of the products encoded by a

significant proportion of imprinted genes are largely unknown, at least seven imprinted genes identified to date are thought to affect growth. *Igf2*, *Mas* and *Ins2* are all paternally expressed and enhance growth rates. By analogy, *Ins1* is also expected to be a growth enhancer. *Igf2r*, *H19*, *p57KIP2* and *Mash2* are all expressed from the maternally-derived genome and reduce growth rates. (Hurst *et al Nature Genetics* **12** 234-237 (1996). In addition, several DNA binding protein genes which undergo imprinting, such as WT1, ZNF127 and Mash2, have been identified as having potential effects at other genomic loci suggesting a further layer of regulation (Moore, T. and Reik, W. *Rev. Reprod.* **1** 73-77 (1996)) and therefore of potential deregulation. One of the components of the imprinting process is the methylation of DNA at CpG dinucleotides (Moore, T. and Reik, W. *Rev. Reprod.* **1** 73-77 (1996)). In addition to their known effects on fetal growth, the imprinted genes (or those identified as imprinted in other species) have been suggested as candidates for involvement in the Large Offspring syndrome in cattle and sheep as many imprinted genes undergo significant, allele-specific changes in their DNA methylation during early embryogenesis when imprints are established or maintained (Szabo P.E. and Mann J.R. *Genes and Development* **9** 3097-3108 (1995)). These changes in DNA methylation of imprinted genes occur during normal pre-implantation embryo development in mice, at a time when virtually all other DNA in the genome is entirely demethylated (Li, E., in *Genomic Imprinting*, pages 1-20, ed.s Riek, W. and Surani, A., IRL Press, Oxford (1997); Kafri *et al Proc. Nat'l Acad. Sci. USA* **90** 10558-10562 (1993)). However, no definitive cause and effect association between expression of a particular gene or genes (whether imprinted or not) and large Offspring syndrome in cattle or sheep has been identified to date.

During the period of *in vitro* maturation, many cattle and sheep oocytes undergo *in vitro* culture in medium containing a variety of sera and co-cultured cells (Trounson

et al Theriogenology **41** 57-66 (1994); Marquant-Leguienne, B. and Humbolt, P. *Theriogenology* **49** 3-11 (1998); Sirard, M. A. and Blondin, P., *Animal Reproduction Science* **42** 417-426 (1996); Thompson J. G. *Reprod. Fertil. Dev.* **9** 341-354 (1997)), i.e. the same conditions which are associated with induction of LOS during exposure of the early embryo (Thompson *et al Biology of Reproduction* **53** 1385-1391 (1995); Sinclair *et al Theriogenology* **47** 380 (1997)). It is not yet clear whether the period of *in vitro* maturation can perturb an oocyte, in ways which induce LOS, since very few studies have addressed this question. However, some evidence is provided by the study of Holm *et al* (*Journal of Reproduction and Fertility* **107** 175-181 (1996)), which indicates that the syndrome may have arisen from oocytes matured and fertilised *in vitro* and then replaced into recipient ewes for the embryo culture period and the remainder of gestation. For many imprinted genes, imprints and associated changes in DNA methylation are established during gametogenesis and any epigenetic alterations at this stage induced by oocyte exposure to *in vitro* maturation may also result in phenotypes typical of LOS, by similar mechanisms to those proposed for embryo culture. Similarly, epigenetic alterations may arise in the oocyte when subjected to *in vitro* culture within an ovarian follicle prior to subsequent oocyte maturation. Follicle culture has recently been suggested as a means of improving the subsequent developmental competence of bovine oocytes matured *in vitro* (Fouladi Nashta *et al Biology of Reproduction* **59** 255-262 (1998)). It is also possible that, in some cases at least, abnormalities in imprinted genes intrinsic to the oocyte are responsible for initiating LOS. By using procedures such as *in vitro* maturation or superovulation, more oocytes are obtained from a single ovary than would normally mature *in vivo* in an oestrous cycle. Most of the oocytes matured by these procedures would die prior to ovulation *in vivo*. These procedures are often used to increase the availability of oocytes for *in vitro* production or nuclear transfer and may "rescue" oocytes with imprinting defects which would not normally survive *in vivo*. This type of

"rescue" may also occur during the period of embryo culture, although it is known that exposure to the period of embryo culture alone is sufficient to induce LOS (Thompson *et al Biology of Reproduction* **53** 1385-1391 (1995); Sinclair *et al Theriogenology* **47** 380 (1997))

The induction of LOS subsequent to nuclear transfer is also poorly understood. For example, it has not been demonstrated whether the perturbation is due to nuclear transfer *per se* or to the associated periods of oocyte or embryo culture. Most nuclear transfer embryos are either cultured subsequently *in vitro* using protocols associated with perturbed growth and development (for example see Yazawa *et al Theriogenology* **48** 641-650 (1997), or ligated in a sheep oviduct with no regard to synchrony of the recipient animal with the developmental stage of the reconstructed embryo (for example see Willadsen *et al Theriogenology* **35** 161-170 (1991). Exposure of sheep embryos to an asynchronous reproductive tract environment has previously been demonstrated to result in fetal oversize (Wilmut, I. and Sales, D. I. *J. Reprod. Fertil.* **61** 179-184 (1981); Young *et al Theriogenology* **45** 231 (1996)). In addition to the possible asynchrony, ligation in an oviduct is in itself an unusual environment. During the period of development to the blastocyst stage (normally used for embryo transfer to gestation recipients in cattle and sheep), embryos would naturally be in the uterus for 3-4 days and exposed to uterine secretions. Ligation of the oviduct may, therefore, expose the embryo to unusual secretions which provide a perturbing milieu, resulting in LOS. In cattle and sheep embryos genetically modified by pronuclear injection, subsequent development also commonly occurs in a ligated oviduct or in *in vitro* embryo culture.

In addition, it has been suggested (Jaenish R. *Trends in Genetics* **13** 323-329 (1997) that the successful use of nuclei for donors in nuclear transfer, in terms of

efficiency and frequency of fetal death may in part depend on the incidence of methylation errors found in imprinted genes in the donor cell. It is argued that incidence of such errors would be related to the age of the animal from which the donor cell was derived, with older cells having more errors. This awaits direct experimental evidence but such errors specifically in imprinted genes would not be erased by the demethylation and *de novo* DNA methylation events which occur during early embryogenesis (Kafri *et al Genes Dev.* **6** 705-714 (1992)), during the time of nuclear reprogramming. In some cases, these errors may provide another possible route for induction of Large Offspring syndrome.

It is believed that, in theory, any manipulation of embryos or cells used to derive embryos which may alter the environment of such cells or the genetic material of these cells, including DNA and RNA, may induce epigenetic change in a vulnerable gene or genes. Procedures involving drilling or penetration of the zona pellucida, for example during enucleation, intracytoplasmic sperm injection (Barnes *et al Human Reproduction* **10** 3243-3247 (1995)) or pre-implantation diagnosis (Handyside, A. H. and Delhanty J. D. *Trends in Genetics* **13** 270-275 (1997)), may expose the genetic material to factors normally excluded by the zona pellucida. Procedures such as cytoplasmic transfer or cytoplasmic reduction (Cohen *et al Mol. Hum. Reprod.* **4** 269-280 (1998); Cohen *et al Lancet* **350** 186-187 (1997); Eviskov *et al Development* **109** 323-328 (1990); Eviskov *et al Roux's Arch. Dev. Biol.* **203** 199-204 (1994)) may alter the concentration of cytoplasmic factors which influence DNA (Moore, T. and Riek, W., *Rev. Reprod.* **1** 73-77 (1996)) or RNA. In addition, procedures such as intracytoplasmic sperm injection or nuclear transfer, which involve introduction of foreign material into a recipient oocyte or cell, may inadvertently introduce factors into the recipient which may be perturbing.

Thus there are several possible routes for initiation of LOS in cattle and sheep, all of which may act through epigenetic alterations in imprinted genes. These include epigenetic alterations induced by in vitro culture of oocytes or embryos, exposure of oocytes or embryos to an unusual environment (including a ligated oviduct), intrinsic defects in oocytes matured in vitro or in vivo by superovulation, intrinsic defects in donor cells used for nuclear transfer and inappropriate reprogramming of imprinting subsequent to nuclear transfer.

Central to the regulation of mammalian fetal growth is insulin-like growth factor II (IGFII). IGFII regulation occurs in a dynamic and hierarchical manner with controls acting at the levels of transcription, translation, protein binding or receptor interactions (Zarrilli *et al Mol. Cell. Endocrinol* **101** R1-R4 (1994)). The *Igf2* gene is imprinted in the mouse (DeChiara *et al Cell* **64** 849-859 (1991)), human (Giannoukakis *et al Nature Genetics* **4** 94 (1993) and sheep (Feil *et al Mammalian Genome* - in press (1998); Hagemann *et al Mol. Reprod. Dev.* **50** 154-162 (1998)) and loss of imprinting consequent to the failure of imprinting mechanisms leads to biallelic expression and therefore overexpression of the *Igf2* gene. Loss of imprinting of this gene leading to fetal overgrowth in both humans and mice is thought to be caused by an increased concentration of insulin-like growth factor-II (IGFII) protein (Eggenschwiler *et al Genes Dev.* **11** 3128-3142 (1997); Sun *et al Nature* **389** 809-815 (1997)). In mice, loss of imprinting of *Igf2* has been induced by deleting the adjacent H19 gene which appears to be involved in the *Igf2* imprinting mechanism (Leighton *et al Nature* **375** 34-39 (1995); Ohlsson *et al Development* **120** 361-368 (1994)). Transgenic overexpression of the mouse *Igf2* gene results in prenatal overgrowth and other effects which are similar to those of Large offspring syndrome in cattle and sheep and Beckwith-Weidmann syndrome in humans. These include polyhydramnios, fetal and neonatal lethality, organ and skeletal abnormalities (Sun *et al Nature* **389** 809-815 (1997)).

In some cases of Beckwith-Weidmann syndrome in humans, fetal overgrowth results from disomies, trisomies or other chromosomal rearrangements of the *H19/Igf2* region. However, in many sporadic cases of the syndrome, the observed loss of imprinting and resulting biallelic overexpression of the *Igf2* gene is not associated with chromosomal abnormalities. Instead, these cases have been suggested to result from "epigenetic accidents" in the *H19/Igf2* domain in the germline or early embryo (Reik *et al Human Molecular Genetics* 4 2379-2385 (1995)), although these have not yet been identified or described and it is not known how they might arise. Environmentally-induced epigenetic alterations to imprinted genes, such as alterations in DNA methylation, were suggested as the possible cause of prenatal and perinatal losses and large size observed in mouse fetuses derived from ES cells in *in vitro* culture (Nagy *et al Proc. Nat'l Acad. Sci USA* **90** 8424-8428 (1993)). Although, it is not yet known which gene(s) is responsible for this effect, it has now recently been shown that epigenetic alterations in DNA methylation and imprinted gene expression arising from derivation or culture of mouse embryonic stem (ES) cells do not become corrected during post-implantation development and are associated with aberrant gene expression in the ES cell-derived fetus (Dean *et al Development* **125** 2273-2282 (1998)). This study found evidence of alterations in all four imprinted genes which were examined, although these were inconsistent between and within the (four) different ES cell lines tested. In particular, loss of methylation of the maternal *U2af1-rs1* allele was commonly detected and resulted in biallelic expression of this gene (which encodes a splicing factor of unknown biological function). Biallelic methylation of *H19* was also common and this resulted in strongly- repressed *H19* expression in the ES cell fetuses. In all ES cell lines studied, the maternal *Igf2* allele was unusually methylated, in addition to the paternal allele. In several fetuses, this resulted in expression of *Igf2* from both alleles, but particularly from

the maternal allele. Finally, the *Igf2r* gene was maternally methylated in all ES cell lines and in most ES cell fetuses. In all ES cell lines the pattern of *Igf2r* expression was biallelic and in the majority of ES cell fetuses was only from the maternal allele. This is consistent with the patterns of *Igf2r* methylation and expression found in normal mouse blastocyst-stage embryos (from which ES cells are derived) and fetuses. These observations confirm previous reports of the stability of *Igf2r* imprinting in mouse ES cell lines (Labosky *et al Development* **120** 3197-3204 (1994)). However, some ES fetuses derived from two ES cell lines showed partial biallelic expression of *Igf2r* (with $<35\%$ of the expression from the normally silent, paternal allele). In addition, in one ES fetus (out of 24), biallelic *Igf2r* expression was observed and in this case methylation of both parental alleles was evident.

