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(54) Title: A METHOD FOR PRODUCING GENETICALLY MODIFIED ANIMALS

(57) Abstract

The present invention relates generally to genetically modified animals and to a method of producing same. More particularly, the present invention provides a method of producing genetically modified animals by introducing embryonic stem cells into blastocysts which have been isolated at an early embryonic stage and then cultured to fully expanded blastocyst stage *in vitro*. The method of the present invention is particularly useful for obtaining blastocysts from animals exhibiting delayed embryonic development and/or from animals which generally yield low numbers of mature blastocysts after superovulation procedures or following natural mating. The subject method also permits the generation of a range of genetically modified animals. These animals are particularly useful in assessing gene function and gene expression and their effects on the modified animal's phenotype.

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A METHOD FOR PRODUCING GENETICALLY MODIFIED ANIMALS

FIELD OF THE INVENTION

The present invention relates generally to genetically modified animals and to a method of producing same. More particularly, the present invention provides a method of producing genetically modified animals by introducing embryonic stem cells into blastocysts which have been isolated at an early embryonic stage and then cultured to fully expanded blastocyst stage *in vitro*. The method of the present invention is particularly useful for obtaining blastocysts from animals exhibiting delayed embryonic development and/or from animals which generally yield low numbers of mature blastocysts after superovulation procedures or following natural mating. The subject method also permits the generation of a range of genetically modified animals. These animals are particularly useful in assessing gene function and gene expression and their effects on the modified animal's phenotype.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BACKGROUND OF THE INVENTION

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating research and development of a vast array of therapeutic and diagnostic agents. This is particularly the case in the development of transgenic and genetically modified animals. Genetically modified mouse strains, for example, generated by means of homologous recombination in embryonic stem (ES) cells, are a major research tool in fields as diverse as neurobiology, developmental biology and immunology.

ES cells are the pluripotent out growths of the inner cell mass of blastocysts and can be cultured and manipulated *in vitro* and then returned to the embryonic environment to contribute normally to all tissues including the germline (1). Generally, ES cells are injected into blastocysts from a selected animal strain which are then implanted into a pseudopregnant female animal for development.

However, for gene targeting, the production of mice and other animals with a pure genetic background is highly desirable since back-crossing potentially causes conflicting and inconsistent results (2-4).

Until the advent of the present invention, biomedical research involving mice virtually exclusively used ES cells from the white coated 129 mouse strain. ES cells from a white mouse (eg. 129) are injected into blastocysts derived from a black strain and implanted into a suitable pseudopregnant mouse. Chimeric pups are produced and these are bred to produce homozygous knockout mice on the 129 strain background. However, mouse strain 129 can be difficult to breed and thus is usually crossed to other strains. Concerns have been raised about the ambiguity of results derived from back-crossed animals (4).

Efforts to generate ES cell lines from mouse strains other than 129 led to the isolation of C57BL/6 (black) and BALB/c (white) ES cells (3, 5, 6).

To successfully generate chimeric mice with C57BL/6 ES cells, BALB/c strain blastocysts are the preferred recipient (5). However, a major disadvantage of using blastocysts from the BALB/c strain is the delayed embryonic development exhibited by this strain. There is also a low yield of embryos per mouse obtained from naturally mated animals when harvested at the anticipated time of blastocyst development. BALB/c mice are also poorly susceptible to superovulation procedures.

There is a need, therefore, for a methodology capable of generating high yields of blastocysts and which enable efficacious levels of germline transmission of the then injected ES cells.

SUMMARY OF THE INVENTION

One aspect of the present invention contemplates a method for producing a chimeric animal, said method comprising isolating embryos from an animal at an early embryonic stage, culturing said embryos *in vitro* to an injectable blastocyst stage, introducing ES cells from another animal to said cultured blastocysts and then implanting the injected blastocysts into a suitable animal for development of chimeric off-spring.

In a related embodiment, the present invention provides a method for producing a chimeric animal capable of germ-line transmission of an introduced genetic trait said method comprising isolating embryos from an animal at an early embryonic stage, culturing said embryos *in vitro* to an injectable blastocyst stage, introducing ES cells from another animal into said cultured blastocysts, implanting the injected blastocysts into a suitable animal for development of chimeric off-spring and then back-crossing a chimeric animal off-spring with the same animal species from which the ES cells were isolated or an F1 hybrid thereof and selecting off-spring exhibiting said introduced genetic trait.

Another aspect of the present invention contemplates a method for producing a chimeric mouse said method comprising introducing ES cells from one mouse into blastocysts from another mouse said blastocysts obtained from embryos isolated at an early embryonic stage and cultured to maturity *in vitro* and then implanting said blastocysts into a suitable mouse for development of chimeric mice off-spring.

In a related embodiment, the present invention relates to a method for producing a chimeric mouse capable of germ-line transmission of an introduced genetic trait, said method comprising introducing ES cells from one mouse into blastocysts from another mouse said blastocysts prepared from embryos obtained at an early embryonic stage and cultured to mature blastocysts *in vitro,* implanting said blastocysts into a suitable mouse for development of chimeric mice off-spring and then back-crossing a chimeric mouse off-spring with the same species of mouse from which the ES cells were isolated or an F1 hybrid thereof and selecting off-spring exhibiting said introduced genetic trait.