The role of imprinting in *Igf2r* expression and the fact that lack of expression results in fetal overgrowth in mice has been used to hypothesise that a corresponding loss of imprinting in *Igf2r* (i.e. biallelic expression) would have the opposite effect to that of *Igf2* and would instead lead to a reduction in fetal growth (Moore, T. and Reik, W. *Rev. Reprod.* **1** 73-77 (1996)). No such phenotype was reported in the ES cell fetuses, neither were there any culture-induced changes in *Igf2r* which would be consistent with fetal overgrowth or other symptoms of Large Offspring Syndrome (Dean *et al Development* **125** 2273-2282 (1998)). Furthermore, the different developmental abnormalities described for the ES cell-derived fetuses, including polyhydramnios, mandibular abnormalities and interstitial bleeding have not so far been related to specific imprinted genes and may of course be associated with genes not examined in the reported study. These observations of aberrant imprinted gene expression in cultured, embryo-derived ES cells, coupled with the known developmental roles of imprinted genes and (in some cases) known phenotypic effects on fetal growth, add credibility to the

hypothesis that imprinted genes and their epigenetic alterations underlie the Large offspring syndrome in cattle and sheep subsequent to embryo culture in an unusual environment and/or nuclear transfer (Moore, T. and Reik, W. *Rev. Reprod.* **1** 73-77 (1996); Walker *et al Theriogenology* **45** 111-120 (1996); Dean *et al Development* **125** 2273-2282 (1998)). However, they shed little light on which gene or genes are directly involved.

A screening method diagnostic of inducing LOS would be of commercial benefit in identifying and improving embryo culture, nuclear transfer and other embryo manipulation protocols associated with abnormal development. There is therefore a pressing need to be able to screen effectively for the presence of abnormal development in an *in vitro* culture animal embryo to allow appropriate intervention or modification of the culture conditions.

Evidence is now provided that altered expression of the Igf2r gene encoding the insulin-like growth factor II receptor (IGFIIR) is predictive of abnormal development of an animal embryo with symptoms typically associated with Large Offspring syndrome (LOS). Embryos at risk from LOS include embryos cultured *in vitro* or in an unusual *in vivo* environment, as well as reconstituted embryos prepared by nuclear transfer.

According to a first aspect of the present invention there is provided a method of screening an animal embryo for Large Offspring Syndrome comprising the step of analysing Insulin-like growth factor-2 receptor (*Igf2r*) gene expression in the animal embryo or in a biological sample from the embryo.

The screening of animal embryos by a method according to the present invention can avoid the problems encountered where an *in vitro* culture animal embryo is

affected by the symptoms of Large Offspring Syndrome in which the embryo can grow to a large size or have associated organ defects or developmental abnormalities. The method is also applicable to an animal embryo cultured in an altered or an unusual environment *in vivo*. The use of the screening method will allow for intervention in the development of the embryo to arrest or to prevent the abnormal development. The detection of pluripotential cells and embryos or cells derived from them which are likely to result in the production of abnormal offspring would allow selection of appropriate lines for generation of chimeras or cell-derived embryos. Methods in accordance with the present invention will also permit the identification of culture conditions which do not initiate the defects leading to Large Offspring syndrome. Such methods may be of considerable commercial benefit in agricultural species and may have future applications in several other species, including humans. This may be of particular relevance to any future use of stem cells or other cells used in human cell-based therapies for medical applications (Solter, D., *Nature* **394** 315-316 (1998)).

The screening method of the present method is generally applicable to any animal embryo, including birds, such as domestic fowl, fish, eutherian and marsupial mammals, including where the embryo is the result of nuclear transfer or *in vitro* production (IVP), comprising *in vitro* culture (IVC) and/or *in vitro* fertilisation (IVF) and/or *in vitro* maturation (IVM) of an oocyte, and further including culture in any environment in which an embryo would not find itself in or exposed to during normal gestation, e.g. in a ligated oviduct in a temporary recipient animal . However, the method may find its main use in screening mammalian embryos, particularly ruminant, human or primate embryos. Other mammalian species to which the method may find application, include but are not limited to, non-human mammals, for example, ungulate species, such as cattle, sheep, pigs, goats, horses, camels or buffalo (including water buffalo), and species such as dogs, cats, horses, llamas, alpacas and rodents including rats, mice, or rabbits, or guinea pigs, and a

variety of animal (wildlife or non-domesticated) species, including pandas, tigers, and cetacean species including whales and dolphins.

The method may also find application to transgenic or genetically modified animal embryos, including chimeras and embryos prepared by nuclear transfer procedures or where the embryo has been the subject of manipulation to alter its genetic content. Such methods may also find utility in cell-based transgenics using ES cells or other cells in culture using nuclear transfer. For example, in the screening of embryos or embryo-derived cells, particularly ES or EG cells (Dinsmore *et al* *Theriogenology* **49** 145-151 (1998)) which involve modifications of the animal's genome where embryonic cells are used to make specific cell types for medical therapeutic uses, including *in vitro* differentiation of such cells.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their genome or germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line or genome has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose genome or germ line an exogenous DNA sequence has been added. In embodiments of the invention in which the animal is transgenic, the genetic modification may be undertaken using physical techniques such as microinjection into the male or female pronucleus of the zygote or into the cytoplasm or nucleus of an oocyte or embryo. Alternatively, the genetic modification can involve the use of mass transformation or transfection techniques such as electroporation, viral transfection (including the use of adenoviruses, retroviruses, adeno-associated means or synthetic

retrotransposons), lipofection, microprojectile cell bombardment, antisense technology, vectors such as YAC and BAC or by using other means such as sperm. Furthermore the modification can benefit from intervention by homologous recombination, DNA repair mechanisms, including the use of restriction enzymes. Cell-mediated transgenesis can employ a variety of cells, including ES cells, EG cells and other stem cells or suitable cells from any mammalian species.

Further applications may be found using nuclear transfer of non-genetically modified animals. For example unmodified clones may be used to multiply genetically superior or useful individuals for agricultural applications, including increased milk and beef yield or quality and increased reproductive performance. It may also be used in situations where endangered species are cloned to maintain a breed, population or species (e.g. tiger, rhino, panda, whale or dolphin). In addition, screening of an embryo for potential development of LOS-associated abnormalities may also be of immense benefit in the potential development and use of pre-implantation human embryos for cell-based therapies or of other embryos for transplantation.

Methods according to the present invention are employed to screen an animal embryo. The culture of embryos *in vitro* is now a well established technique in *in vitro* production of animals, including *in vitro* maturation of oocytes. It has found application in overcoming infertility problems in humans and in controlled breeding in agricultural farm animals commonly using *in vitro* fertilisation where an oocyte and sperm are brought together in a controlled and observable environment outside the body. *In vitro* culture of animal embryos also includes animal embryos prepared by nuclear transfer procedures or animal embryos which have been the subject of manipulation to alter their genetic content. *In vivo* culture of embryos is also commonly employed, especially after reconstruction of an embryo by nuclear

transfer or genetic modification by pronuclear injection. This generally involves surgical ligation of the embryos in the oviduct of a recipient animal, where the embryos may be embedded in a protective, permeable but inert medium, e.g. agar or an equivalent substance (Campbell *et al Biol. Reprod.* **50** 1385-1393 (1994)). The recipient animal is not always of the same species as the *in vivo* cultured animal embryo (Wilson *et al Anim. Reprod. Soc.* **38** 73-83 (1995)). The method is also applicable to the screening of any animal embryo cultured in an altered or unusual environment which may result in the development of Large Offspring Syndrome (LOS), comprising any environment in which an embryo would not find itself in or be exposed to during normal gestation. This may include transfer of the embryo from the reproductive tract from one animal to another, or to another part of the reproductive tract of the same animal. It may also include direct or indirect alterations to the reproductive tract environment. Examples of direct alterations include, but are not limited to, flushing the tract with a suitable fluid or addition of any implant, foreign substance or object. Indirect alterations may include, but are not limited to, dietary, endocrine or drug administration to the recipient animal.

The term "embryo" is used to describe the developing animal following conception and the first division of the zygote until the birth of the new-born animal. The term therefore includes more specialised descriptive terms "blastula", "gastrula", and "foetus" (or "fetus"). Fetus can be used to describe an embryo when the first bone cells appear in the cartilage, after implantation. The term also includes "blastocyst" which describes the stage of development in humans at which implantation into the uterine wall occurs in normal gestation which occurs when the inner cell mass (ICM) spreads inside the blastocoele as a flat disc. In other animal species, such as sheep and cattle, implantation may not occur until after the blastocyst stage and so this definition should be understood to extend to the stage of development which consists of inner cell mass cells and trophectoderm cells around

a central blastocoele cavity.

The method of the present invention is directed to screening for Large Offspring Syndrome (LOS). The syndrome is most commonly seen in agricultural farm animals produced by nuclear transfer procedures, *in vitro* genetic manipulation and/or *in vitro* production techniques, including the use of *in vitro* maturation, *in vitro* fertilisation and/or *in vitro* embryo culture. The phenomenon of Large Offspring Syndrome is often difficult to characterise as it is not only exhibited in terms of birthweight. There are also other associated abnormalities including increased rates of abortion and physical abnormalities, increased gestation length and increased levels of mortality and morbidity (Walker *et al Theriogenology* **45** 111-120 (1996)). The abnormal development is with respect to that of a normal animal embryo at the equivalent stage of development.

The presence of Large Offspring Syndrome (LOS) in an embryo screened using a method according to the present invention is diagnosed following an analysis of the expression of the gene coding for the insulin-like growth factor-2 receptor (IGF2R) in the animal embryo. The IGF2R is also sometimes referred to as the mannose 6-phosphate receptor, the Type II IGF receptor and the cation-independent mannose 6-phosphate receptor. References to IGF2R protein and *Igf2r* gene should therefore be understood to include these definitions. The altered or perturbed expression of the *Igf2r* gene is indicative of abnormal development in the animal embryo compared to normal levels of gene expression. The altered gene expression may be seen as a reduced level of transcription of the DNA encoding the IGF2R. This change may also be demonstrated by an altered pattern of DNA methylation in the gene coding for IGF2R in the affected embryo. Other changes in DNA, including alterations in chromatin structure or histone acetylation may also be associated with altered *Igf2r* expression. The change may also be indicated by a

reduction in the levels of IGF2R protein compared to normal embryos of the same gestational age. Where the expression of *Igf2r* in the animal is reduced this is predictive of the embryo being at risk of developing Large Offspring Syndrome (LOS). Without wishing to be bound by theory, it is believed that the reduction in the expression of the *Igf2r* gene is accompanied by no alteration in the expression of the *Igf2* gene. It will also be appreciated that a method of the present invention may also find use in screening a neonate or new-born animal as some features of LOS are not readily apparent and there may be circumstances in which a screen at this stage shortly after birth would be useful.

The analysis of gene expression is carried out on a biological sample from the embryo. The sample may comprise a tissue biopsy, cells or cellular material or a biological fluid, such as for example, blood, taken from the embryo, or amniotic or allantoic fluid from the placenta. The tissue or cell sample may be taken from an organ, muscle, cartilage, bone or skin. However, in the early stage embryo it is likely that the sample will conveniently be taken from a cell of the developing embryo. In some cases, the entire embryo may be screened. The sample may also be an oocyte or an ovarian follicle which incorporates an oocyte at any stage of development from the oogonium, through the primary oocyte stage to the secondary oocyte stage and the mature ovum.

Analysis of *Igf2r* gene expression may be achieved by any standard means including PCR based methods, e.g. RT-PCR. *Igf2r* primers can be designed from Genbank bovine sequences, for example sequence J03527 (5'-nucleotides 530-551, 3'-nucleotides 769-750; Lobel *et al J. Biol. Chem.* **263** (5) 2563-2570 (1988)).

In some cases, a comparison for control purposes can be made in a method of the invention between levels of expression of *Igf2* and *Igf2r*. Primers for *Igf2* exon-

specific transcripts can be obtained as described by Ohlsen *et al* in *DNA Cell Biol.* **13** (4) 377-388 (1994), IGFBP2, IGFBP3 as described in Winger *et al Biol. Reprod.* **56** 1415-1423 (1997) and IGFBP4 as described in Armstrong *et al Endocrinology* **139** (4) 2146-2154 (1998). *Igf2* is expressed as a series of alternatively-spliced, exon-specific transcripts in a tissue-specific and developmental stage-specific manner (Ohlsen *et al DNA Cell Biol.* **13** (4) 377-388 (1994))

A method of the present invention can therefore be considered to comprise the use of a nucleic acid sequence encoding IGF2R or a fragment thereof or a sequence complementary or homologous thereto in the preparation of an agent for the diagnosis of Large Offspring Syndrome (LOS) in an animal embryo or in a sample from the embryo. The sequence of the gene *Igf2r* encoding IGF2R is known in humans (Oshima *et al J. Biol. Chem.* **263** (5) 2553-2562 (1988); Morgan *et al Nature* **329** 301-307 (1987)), in bovines (Lobel *et al J. Biol. Chem.* **263** (5) 2563-2570 (1988)) and in mice (Ludwig *et al Gene* **142** (2) 311-312 (1994)) and the skilled person can select appropriate primer sequences based on standard molecular biological techniques (Sambrook *et al Molecular Cloning: A Laboratory Manual*, Second edition (1989)). Such nucleic acid sequences may be used directly, or more commonly they will be used to design appropriate primers for use in PCR based assays.

A nucleic acid sequence which is complementary to a nucleic acid sequence useful in a method of the present invention is a sequence which hybridises to such a sequence under stringent conditions, or a nucleic acid sequence which is homologous to or would hybridise under stringent conditions to such a sequence but for the degeneracy of the genetic code, or an oligonucleotide sequence specific for any such sequence. The nucleic acid sequences include oligonucleotides composed of nucleotides and also those composed of peptide nucleic acids. Where the nucleic sequence is based

on a fragment of the gene encoding IGF2R, the fragment may be at least any ten consecutive nucleotides from the gene, or for example an oligonucleotide composed of from 20, 30, 40, or 50 nucleotides.

Stringent conditions of hybridisation may be characterised by low salt concentrations or high temperature conditions. For example, highly stringent conditions can be defined as being hybridisation to DNA bound to a solid support in 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1mM EDTA at 65°C, and washing in 0.1xSSC/ 0.1%SDS at 68°C (Ausubel *et al* eds. "*Current Protocols in Molecular Biology*" **1**, page 2.10.3, published by Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, (1989)). In some circumstances less stringent conditions may be required. As used in the present application, moderately stringent conditions can be defined as comprising washing in 0.2xSSC/0.1%SDS at 42°C (Ausubel *et al* (1989) *supra*). Hybridisation can also be made more stringent by the addition of increasing amounts of formamide to destabilise the hybrid nucleic acid duplex. Thus particular hybridisation conditions can readily be manipulated, and will generally be selected according to the desired results. In general, convenient hybridisation temperatures in the presence of 50% formamide are 42°C for a probe which is 95 to 100% homologous to the target DNA, 37°C for 90 to 95% homology, and 32°C for 70 to 90% homology.

Examples of preferred nucleic acid sequences for use in a method of the present invention are oligonucleotide primers based on 5'-nucleotides 530-551, 3'-nucleotides 769-750 of the bovine sequence of the *Igf2r* gene (Lobel *et al J. Biol. Chem.* **263** (5) 2563-2570 (1988)).

Methods in accordance with the present invention have advantages over the techniques for *in vitro* production (including *in vitro* fertilisation and *in vitro* maturation), genetic modification and nuclear transfer previously described. The

ability to screen for abnormal development should permit the efficiency of the techniques to be enhanced by reducing the number of unsuccessful pregnancies where an embryo develops abnormally and dies during embryonic development, or at birth or shortly thereafter. The identification of embryos at risk from developing abnormally should also permit measures to be taken to safeguard the health of the female carrying the embryo.

According to a second aspect of the present invention there is provided a method for *in vitro* production of an animal embryo comprising the steps of introducing a sperm cell to an oocyte in an *in vitro* culture system, subsequently culturing the resulting embryo and screening the embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in a biological sample from the embryo.

Generally, the oocyte to be fertilised will be subjected to *in vitro* maturation step prior to being brought into proximity with the sperm cell. *In vitro* production of an animal embryo will typically involve *in vitro* fertilisation of animal oocytes and can be achieved by any suitable method depending upon the animal species. Examples of such methods include, but are not limited to those of Trounson *et al* *Theriogenology* **41** 57-66 (1994); Thompson, J. G. *Reprod. Fertil. Dev.* **9** 341-354 (1997); Wilmut *et al* in *Genetics of the Sheep*, pages 395-412 ed.s Piper, L. and Ruvinsky, A., CAB International, Oxford, UK (1997); Gardner, D. K. and Lane, M, *Human Reproduction Update* **3** 367-382 (1997); Summers *et al Biol. Reprod.* **53** 431-437 (1995); Weston, A. M. and Wolf, D. P. *Mol. Reprod. Dev.* **44** 88-92 (1996); Liu *et al Mol. Reprod. Dev.* **45** 157-162 (1996); Li *et al Theriogenology* **47** 1103-1113 (1997); Biggers *et al Human Reproduction Update* **3** 125-135 (1997); Trounson, A. and Gardner, D. K. ed.s *Handbook of in vitro Fertilisation*, CRC Press Inc. Salem, USA (1993)). This aspect of the invention may also involve *in vitro* culture of the resultant animal embryos. It will also be readily appreciated

that a method according to this aspect of the invention may also include further screening steps for the viability of the embryo and its suitability for transfer to the final female recipient. Additionally, the method also extends to an embryo which is the result of normal fertilisation but which is subsequently removed from the female animal and cultured *in vitro* for a period of time prior to re-implantation in a receptive female animal.

Oocytes suitable for *in vitro* maturation are commonly derived from either aspirating fresh abattoir ovaries, or from donor animals or patients, using surgical laparoscopy or ultrasound-guided "ovum pick-up" (OPU) (Wilmut *et al* in *Genetics of the Sheep*, pages 395-412 ed.s Piper, L. and Ruvinsky, A., CAB International, Oxford, UK (1997); Trounson, A. and Gardner, D. K. ed.s *Handbook of in vitro Fertilisation*, CRC Press Inc. Salem, USA (1993)). In some cases the donors may have been pre-treated with hormones or by other agents. *In vitro* maturation is most commonly performed in tissue culture medium 199 with a variety of supplements, including co-cultured support cells, gonadotrophic hormones, oestradiol, growth factors, pyruvate, serum and polyvinyl alcohol (Thompson, J. G. *Reprod. Fertil. Dev.* **9** 341-354 (1997); Barnes *et al Human Reproduction* **10** 3423-3247 (1995)) which allows development of an oocyte to the stage at which it may be fertilised.

In vitro fertilisation can occur in any conditions which support both motility and capacitation of spermatozoa, yet also maintain the viability of the oocytes to be fertilised. These conditions vary widely between species. As distinct from ruminants, for example, human sperm do not require any particular induction of capacitation (Trounson *et al Theriogenology* **41** 57-66 (1994)). For bovine oocytes, the most commonly used fertilisation medium is Tyrode's albumin lactate pyruvate medium (TALP) usually supplemented with several capacitating and motility

stimulating agents such as heparin, epinephrine, hypotaurine and penicillamine (Thompson, J. G. *Reprod. Fertil. Dev.* **9** 341-354 (1997)). In contrast to the cow, simple media such as synthetic oviduct fluid (SOF) are routinely used for sheep *in vitro* fertilisation. Motile sperm are prepared by several methods, including "swimming-up" and using Percoll gradients (Wilmut *et al* in *Genetics of the Sheep*, pages 395-412 ed.s Piper, L. and Ruvinsky, A., CAB International, Oxford, UK (1997)).

In vitro methods for the culture of embryos produced from either *in vitro* maturation and/or fertilisation protocols or from superovulation regimes and donor embryo recovery vary widely both between and within species. There are essentially two culture systems: co-culture (or medium previously "conditioned" on cells) and defined (or semi-defined). Co-culture of cattle and sheep embryos is normally performed in either tissue culture medium 199 or Menezo's B2 medium, usually supplemented with serum. The support cells are usually bovine granulosa, bovine oviduct epithelial or buffalo rat liver cells. Embryo culture systems without somatic cell support are commonly referred to as semi-defined or defined systems, depending on whether sera (or a protein source such as bovine serum albumin) or synthetic macromolecule (such as polyvinyl alcohol) supplementation is used. Widely-used defined media include synthetic oviduct fluid (SOF) and CR1, to which a wide variety of sera and other supplements (such as growth factors, hormones, vitamins, amino acids, enzymes and antioxidants) may be added (Thompson, J. G. *Reprod. Fertil. Dev.* **9** 341-354 (1997)). Embryos have also been cultured in bodily fluids or media which contain bodily fluids such as follicular fluid and human amniotic fluid (Trounson *et al Theriogenology* **41** 57-66 (1994)). In addition to static embryo culture systems, the use of perfusion systems for continuous delivery of fresh culture medium are being developed (Thompson, J. G. *Theriogenology* **45** 27-40 (1996)).

Cattle and sheep embryos are routinely cultured to the blastocyst stage (after 5-7 days in embryo culture) prior to transfer into a suitable recipient animal while human embryos are generally transferred to the uterus on day 2 or 3 of development. However, culture of human embryos to the blastocyst stage is becoming more widely contemplated (Gardner, D. K. and Lane, M, *Human Reproduction Update* 3 367-382 (1997)) or at least up until development of the primitive streak. The primitive streak can be taken to have appeared in an embryo not later than the end of the period of 14 days beginning with the day the gametes are mixed, not counting any time during which the embryo is stored. *In vitro* embryo culture is required as part of many current and potential applications as well as for assisted reproduction in humans and other species. Many genetically-manipulated embryos produced by pronuclear injection have been cultured prior to transfer into a recipient, as have many embryos reconstructed by nuclear transfer. *In vitro* embryo culture has been used in protocols for the derivation of embryonic stem cells (ES cells) and ES cell-like cells and may be important in the development of cell-based therapies for clinical use. Despite a considerable research effort and great improvement in effectiveness of the techniques, there is still not a method of producing embryos at a comparable rate to *in vivo* (Wilmut *et al* in *Genetics of the Sheep*, pages 395-412 ed.s Piper, L. and Ruvinsky, A., CAB International, Oxford, UK (1997)).

According to a third aspect of the invention there is provided a method for reconstituting an animal embryo comprising the step of transferring a nucleus from a donor cell into a suitable recipient cell and screening the resulting embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in a biological sample from the embryo.

In the method of the invention described in the above aspect, a nucleus is transferred from a donor cell to a recipient cell. The use of this method is not restricted to a particular donor cell type. The donor cell may be as described in Wilmut *et al Nature* **385** 810 (1997); Campbell *et al Nature* **380** 64-66 (1996); Cibelli *et al Science* **280** 1256-1258 (1998); or Wakayama *et al Nature* **394** 369-373 (1998)). All cells of normal karyotype, including embryonic, foetal and adult somatic cells which can be used successfully in nuclear transfer may in principle be employed in a method according to the present invention. Foetal fibroblasts are a particularly useful class of donor cells. Generally suitable methods of nuclear transfer are described in Campbell *et al Theriogenology* **43** 181 (1995), Collas *et al Mol. Reprod. Dev.* **38** 264-267 (1994), Keefer *et al Biol. Reprod.* **50** 935-939 (1994), Sims *et al Proc. Nat'l. Acad. Sci. USA* **90** 6143-6147 (1993), Wakayama *et al Nature* **394** 369-373 (1998), WO-A-9426884, WO-A-9424274, WO-A-9807841, WO-A-9827214, WO-A-9003432, US-A-4994384 and US-A-5057420. The invention therefore contemplates the use of an at least partially differentiated cell, including a fully differentiated cell. Donor cells may be, but do not have to be, in culture and may be quiescent. Nuclear donor cells which are quiescent are cells which can be induced to enter quiescence or exist in a quiescent state *in vivo*. Cultured bovine primary fibroblasts, an embryo-derived ovine cell line (TNT4), an ovine mammary epithelial cell derived cell line (OME) from a 6 year old adult sheep, a fibroblast cell line derived from foetal ovine tissue (BLWF1) and an epithelial-like cell line derived from a 9-day old sheep embryo (SEC1) are described in WO-A-9707669 and WO-A-9707668. A class of embryo-derived cell lines useful in the invention which includes the TNT4 cell line described in WO-A-9607732. Cultured inner cell mass (CICM) cells are described in WO-A-9737009 and WO-A-9827214 and embryonic or stem-like cell lines are described in WO-A-9807841. Transgenic bovine fibroblasts for use as nuclear donors are described in Zawada *et al* (*Nature Medicine* **4** (5) 569-574 (1998) and in Cibelli *et al* (*Science* **280** 1256-1258 (1998)).

Where the donor cells are described as being quiescent, such cells may not be actively proliferating by means of the mitotic cell cycle. The use of a quiescent donor cell is described in WO-A-9707669. The mitotic cell cycle has four distinct phases, G1, S, G2 and M. The beginning event in the cell cycle, called *start*, takes place in the G1 phase and has a unique function. The decision or commitment to undergo another cell cycle is made at *start*. Once a cell has passed through *start*, it passes through the remainder of the G1 phase, which is the pre-DNA synthesis phase. The second stage, the S phase, is when DNA synthesis takes place. This is followed by the G2 phase, which is the period between DNA synthesis and mitosis. Mitosis itself occurs at the M phase. Quiescent cells (which include cells in which quiescence has been induced as well as those cells which are naturally quiescent, such as certain fully differentiated cells, e.g. Sertoli cells, neurons or cumulus cells) are generally regarded as not being in any of these four phases of the cycle; they are usually described as being in a G0 state, so as to indicate that they would not normally progress through the cycle, or are not actively dividing by means of the cell cycle. The nuclei of quiescent G0 cells have a diploid DNA content.

Cultured cells can be induced to enter the quiescent state by various methods including chemical treatments, nutrient deprivation, growth inhibition or manipulation of gene or protein expression. Presently the reduction of serum levels in the culture medium has been used successfully to induce quiescence in both ovine and bovine cell lines. In this situation, the cells exit the growth cycle during the G1 phase and arrest, as explained above, in the so-called G0 stage. Such cells can remain in this state for several days (possibly longer depending upon the cell) until re-stimulated when they re-enter the growth cycle. Quiescent cells arrested in the G0 state are diploid. The G0 state is the point in the cell cycle from which cells are able to differentiate. On quiescence a number of metabolic changes have been reported and these include:

monophosphorylated histones, ciliated centrioles, reduction or complete cessation in all protein synthesis, increased proteolysis, decrease in transcription and increased turnover of RNA resulting in a reduction in total cell RNA, disaggregation of polyribosomes, accumulation of inactive 80S ribosomes and chromatin condensation (reviewed Whitfield *et al.*, *Control of Animal Cell Proliferation*, **1** 331-365 (1985)).

Many of these features are those which are required to occur following transfer of a nucleus to an enucleated oocyte. The fact that the G0 state is associated with cell differentiation suggests that this may provide a nuclear/chromatin structure which is more amenable to either remodelling and/or reprogramming by the recipient cell cytoplasm. In this way, by virtue of the nuclear donor cells being in the quiescent state, the chromatin of the nuclei of the donors may be modified before embryo reconstitution or reconstruction such that the nuclei are able to direct development. This differs from all previously reported methods of nuclear transfer in that the chromatin of donor cells is modified prior to the use of the cells as nuclear donors.

The recipient cell to which the nucleus from the donor cell is transferred may be an oocyte or another suitable cell. Reprogramming of cell other than oocytes has been demonstrated, including embryonic stem cells (Matveeva *et al Mol. Reprod. Dev.* **50** 128-138 (1998) and embryonic germ cells (Tada *et al EMBO J.* **16** 6510-6520 (1997)).

Recipient cells at a variety of different stages of development may be used, from oocytes at metaphase I through metaphase II, to zygotes and two-cell embryos (Cheong *et al Jpn. J. Vet. Res.* **40** 149-150 (1992)). Each has its advantages and disadvantages. The use of fertilised eggs ensures efficient activation whereas parthenogenetic activation is required with oocytes (see below). Another mechanism that may favour the use of cleavage-stage embryos in some species is the extent to

which reprogramming of gene expression is required. Transcription is initiated during the second cell cycle in the mouse and no major changes in the nature of the proteins being synthesised are revealed by two-dimensional electrophoresis until the blastocyst stage (Howlett & Bolton *J. Embryol. Exp. Morphol.* **87** 175-206 (1985)). In most cases, though, the recipient cells will be oocytes. In cattle and sheep embryos, transcription is initiated later, during the fourth cell cycle, and so later cleavage-stage embryos may be suitable in these species. In most cases though, the recipient cells will be oocytes.

It is preferred that the recipient be enucleate. While it has been generally assumed that enucleation of recipient oocytes in nuclear transfer procedures is essential, there is no published experimental confirmation of this judgement. The original procedure described for ungulates involved splitting the cell into two halves, one of which was likely to be enucleated (Willadsen *Nature* **320** (6) 63-65 (1986)). This procedure has the disadvantage that the other unknown half will still have the metaphase apparatus and that the reduction in volume of the cytoplasm is believed to accelerate the pattern of differentiation of the new embryo (Eviskov *et al., Development* **109** 322-328 (1990)).

More recently, different procedures have been used in attempts to remove the chromosomes with a minimum of cytoplasm. Aspiration of the first polar body and neighbouring cytoplasm was found to remove the metaphase II apparatus in 67% of sheep oocytes (Smith & Wilmut *Biol. Reprod.* **40** 1027-1035 (1989)). Only with the use of DNA-specific fluorochrome (Hoechst 33342) was a method provided by which enucleation would be guaranteed with the minimum reduction in cytoplasmic volume (Tsunoda *et al., J. Reprod. Fertil.* **82** 173 (1988)). In livestock species, this is probably the method of routine use at present (Prather & First *J. Reprod. Fertil. Suppl.* **41** 125 (1990), Westhusin *et al., Biol. Reprod. (Suppl.)* **42** 176 (1990)).

There have been very few reports of non-invasive approaches to enucleation in mammals, whereas in amphibians, irradiation with ultraviolet light is used as a routine procedure (Gurdon *Q. J. Microsc. Soc.* **101** 299-311 (1960)). There are no detailed reports of the use of this approach in mammals, although during the use of DNA-specific fluorochrome it was noted that exposure of mouse oocytes to ultraviolet light for more than 30 seconds reduced the developmental potential of the cell (Tsunoda *et al., J. Reprod. Fertil.* **82** 173 (1988)).

Transfer of the donor nucleus into the recipient oocyte may be by any suitable procedure known in the art. Techniques, include but are not limited to, fusion or injection, i.e. microinjection as described in Wilmut *et al Nature* **385** 810 (1997); Campbell *et al Nature* **380** 64-66 (1996); Cibelli *et al Science* **280** 1256-1258 (1998); or Wakayama *et al Nature* **394** 369-373 (1998)).

The reconstituted animal embryo may be cultured *in vitro* under suitable conditions prior to transfer into a recipient animal. Culture may up to and including any convenient stage in embryo development, e.g. to the morula, gastrula or blastocyst stage, or to a defined number of cells, e.g. 16-cell, 32-cell or 64-cell. Alternatively, culture may be up to development of the primitive streak. The primitive streak can be taken to have appeared in a human embryo not later than the end of the period of 14 days beginning with the day when nuclear transfer was carried out not counting any time during which the embryo is stored. The process may also involve temporary embryo culture *in vivo*. Experience suggests that embryos derived by nuclear transfer are different from normal embryos and sometimes benefit from or even require culture conditions *in vivo* other than those in which embryos are usually cultured (at least *in vivo*). The reason for this is not known. In routine multiplication of bovine embryos, for example, reconstituted

embryos (many of them at once) have been cultured in sheep oviducts for 5 to 6 days (as described by Willadsen, in Mammalian Egg Transfer (Adams, E. E., ed.) 185 CRC Press, Boca Raton, Florida (1982)). In order to protect a embryo derived from nuclear transfer or *in vitro* fertilisation procedures, it may be necessary to embed the embryo in a protective medium such as agar before implantation and then dissected from the agar after recovery from the temporary recipient. The function of the protective agar or other medium is twofold: first, it acts as a structural aid for the embryo by holding the zona pellucida together; and secondly it acts as a barrier to cells of the recipient animal's immune system.

If development up to the blastocyst stage has taken place *in vitro*, then transfer into the final recipient animal takes place at this stage. If, however, blastocyst development has taken place *in vivo*, although in principle the blastocyst can be allowed to develop to term in the pre-blastocyst host, in practice the blastocyst will usually be removed from the (temporary) pre-blastocyst recipient and, after dissection from the protective medium, will be transferred to the (permanent) post-blastocyst recipient to allow development to term. Culture of a large number of embryos in a single temporary recipient reduces the numbers of such animals required but a smaller number of embryos suitable to establish a successful pregnancy in the relevant species is usually transferred to permanent recipients.

According to a fourth aspect of the invention there is provided a method of reconstituting an animal embryo, the process comprising transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division without concomitantly activating the oocyte, keeping the nucleus exposed to the cytoplasm of the recipient for a period of time sufficient for the embryo to become capable of giving rise to a live birth and subsequently activating the reconstituted embryo while maintaining correct ploidy, including screening the

embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in the embryo or in a biological sample from the embryo. Recipient oocytes according to this aspect of the invention are fully described in WO-A-9707668. Other features are as described previously above.

According to a fifth aspect of the present invention there is provided a reconstituted animal embryo prepared by a method as described previously in the third or fourth aspects of the invention or an animal embryo prepared by *in vitro* production as described in accordance with the second aspect of the invention.

According to a sixth aspect of the present invention there is provided a method for preparing an animal, the method comprising:

(a) culturing an animal embryo *in vitro* or *in vivo* environments including where the embryo has been prepared by *in vitro* techniques such as maturation, fertilisation and/or culture, where the embryo has been subjected to pronuclear injection, or by reconstituting an animal embryo as described above;

(b) screening the embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in the embryo or in a biological sample from the embryo;

(c) causing an animal to develop to term from the embryo; and

(d) optionally breeding from the animal so formed.

Steps (a) and (b) have been described in depth above. The third step (c), in this

method of the present invention is to cause an animal to develop to term from the embryo. This may be done directly or indirectly. In direct development, the reconstituted embryo from step (b) is simply allowed to develop without further intervention beyond any that may be necessary to allow the development to take place. In indirect development, however, the embryo may be manipulated before full development takes place. For example, the embryo may be split and the cells clonally expanded, for the purpose of improving yield. Alternatively, or additionally, it may be possible for increased yields of viable embryos to be achieved by means of any aspect of the present invention by clonal expansion or by serial nuclear transfer. Serial nuclear transfer may be useful if a transgenic or chimeric animal embryo is to be prepared by nuclear transfer where more than one transgene is to be inserted into the animal embryo's DNA content. For example, after nuclear transfer from a donor cell (which may itself be transgenic) and embryo reconstitution, the resulting embryo can be split and its cells subject to insertion of a further transgene or transgenes to be used as a donor cell in further nuclear transfer. At each stage in the process, the embryo can be screened by a method according to the present invention to check for abnormal development. In optional step (d) of this aspect of the invention, animals may be bred from the animal prepared by the preceding steps. In this way an animal may be used to establish a herd or a flock of animals having the desired genetic characteristic(s). Where an animal is transgenic, further screening of the reconstituted embryo may be carried out to select for the stable integration of the transgene(s) and correct genotype/phenotype.

By way of illustration and summary, the following scheme sets out a typical process by which transgenic and non-transgenic animals may be prepared. The process can be regarded as involving four steps:

(1) preparation of *in vitro* culture embryo by nuclear transfer or *in vitro* production;

(2) screening the embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in a biological sample from the embryo;

(3) select embryos which show normal patterns of *Igf2r* gene expression

(4) transfer embryo to recipient female animal.

According to a seventh aspect of the invention, there is provided an animal prepared as described above.

According to an eighth aspect of the present invention there is provided a method of screening an animal cell for Large Offspring Syndrome comprising the step of analysing *Igf2r* gene expression in the animal cell.

A screening method as described above allows the identification of animal cells which are at risk from contributing to the development of Large Offspring Syndrome when they are used in the *in vitro* production of an animal embryo or in a nuclear transfer procedure. The animal cell may obtained from an *in vitro* or an *in vivo* source.

Cells which can be screened in accordance with this aspect of the present invention include any animal cell which may be cultured *in vitro*. The definition includes a reconstituted single-celled embryo or a zygote. For example, embryonic stem cells (ES cells), embryonic germ cells (EG cells), sperm derived stem cells or other stem

cells derived from an embryo, neonate, infant, adolescent or adult animal may be screened. In the context of the present invention, the animal stem cells may be pluripotent stem cells, embryonic stem (ES) cells, embryonic germ (EG) cells (primordial germ cell-derived or PGC-derived cells), somatic stem/progenitor cells, haematopoietic stem cells, epidermal stem cells or neuronal stem cells. A totipotent cell can direct the development of a whole animal (when constructing embryos by nuclear transfer from a donor cell into a recipient cell, such as an enucleated oocyte, it is the nucleus of the donor cell which is totipotent). This includes directing the development of extra-embryonic lineages, i.e. the placenta. In this definition, a fertilised zygote and in some species individual blastomeres are also totipotent. In contradistinction, a pluripotent or multipotent cell (i.e. an embryonic stem cell) type has been defined as one which can form all tissues in the conceptus/offspring after injection into the blastocoele cavity. In a preferred embodiment of the present invention, the animal stem cells may be embryonic stem (ES) cells or embryonic germ (EG) cells. In this definition, the terms embryonic stem (ES) and embryonic germ (EG) cells are not limited to those derived from mice but relate to any equivalent cell, such as those described as embryonic stem cell-like cells in other species.

Other cells which may be usefully screened by a method of this aspect of the invention include any cell used in or prepared by nuclear transfer or embryo reconstruction using tetraploid aggregation, blastocyst injection etc., or a cell which is the result of serial nuclear transfer. Typically such cell types include, but are not limited to, differentiated or undifferentiated cells which may also be transgenic, suitably fibroblasts. Undifferentiated cells may further be induced to differentiate by standard techniques known in the art.

It will be appreciated that a particular application of a method according to the first

aspect of the invention will be in the testing of culture environments, including culture media and conditions, *in vivo* environments and embryo manipulation procedures, to determine whether an animal embryo cultured in such a system might be at risk from developing Large Offspring Syndrome (LOS). According to a ninth aspect of the present invention there is provided a method of testing an embryo culture environment for the induction of Large Offspring Syndrome in an animal embryo comprising the step of analysing *Igf2r* gene expression in a biological sample from the embryo.

A method of testing for the ability of an embryo culture environment to induce LOS in an animal embryo is especially advantageous in providing the means to determine which features of the system are responsible for the perturbation in the development of an animal embryo in embryo culture. In this regard, it is likely to find use in optimising embryo culture protocols for commercial application. This aspect of the present invention therefore also extends to the use of a method in accordance with the first aspect of the invention to test an embryo culture environment for the induction of Large Offspring Syndrome in an animal embryo.

An embryo culture environment can include embryo manipulation procedures, embryo culture procedures and nuclear transfer procedures. Typically, embryo manipulation procedures involve drilling or penetration of the zona pellucida to assist in blastocyst hatching, for sperm introduction techniques such as ICSI (intracytoplasmic sperm injection; Barnes *et al Human Reproduction* **10** 3243-3247 (1995)), for cytoplasmic reduction or replacement (Cohen *et al Mol. Hum. Reprod.* **4** 269-280 (1998)); Cohen *et al Lancet* **350** 186-187 (1997)), for enucleation, transfer or removal of nuclei, pronuclei, mitochondria or any genetic material and for pre-implantation diagnosis (Handyside, A. H. and Delhanty, J. D. *Trends in Genetics* **13** 270-275 (1997)). Other techniques of embryo manipulation include

transfer of an embryo from the reproductive tract of one animal to another or to another part of the same reproductive tract. In some procedures, the oviduct is ligated with or without the embryos in agar blocks or an equivalent. The embryo's environment may also be modified by flushing or addition of a foreign substance or implant, e.g. hormones, dietary supplements or any change in the maternal environment. Embryo culture procedures include methods for the preparation and maintenance of stem cells or any other cells used for nuclear transfer or embryo reconstruction using methods such as tetraploid aggregation and blastocyst injection. Culture procedures may also include co-culture or the use of perfusion systems as well as static culture. Nuclear transfer procedures and pro-nuclear or intracytoplasmic injection of DNA are described above. Embryo culture media are well known in the art and typically contain nutrients, serum, growth factors, hormones, vitamins, anti-oxidants, enzymes and other components. The culture media may also include body fluids, e.g. follicular fluid.

The results of a method in accordance with this aspect of the invention will enable the skilled person to alter the conditions of the embryo culture environment to prevent the induction of LOS by adding or removing one or more components, or altering the concentration of a component. For example, changes in temperature, pH or gas environment; changes in levels of components such as methylating or demethylating agents, e.g. methyl transferases, 5-azacytidine, polyamines etc; changes in the expression of the *Igf2r* gene or the IGFIIR protein by genetic or chemical modifications, e.g. transgenesis or use of antisense oligonucleotides; addition of enzymic or other scavengers of toxic substances e.g. use of glutamate dehydrogenase to remove ammonia (Gardner, D. K. *Cell Biol. Int.* **18** 1163-1179)).

The use of such a screening method will enable the identification of the factor(s)

which act on embryos, cells and genetic material, including DNA or RNA, to perturb the development of an animal embryo by affecting expression of the *Igf2r* gene. This will allow the design of new or modified *in vitro* culture systems, embryo environments, including temporary *in vivo* systems, and nuclear transfer procedures which avoid the syndrome. It may also be possible to use the screen to determine the stage in development at which the perturbation of growth occurs which may provide for alternative means for changing the culture protocol.

Embryos which have been screened according to a method of the present invention or prepared by a method which incorporates a step for screening for LOS can be used as sources of additional nuclear donor cells as described above. The embryos can be further utilised as a source of cells to produce a cell line which may be an ES cell or ES cell-like cell. Animal cells or cell lines derived from such embryos could also be used in cell-transplantation therapy. Accordingly, there is provided in a further aspect of the invention a method of therapy comprising the administration of animal cells to a patient, wherein the cells have been prepared from an embryo screened according to a method as described above, or which has been prepared according to a method incorporating such a screening step. This aspect of the invention extends to the use of such cells in medicine, e.g. cell-transplantation therapy, and also to the use of cells derived from such embryos in the preparation of a cell or tissue graft for transplantation. The cells may be organised into tissues, for example, heart, lung, liver, kidney, pancreas, corneas, nervous (e.g. brain, central nervous system, spinal cord), skin, or the cells may be blood cells (e.g. haemocytes, i.e. red blood cells, leucocytes) or haematopoietic stem cells or other stem cells (e.g. bone marrow). For example, an autograft may be prepared where the cells are removed from the patient prior to modification and returned subsequently. However, a method of the present invention will also find utility in the screening of embryos in the preparation of syngrafts (isografts),

allografts and/or xenografts. These methods might include *in vitro* differentiation of embryonic cells for therapeutic transplantation into a patient, including situations where the cells are genetically modified to correct a medical defect. Such applications might include treatment of diseases such as diabetes, Parkinson's disease, motor neurone disease, multiple sclerosis, AIDS etc, or disease conditions characterised by a loss of function in the cells or an organ of an affected individual.

The present invention therefore also extends to the provision of a kit for the diagnosis of Large Offspring Syndrome (LOS) in an animal embryo or in a sample from the embryo comprising a means for the detection of *Igf2r* gene expression. Such kits may also be used to carry out a method in accordance with the ninth aspect of the present invention of a method of testing an embryo culture environment for the induction of Large Offspring Syndrome in an animal embryo. Analysis or detection of *Igf2r* expression may be carried out as described above.

Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

The invention will now be further described by way of reference to the following Examples and Figures which are provided for the purposes of illustration only and are not to be construed as being limiting on the invention. Reference is made to a number of Figures in which:

FIGURE 1 shows differential organ growth of the fetal liver (FIGURE 1(a)), heart (FIGURE 1(b)), kidneys (FIGURE 1(c)) at day 125 of gestation following *in vitro* culture. Control (CONT) fetuses are represented by the solid (fit to data) and dotted (predicted growth to term) lines, generated by the linear allometric equation $\log_e y = \log_e a + b(\log_e)x$, where y = organ

weight and x = fetal weight. Points (i) and (ii) represent, respectively, the minimum and maximum weights of CONT fetuses at day 125 of gestation, and point (iii) represents their birth weights predicted from the Gompertz equation. Allometric coefficients (b±s.e.m.) are for heart (CONT b = 0.91±0.18, Culture b = 1.47±0.12), liver (CONT b = 0.89±0.13, Culture b = 1.16±0.10) and kidneys (CONT b = 1.10±0.29, Culture b = 1.09±0.26), (●, ○). Open data points (○) represent pregnancies where polyhydramnios was present.

FIGURE 2 shows the results of the validation of *Igf2r* PCR assay and identity of IGFIIr protein.

FIGURE 2(a) shows a representative validation of reverse-transcription polymerase chain reaction (RT-PCR) assay to quantify levels of gene transcription (in this case liver *Igf2r*) relative to the 18S ribosomal subunit. Both *Igf2r* and 18S were amplified in a single tube, with an appropriate cycle number and ratio of 18S primer to attenuating Competimer™ to ensure linear amplification over at least a 10-fold dilution of cDNA. Values are arbitrary units of signal intensity.

FIGURE 2(b) shows RT-PCR amplifications representing *Igf2r* and 18S in CONT and LO liver samples.

FIGURE 2(c) shows confirmation of a >200kDa band on IGFII Western ligand blot as IGFII receptor. Immunoblotting of sheep fetal plasma using a polyclonal antibody against IGFIIr protein reveals a single band (a) which co-migrates with purified bovine

IGFIIr (b) and the >200 kDa band identified by Western ligand blot of fetal plasma.

FIGURE 2(d) shows IGFII Western ligand blots representing CONT and LO plasma samples, identifying IGFIIr protein and the IGF binding proteins (IGFBP).

Materials and Methods

Model system for LOS in sheep

A variety of *in vitro* embryo culture systems have been associated with programming of the syndrome (Lau *et al*, *Genes Dev.* 8 2953-2963 (1994)) although the incidence has been neither predictable nor reproducible. In the present studies fertilised eggs from superovulated Scottish Blackface ewes were cultured for five days in four *in vitro* treatments. These comprised two cultured systems, synthetic oviduct fluid (SOF) and TCM 199 co-culture with bovine granulosa cell monolayers (Cocult) (Lau *et al*, *Genes Dev.* 8 2953-2963 (1994)), supplemented with one of two sera (A and B) obtained from steers on different dietary regimes (treatments SOFA, SOFB, CocultA and CocultB). Blastocyst stage embryos were transferred singly to Scottish Blackface recipient ewes and conceptuses were recovered at day 125 of gestation (11 SOFA, 13 SOFB, 11 CocultA and 13 CocultB). Day 6 control (CONT) embryos were transferred to recipients 1-6 hours after recovery, with 22 conceptuses recovered.

Embryo Culture and Transfer

Superovulation was induced in mature Scottish Blackface ewes using 400 i.u. PMSG (Intervet) and Ovagen (ICP) after oestrous synchronisation with Chronogest intravaginal sponges (Intervet). Estrous cycles were synchronised in both donor and recipient ewes using progesterone-releasing intravaginal sponges (30mg fluorogestone acetate, Chronogest, Intervet) for 12 days. For recipient ewes the

same sponge remained in position for 12 days whereas for donor ewes it was removed and a second sponge inserted after 7 days. Recipients received 400 i.u. PMSG (Intervet) at sponge withdrawal. Estrous observations began 24 hours after progesterone withdrawal and were carried out at 08.00, 12.00, 16.00 and 24.00 h during the next three days, using raddled vasectomised rams. Superovulation was induced in donor ewes using a total dose of 9 mg of oFSH (Ovagen, ICP Ltd, New Zealand) administered twice daily in equal doses over four days beginning on day 10 of the 12 day progesterone priming period.

Laparoscopic intrauterine insemination was carried out 44 hours after sponge withdrawal using fresh semen from a single Suffolk sire to minimise genetic variation. Semen was diluted 3:1 in phosphate-buffered saline containing 1000 i.u. sodium penicillin and 1mg streptomycin sulphate per ml. The diluted semen was held at 30°C prior to insemination and approximately $100x10^6$ motile spermatozoa were placed in each uterine horn.

Embryos were recovered by laparotomy 36 hours after insemination following induction of general anaesthesia (Halothane; Oxygen; Nitrous oxide). Each oviduct was flushed with 20ml HEPES-buffered synthetic oviductal fluid (HSOF), derived from the formulation of Tervit *et al* (1972), and containing 20.0mM HEPES buffer, 5.0mM sodium bicarbonate, 1.5mM glucose, 3.3mM sodium lactate, 0.33mM sodium pyruvate, 1.0mM L-glutamine and 30 mg/ml bovine serum albumin.

Embryos were then allocated to the four culture treatments. Allocation across treatments aimed to ensure that, in so far as egg yields permitted, each donor was equally represented. Embryo culture was in SOF and granulosa cell co-culture (Cocult) according to standard techniques. The co-culture medium used was

Medium 199 with Earle's salts (Life Technologies, UK) together with 50 i.u./ml penicillin, 50μg/ml streptomycin sulphate and 10% heat-inactivated (56°C; 30 min) steer serum (Globepharm Ltd., UK). The pH was adjusted to 7.3 and osmolarity to between 390 and 310 mOsm. SOF was derived from the original formulations of Tervit *et al* (1972) excluding HEPES buffer and including 25.0mM sodium bicarbonate, 9.9mM sodium lactate, 0.99mM sodium pyruvate, 1.5mM glucose, 1.0mM L-glutamine, 50 i.u./ml penicillin and 50μg/ml streptomycin sulphate. The pH of SOF medium was adjusted to 7.4 and osmolarity to between 270 and 280 mOsm or 290 and 310mOsm.

Sera A and B were obtained from steers fed on diets including 120 and 190g crude protein/kg dry matter, respectively. These sera were used (4:1 v/v) to supplement both SOF and Cocult, forming four embryo culture treatments (SOFA, SOFB, CocultA and CocultB). Each 60mm diameter tissue culture dish (Bibby Sterilin) used for co-culture contained 50μl droplets (n=8) of M199 and serum overlaid with mineral oil (Cat. No. M8410 Sigma, UK). At the time of zygote introduction (Day 1), 4-day old bovine granulosa cell layers were established in these droplets. The culture medium was refreshed (50% replacement, by volume) just before the zygotes were introduced and thereafter at 48-hour intervals during the culture period, which proceeded for 5 days in a humidified atmosphere (5% CO_2, 95% air) at 38.5°C. Serum-supplemented SOF droplets (20μl under oil) were prepared on the day of zygote collection, with 8 droplets used per 60mm culture dish. Up to 4 zygotes were cultured per drop in a reduced-oxygen atmosphere (5% CO_2, 5% O_2, 90%N_2) at 38.5°C. At 48-hour intervals thereafter, rather than refreshing the media in each droplet, zygotes were moved to freshly prepared 20μl droplets under identical conditions.

After five days in embryo culture, embryos which had developed to late morula/blastocyst stage were transferred singly to uteri of oestrus synchronised ewes by laparoscopy. The transfer technique involved temporary exposure of the tip of the uterine horn through a small ventral incision of the abdominal wall by laparoscopically-guided pick-up. Pregnant ewes were euthanased at day 125 of gestation by administration of 25ml of a 20% w/v solution of pentobarbitone sodium (Euthatal, Rhone Merieux Ltd, UK). The gravid uterus was then recovered and weighed. The fetus was removed and weighed and the liver, heart, kidneys and individual muscles were excised and weighed.

All ewes in this study were maintained on hay treated with 2.5% urea. Procedures were carried out in strict accordance with Home Office regulations. Fetal weights were analysed by ANOVA. Differences between means and allometric coefficients were then assessed by t-tests.

Results

Fetal weights in all embryo culture treatments were greater than in the control group at day 125 of gestation ($P < 0.01$; mean ± s.e. CONT 3907 ±68, SOFA 4594 ±248, SOFB 4856 ±309, CocultA 4495 ±291 and Cocult 5302 ±422) and the percentages of fetuses heavier than the heaviest control group fetus were 46, 54, 36 and 54% respectively. Polyhydramnios (defined as fetal fluid weights more than three standard deviations above the mean for control group conceptuses) was evident in 54% of pregnancies derived from *in vitro* cultured embryos and 15% displayed severe fetal edema (Hydrops fetalis). Three fetuses from culture treatments were twice the mean weight of the control group, indicating extreme overgrowth. Furthermore, 13% of fetuses from cultured embryos were heavier at day 125 of gestation then the predicted birthweight of the control fetuses three weeks later at day 147. The external appearance of many large fetuses from the

culture treatments was similar to that of a term fetus, both in general conformation and extent of coat growth. This suggests that development is considerably advanced by LOS.

The development of key organs in the oversized fetuses was also found to be fundamentally perturbed. Development at day 125 was assessed by relating organ weight to fetal body weight for all treatment groups using the linear allometric equation:

$$\log_e y = \log_e a + b\log_e x$$

where y = organ weight and x = fetal weight.

Allometric coefficients (b ±s.e.m.) established for the control (CONT) group shown in Figure 1 were similar to those derived from 121 normal fetuses of the same genotype recovered between days 35 and 135 of gestation in an earlier study (Sinclair *et al Theriogenology* **45** 223 (1996)) (heart b= 0.92 ±0.01, liver b= 0.86 ±0.01, kidneys b= 0.99 ±0.01). Corresponding coefficients for fetuses derived from all *in vitro* cultured embryos (Culture: Figure 1) were compared to those from the previous study since the larger weight range of fetuses observed therein results in more precise estimates of allometric coefficients. These growth coefficients were significantly greater for liver (Figure 1(a), $P<0.05$) and heart (Figure 1(b), $P<0.0001$) from fetuses derived from *in vitro* cultured embryos, indicating that these organs were growing at a higher rate than expected from both normal fetuses at day 125 of gestation and from older, normally-developing fetuses of equivalent weights. Kidney weights from cultured embryos were highly variable (Figure 1(c)). Several kidneys were grossly enlarged and these were generally associated with polyhydramnios (defined as fetal fluid weights more than three standard

deviations above the mean for CONT pregnancies; Figure 1(c)) and severe fetal oedema (Hydrops fetalis; data not shown). Such alterations in development of vital organs may contribute to the increased prenatal and perinatal losses associated with the Large Offspring Syndrome.

Example 1: Analysis of *Igf2r* gene expression

Transcript levels were quantified relative to 18S ribosomal RNA in a single tube assay. Amplification by 18S primers was attenuated by competition with 18S PCR Competimer™'s (Ambion) which block extension by Taq DNA polymerise. Total RNA was extracted from tissues using RNA Isolator (TM) (Genosys) and 2.5µg reverse transcribed using pd(N)6 random hexamer primer and First-strand cDNA synthesis kit (Pharmacia Biotech). In a 25µl PCR reaction, 125ng of cDNA template was mixed with 0.5µM gene-specific primers, 0.5µM alternative 18S PCR Primers and 18S PCR Competimer™'s (Ambion), 10x buffer with 2.5mM $MgCl_2$, 1mM dNTPs (InVitrogen) and 0.635 units Taq DNA Polymerase (Boehringer). Primers for *Igf2* exon-specific transcripts (Ohlsen *et al DNA Cell Biol.* **13** (4) 377-388 (1994)), IGFBP2, IGFBP3 (Winger *et al Biol. Reprod.* **56** 1415-1423 (1997)) and IGFBP4 (Armstrong *et al Endocrinology* **139** (4) 2146-2154 (1998)) were as described previously while *Igf2r* primers were designed from Genbank bovine sequence J03527 (5'-nucleotides 530-551, 3'-nucleotides 769-750; Lobel *et al J. Biol. Chem.* **263** (5) 2563-2570 (1988)). To ensure that the assay was in the linear range of amplification the ratio of 18S Competimer™: 18S Primer was varied for each tissue and gene-specific primer set. All of the *Igf2* primers span introns, providing a control for contaminating DNA and tubes with no cDNA were also included in each PCR reaction.

In the protocol used, cDNA was denatured for 5 mins at 95°C, then subjected to PCR (94°C for 1 min, 60-65°C for 30s, 72°C for 1 min) for the minimum number

of cycles required to detect transcripts on a 1.7% agarose gel stained with ethidium bromide (typically 25-27 cycles). Band intensities were quantified using a molecular Imager image analysis system with Molecular Analyst™ software (Bio-Rad). Results are exposed as ratios of transcript: 18S and statistical differences determined using TWO-SAMPLE t-test (Minitab). PCR product identify was confirmed by sequencing (Sequence Version 2.0). Amersham).

Results

Fetuses exceeding 5.5 kg in all treatment groups were defined as large offspring (LO) for analysis of gene expression. Using relative RT-PCR (Figure 2(a)), the levels of expression of *Igf2* and *Igf2r* in the liver, kidney and heart were compared from the CONT and LO offspring fetuses. Tissue from the forelimb extensor carpi radialis forelimb was also used for the analysis of gene expression in a skeletal muscle. *Igf2* is expressed as a series of alternatively–spliced, exon-specific transcripts in a tissue-specific and developmental stage-specific manner (Ohlsen *et al DNA Cell Biol.* **13** (4) 377-388 (1994)). Expression of all possible transcripts was examined using 5'-primers representing each non-coding exon and a 3'-primer from the first coding exon, exon 8. No quantitative changes were detected in the level of any exon-specific transcript normally expressed in the day 125 sheep fetus (transcripts from leader exons 5, 6 and 7) shown in Table 1. Furthermore, no qualitative changes were observed which would have indicated activation of exon-specific transcript forms not normally transcribed in the tissues examined at day 125 of gestation (i.e. exons 1 and 3-postnatal transcripts and exon 4-placental transcripts; data not shown). Thus the mechanism underlying ovine Large Offspring syndrome does not appear analogous to the fetal overgrowth typical of Beckwith-Weidmann syndrome in humans, induced by overexpression of *Igf2*. In contrast, expression of the *Igf2r* gene was significantly reduced by ~50% in the liver and muscle and by ~30% in the kidney and heart (Table 1).

Table 1 shows the expression of gene transcripts in day 125 fetal tissues. Values are arbitrary absorbance units expressed as a ratio of transcript:18S. All values are mean ± se for at least 21 CONT and 10 LO fetuses. The asterisks *,**,*** indicate values for which there is a significant difference between the CONT and LO values (Students t-test $P<0.05$, 0.01, 0.001 respectively). The abbreviation BQ indicates that amplification of gene transcripts were below detection limit of assay (18S amplification could not be modulated sufficiently by competitors to ensure linear amplification of both transcript and 18S).

Example 2: Western blot analysis

To investigate whether decreased *Igf2r* gene expression resulted in reduced IGFIIr protein levels, Western ligand blots of tissue protein extracts and plasma samples were probed with ^{125}I-IGFII. Western ligand blotting using ^{125}I-IGFII was performed as described previously (Armstrong *et al J. Endocrinol.* **120** 373-378 (1989)), with the modification that the cathode buffer used for transfer to nitrocellulose contained 0.5mg/ml BSA (Bhavsar *et al Anal. Biochem.* **221** 234-242 (1994)). IGFBP's were identified as previously described (Delhanty, P. J. D. and Van, V. K. M., *Endocrinology* **132** (1) 41-52 (1993)). Immunoblotting was performed with an anti-bovine IGFII receptor antibody using a peroxidase detection system (Vector Labs, UK). The IGFIIr antibody and purified IGFIIr were provided by Dr P Lobel, Centre for Advanced Biotechnology and Medicine, Piscataway, NJ, USA.

Previous authors have speculated that a ~250kDa protein evident on ligand blots was the IGFIIr (Butler, J. H. and Gluckman, P. D., *J. Endocrinol.* **109** 333-338 (1986); Delhanty, P. J. D. and Han, V. K. M., *Endocrinol.* **145** 545-557 (1993)) and this was confirmed using Western blotting with an anti-bovine IGFIIr antibody

(Figure 2(b)). Reductions of 61% and 81% in muscle and liver IGFIIr protein levels were observed, respectively ($P<0.001$, Table 2) and 67% reduction in the circulating form ($P<0.001$, Table 2). In view of the reduction in *Igf2r* expression and resulting overgrowth phenotype in mice with disrupted *Igf2r* expression, these findings provide strong evidence for a direct causative role for IGF2R in LOS in sheep. The dramatic decrease in both circulatory and tissue IGFIIr levels could account for the widespread organ effects as well as the general overgrowth.

Table 2 shows the levels of IGFII receptor protein, IGFBP2 and IGFBP3 in day 125 fetal plasma and tissues. All values are mean ±s.e x1000 from at least 21 Control and 10 LO fetuses. Values are phosphorimager absorbance units of signal intensity from IGFII Western ligand blots. The asterisks (*,**,***) indicate values for which there is a significant difference between the CONT and LO values (Students t-test $P<0.05$, 0.01, 0.001 respectively).

Example 3: Assay of plasma levels of IGF-II

The mechanisms by which IGFIIr can influence fetal growth have not been fully established. One function of IGFIIr is to target IGFII to the lysosymes for degradation. Thus it is widely assumed from mouse studies that a reduction in IGFIIr impairs clearance of IGFII from the circulation. The resulting elevation in levels of this potent fetal mitogen provides a well-established mechanism for increasing fetal growth through cell proliferation and inhibition of apoptosis. Plasma IGFII was measured after acid HPLC (Gutierrez *et al J. Endocrinol.* **153** 231-240 (1997)) using an anti-human polyclonal IGFII antiserum and human recombinant IGFII as standard (GroPep Pty Ltd). The detection limit of the assay was approximately 30 pg/tube.

Results

However, it was surprisingly found in the present studies that plasma levels of IGFII were not significantly different between the CONT and LO sheep fetuses (6.08 ±0.57 and 4.63 ±0.67 ug/ml respectively; p = 0.1). This result may be attributable to a species-specific difference in response to depressed IGFIIr, since *Igf2r* null mutant mice at an equivalent stage of gestation (E18.5) were reported to have a four-fold higher level of serum IGFII than wild-types (Ludwig *et al Dev. Biol.* **177** 517-535 (1996)). However, this difference could have been introduced by inefficient removal of the increased circulating IGF binding proteins observed in the mutant mice. In addition, none of the differences in serum IGFII reported in this and other mouse studies were statistically significant (Eggenschwiler *et al Genes Dev.* **11** 3128-3142 (1997); Lau *et al Genes Dev.* **8** 2953-2936 (1994)). Furthermore in H19 null mutant mice, where elevated expression of *Igf2* is responsible for the fetal overgrowth, serum IGFII is not elevated, although tissue levels are increased (Eggenschwiler *et al Genes Dev.* **11** 3128-3142 (1997)).

Discussion

Absolute changes in IGFII levels are not essential for altered biological activity of this growth factor. Action of the insulin-like growth factors is complex, mediated by a series of binding proteins (IGFBP's) and proteases (Jones, J. I. and Clemmons, D. R. *Endocrine Reviews* **16** 3-34 (1995)). The Western ligand blots in the present study revealed that circulating levels of IGFBP2, which binds most avidly to IGFII (Delhanty, P. J. D. and Han, V. K. M. *Endocrinology* **132** (1) 41-52 (1993)), were increased by 102% in the LO fetuses (Table 2) and this was accompanied by an 86% increase in liver gene expression (Table 1). IGFBP2 has been suggested to prolong IGFII half-life in the circulation and to transport IGFII between blood and tissue fluid, modifying local concentrations and access to receptors. Thus increased IGFBP2 in LO fetuses may increase bioactivity of IGFII

without a change in ligand concentration. Plasma levels of IGFBP3, previously reported to be increased in the plasma of oversized E18/19 fetal mice (Lau *et al Genes Dev.* **8** 2953-2963 (1994)), were not changed in the large ovine fetuses and expression levels remained unchanged in all tissues examined except the kidney, where there was a 23% reduction in transcript levels. No differences in IGFBP4 expression was detected in any of the tissues examined.

It is also possible that reduced *Igf2r* expression in large fetuses increases fetal growth by a mechanism independent of IGFII. In addition to effects on clearance of IGFII, IGFIIr is essential for activation of TGFB1, which exerts potent growth suppressive effects (Wang *et al Cancer Res.* **57** 2543-2546 (1997)), and may account for some or all of the overgrowth observed in LOS. The IGFIIr also has a mannose 6-phosphate binding domain and delivers approximately fifty mannose 6-phosphate tagged lysosomal enzymes to lysosomes (Sklar *et al J. Biol. Chem.* **264** 16733-16738 (1989)). Reduced levels of several lysosomal enzymes was reported in cells from *Igf2r* null mice (Wang *et al Nature* **372** 464-467 (1994)), with likely consequences for tissue remodelling during fetal development (Sklar *et al* (1989)). Thus any disruption to lysosomal enzyme function caused by reduced IGFIIr in LOS may explain some of the observed organ defects, and may also contribute to the gross overgrowth. Finally, of course, a combination of effects of the multifunctional IGFII/ mannose-6 phosphate receptor may be responsible for the complex phenotype observed in LOS in sheep.

In this study, a model for large offspring syndrome in sheep has been established, which is likely to be applicable to a similar syndrome in cattle (Kruip, T. A. M. and den Daas, J. H. G. *Theriogenology* **47** 43-52 (1996)). The high incidence of large offspring observed from the embryo culture treatments in the present studies has allowed a comprehensive phenotypic description and the first report of genes

implicated in the perturbing mechanism. Identification of downregulated *Igf2r* expression makes this imprinted gene a strong candidate for initiating the syndrome due to epigenetic change induced by culture conditions in the pre-implantation embryo and this is currently under investigation. Further understanding of the mechanism should lead to the development of embryo manipulation protocols which avoid the problem. This is of critical importance to the development of both cloning technologies and *in vitro* embryo production in domestic species. Furthermore, since it is not known why similar perturbations have not been described after embryo manipulations in non-ruminants, large offspring syndrome has implications for new assisted reproduction protocols in humans.

Table 1

Gene Transcript Levels in day 125 fetal tissues

Organ	Embryo	Igf2 exon5	Igf2 exon6	Igf2 exon7	Igf2r	IgfBP2	IgfBP3	IgfBP4
Liver	Control	1.79 ±0.11	0.77 ±0.05	1.03 ±0.07	0.63 ±0.09	0.40 ±0.08	0.48±0.05	0.45±0.09
	LO	1.57 ±0.25	1.00 ±0.12	1.05 ±0.04	0.26 ±0.06 ***	0.75 ±0.13 *	0.53±0.06	0.31±0.07
Kidney	Control	BQ	0.60 ±0.03	1.55 ±0.12	0.46 ±0.03	0.51 ±0.05	1.01±0.06	0.56±0.03
	LO	BQ	0.57 ±0.03	1.41 ±0.18	0.31 ±0.04 *	0.38 ±0.05	0.78±0.07 *	0.61±0.04
Heart	Control	BQ	BQ	1.89 ±0.17	0.82 ±0.03	BQ	0.78±0.05	0.35±0.05
	LO	BQ	BQ	1.98 ±0.24	0.57 ±0.05 ***	BQ	0.84±0.08	0.32±0.06
Muscle	Control	1.86 ±0.12	1.14 ±0.06	0.63 ±0.05	0.41 ±0.03	BQ	0.46±0.06	0.82±0.07
	LO	2.30 ±0.23	1.19 ±0.07	0.63 ±0.07	0.21 ±0.03 ***	BQ	0.56±0.15	0.96±0.11

Table 2

IGF-IIR and IGF Binding Proteins in day 125 fetal tissues

Organ	Embryo	IGF-IIR	IGFBP2	IGFBP3
Plasma	Control	116.9 ±15.7	127.3 ±77.0	198.8 ±23.1
	LO	385.3 ±10.6 ***	256.6 ±167.2 **	281.3 ±58.1
Liver	Control	1297.5 ±152.0	158.0 ±19.0	427.7 ±47.7
	LO	242.8 ±42.2 ***	188.1 ±31.6	329.4 ±35.3
Kidney	Control	2537.3 ±608.7	434.8 ±84.5	333.3 ±68.5
	LO	2026.1 ±889.0	471.7 ±130.2	262.4 ±61.8
Heart	Control	1220.6 ±120.1	307.3 ±39.3	379.9 ±43.6
	LO	836.4 ±170.0	263.9 ±49.7	308.6 ±33.3
Muscle	Control	174.7 ±12.6	94.8 ±10.0	110.4 ±11.2
	LO	68.3 ±9.4 ***	91.3 ±12.5	105.1 ±12.7

CLAIMS

1. A method of screening an animal embryo for Large Offspring Syndrome (LOS) comprising the step of analysing Insulin-like growth factor-2 receptor (*Igf2r*) gene expression in the animal embryo or in a biological sample from the embryo.

2. A method as claimed in claim 1, in which the animal is a mammal.

3. A method as claimed in claim 2, in which the mammal is a human.

4. A method as claimed in claim 2, in which the mammal is of an ungulate species.

5. A method as claimed in claim 4, in which the ungulate species is cattle, sheep, pigs, goats, horses, camels or buffalo.

6. A method as claimed in claim 4 or claim 5, in which the ungulate mammal is a transgenic animal.

7. A method as claimed in any one of claims 1 to 6, in which the biological sample comprises a tissue biopsy, cells or cellular material or a biological fluid.

8. A method for *in vitro* production of an animal embryo comprising the steps of introducing a sperm cell to the oocyte in an *in vitro* culture system, subsequently culturing the resulting embryo and screening the embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in the animal embryo or in a biological sample from the embryo.

9. A method as claimed in claim 8, in which the method includes the additional

step of maturing the oocyte *in vitro* prior to fertilisation.

10. A method for reconstituting an animal embryo comprising the step of transferring a nucleus from a donor cell into a suitable recipient cell and screening the resulting embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in the animal embryo or in a biological sample from the embryo.

11. A method of reconstituting an animal embryo, the process comprising transferring a diploid nucleus into an oocyte which is arrested in the metaphase of the second meiotic division without concomitantly activating the oocyte, keeping the nucleus exposed to the cytoplasm of the recipient for a period of time sufficient for the embryo to become capable of giving rise to a live birth and subsequently activating the reconstituted embryo while maintaining correct ploidy, including screening the embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in the animal embryo or in a biological sample from the embryo.

12. A reconstituted animal embryo prepared by a method as defined in claim 10 or claim 11.

13. A method for preparing a non-human animal, the method comprising:

(a) culturing an animal embryo *in vitro* where the embryo has been prepared by *in vitro* production or by reconstituting an animal embryo as defined in any one of claims 8 to 11;

(b) screening the embryo for Large Offspring Syndrome (LOS) by analysing *Igf2r* gene expression in the embryo or in a biological sample from the embryo;

(c) causing an animal to develop to term from the embryo; and

(d) optionally breeding from the animal so formed.

14. A non-human animal prepared as defined in claim 13.

15. A method of screening an animal cell for Large Offspring Syndrome comprising the step of analysing *Igf2r* gene expression in the animal cell.

16. A method of testing an embryo culture environment for the induction of Large Offspring Syndrome in an animal embryo comprising the step of analysing *Igf2r* gene expression in the animal embryo or in a biological sample from the embryo.

17. A method of therapy comprising the administration of animal cells to a patient wherein the cells have been prepared from an embryo screened by a method as defined in any one of claims 1 to 7.

18. The use of a nucleic acid sequence encoding Insulin-like growth factor-2 receptor (IGF2R) or a fragment thereof or a sequence complementary or homologous thereto in the preparation of an agent for the diagnosis of Large Offspring Syndrome (LOS) in an animal embryo or in a sample from the embryo.

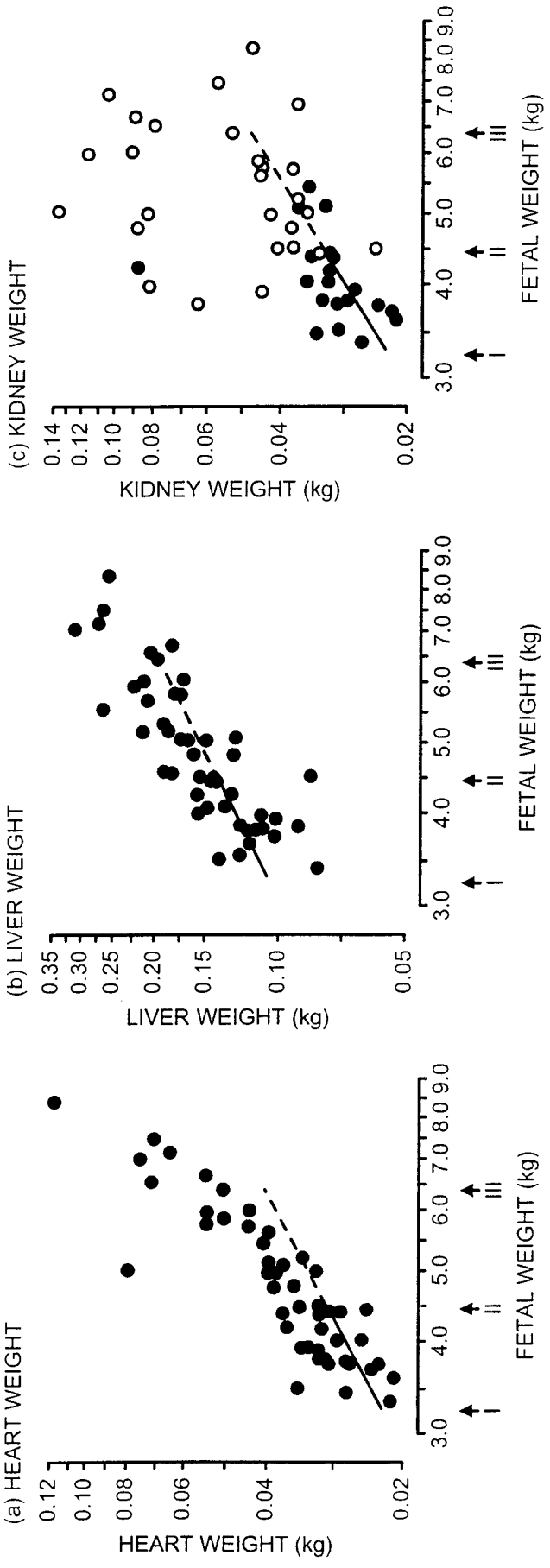
19. The use of cell or a cell line derived from an embryo screened by a method as defined in any one of claims 1 to 7 in medicine.

20. The use of a cell or a cell line derived from an embryo screened by a method as defined in any one of claims 1 to 7 in the preparation of a cell graft for

transplantation.

21. A kit for the diagnosis of Large Offspring Syndrome (LOS) in an animal embryo or in a sample from the embryo comprising a means for the detection of *Igf2r* gene expression.

FIG. 1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0.05
LIVER WEIGHT (kg)
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0.10
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0.06
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0.02
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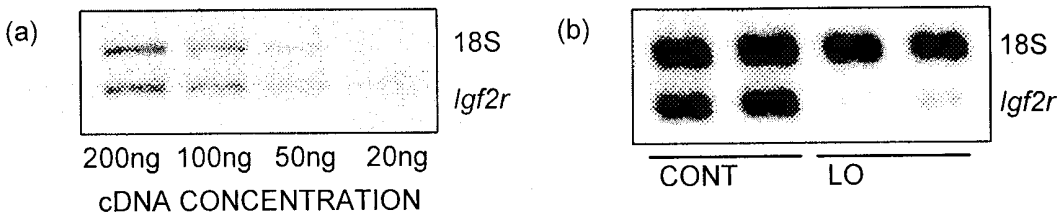
II
III
FETAL WEIGHT (kg)

FIG. 2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200ng 100ng 50ng 20ng
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Igf2r
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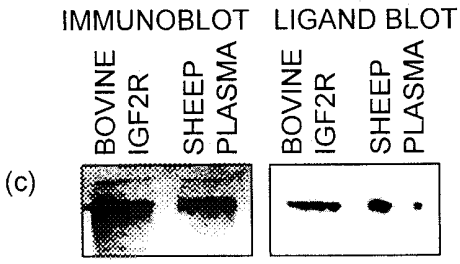
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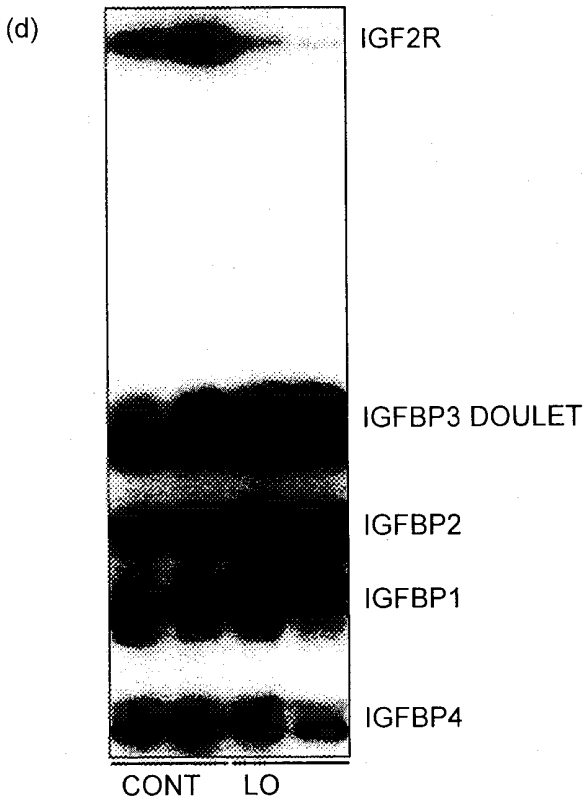
LIGAND BLOT
BOVINE IGF2R
SHEEP PLASMA
BOVINE IGF2R
SHEEP PLASMA
(c)


(d)
IGF2R
IGFBP3 DOULET
IGFBP2
IGFBP1
IGFBP4
CONT
LO

INTERNATIONAL SEARCH REPORT

Inte. ional Application No
PCT/GB 99/03185

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/00 A01K67/027 A61K48/00 C07K14/72 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01K C07K C12N A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	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 JUN;125(12):2273-82, vol. 125, no. 12, June 1998 (1998-06), pages 2273-2282, XP000866564 cited in the application the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2000	14/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	STURM, K.S. ET AL.: "Unrestricted lineage differentiation of pathenogenetic ES cells" DEV GENES EVOL., vol. 206, no. 6, 1997, pages 377-388, XP000866563 the whole document	1
A	MCEVOY T.G. ET AL.: "Post-natal growth and development of Simmental calves derived from in vivo or in vitro embryos." REPROD FERTIL DEV. , vol. 10, no. 6, 1998, pages 459-464, XP000866352 the whole document	1
A	WO 97 07668 A (CAMPBELL KEITH HENRY STOCKMAN ;ROSLIN INST EDINBURGH (GB); WILMUT) 6 March 1997 (1997-03-06) cited in the application the whole document	11
T	YOUNG L E; SINCLAIR K D; WILMUT I: "Large offspring syndrome in cattle and sheep" REVIEWS OF REPRODUCTION , vol. 3, no. 3, September 1998 (1998-09), pages 155-163, XP000866350 cited in the application the whole document	1
P,X	YOUNG, L. E. ET AL.: "Altered IGF binding protein expression is associated with large offspring syndrome in fetal sheep." THERIOGENOLOGY, vol. 51, no. 1, 1 January 1999 (1999-01-01), page 196 XP000866567 the whole document	1
O,P, X	& Meeting Info.: International Workshop on Embryogenesis and Implantation Kamuela, Hawaii, USA February 2-4, 1999	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/03185

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

As far as claims 1 to 6 , 8 to 11, 13, 15 and 16 are directed to a diagnostic method practised on the animal embryo, the search has been carried out and based on the alleged effects of the compound.

Although claim 17 and partially claim 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/GB 99/03185

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9707668 A	06-03-1997	AU 6830996 A	19-03-1997
		CA 2229657 A	06-03-1997
		CN 1202085 A	16-12-1998
		CZ 9800604 A	15-07-1998
		EP 0847237 A	17-06-1998
		GB 2318792 A	06-05-1998
		HU 9802485 A	01-02-1999
		NO 980846 A	29-04-1998
		PL 325336 A	20-07-1998