Yet another aspect of the present invention provides a method for producing a genetically modified animal, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts, and introducing into said blastocysts, ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant animal for development of genetically modified off-spring.

In a related embodiment, the present invention is directed to a method for producing a genetically modified animal capable of germ-line transmission of a genetic modification, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, ES cells carrying a modified genomic sequence, implanting the blastocysts into a suitable implant animal for development of genetically modified off-spring and then back-crossing a genetically modified off-spring with the same species of animal from which the ES cells were isolated or an F1 hybrid thereof and selecting an off-spring exhibiting said genetic modification.

Yet another aspect of the present invention provides a method for producing a genetically modified mouse, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts, and introducing into said blastocysts, ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant mouse for development of genetically modified off-spring.

In a related embodiment, the present invention is directed to a method for producing a genetically modified mouse, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts, and introducing into said blastocysts, ES cells carrying a modified genomic sequence, implanting the blastocysts into a suitable implant mouse for development of genetically modified off-spring and then back-crossing a genetically modified off-spring with the same species of mouse from which the ES cells were isolated or an F1 hybrid thereof and selecting an off-spring exhibiting said genetic modification.

Still yet another aspect of the present invention provides a genetically modified animal produced by isolating animal embryos at an early embryonic stage, culturing said embryos *in vitro* until

mature blastocysts and introducing into said blastocysts, animal ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant animal for development of genetically modified offspring.

In a related embodiment, the present invention relates to a genetically modified animal produced by isolating animal embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, animal ES cells carrying a modified genomic sequence, implanting the blastocysts into a suitable implant animal for development of genetically modified offspring and then back-crossing a genetically modified off-spring with the same species of animal from which the ES cells were isolated or an F1 hybrid thereof and then selecting an off-spring exhibiting said modification.

Yet a further aspect of the present invention provides a genetically modified mouse produced by isolating mouse embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, mouse ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant mouse for development of genetically modified offspring.

Yet a further aspect of the present invention provides a genetically modified mouse produced by isolating mouse embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, mouse ES cells carrying a modified genomic sequence, then implanting the blastocysts into a suitable implant mouse for development of genetically modified offspring and then back-crossing a genetically modified off-spring with the same species of mouse from which the ES cells were isolated or an F1 hybrid thereof and then selecting an off-spring exhibiting said genetic modification.

Still a further aspect of the present invention provides a genetically modified mouse produced by isolating embryos from BALB/c or related mice at an early embryonic stage, culturing said embryos *in vitro* until maturation to blastocyst stage introducing into said blastocysts, ES cells isolated from C57BL/6 or related mice wherein said ES cells carry a modified genomic sequence and then implanting said blastocysts into an implant mouse such as but not limited to C57BL/6

or an F1 hybrid thereof and then selecting an off-spring exhibiting said genetic modification.

In a related aspect, the present invention is related to a genetically modified mouse produced by isolating embryos from BALB/c or related mice at an early embryonic stage, culturing said embryos *in vitro* until maturation of said blastocysts, introducing into said blastocysts, ES cells isolated from C57BL/6 or related mice wherein said ES cells are genetically modified by insertion, deletion and/or substitution of a genetic sequence and then implanting said blastocysts into an implant mouse such as but not limited to C57BL/6 or an F1 hybrid thereof and then back-crossing a chimeric mouse produced by said method with a C57BL/6 mouse or a C57BL/6 x BALB/c F1 hybrid mouse and selecting an off-spring exhibiting said genetic modification.

Another aspect of the present invention contemplates the method for isolating embryos from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric animals, said method comprising isolating said embryos from an animal at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

More particularly, the present invention contemplates the method for isolating embryos from mice exhibiting delayed embryonic development or from mice which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric mice, said method comprising isolating said embryos from a mouse at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

Even more particularly, the present invention is directed to a method for isolating embryos from BALB/c mice or other mice exhibiting delayed embryonic development or from mice which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric mice, said method comprising isolating said embryos from a mouse at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

Preferably, with respect to back-crossing, the same species of animal is in fact the same strain of animal.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to genetically modified mice and a method of producing same. The subject invention provides methodology to permit the generation of high yields of blastocysts from animals exhibiting delayed embryonic development and/or from animals which generally yield low numbers of mature blastocysts after superovulation procedures or following natural mating. ES cells carrying genetic modifications to their genomes can then be introduced into the blastocysts for introduction into a suitable implant animal.

Accordingly, one aspect of the present invention contemplates a method for producing a chimeric animal, said method comprising isolating embryos from an animal at an early embryonic stage, culturing said embryos *in vitro* to an injectable blastocyst stage, introducing ES cells from another animal into said cultured blastocysts and then implanting the injected blastocysts into a suitable animal for development of chimeric off-spring.

Generally, the preferred chimeric animals contain a genetic trait capable of transmission through their germ-line. To ensure germ-line transmission, the resulting chimeric off-spring can be back-crossed, generally with the same species and, more preferably, the same strain of animal from which the ES cells are isolated or an F1 hybrid thereof and selecting off-spring which exhibit the genetic trait.

Accordingly, another aspect of the present invention provides a method for producing a chimeric animal capable of germ-line transmission of an introduced genetic trait said method comprising isolating embryos from an animal at an early embryonic stage, culturing said embryos *in vitro* to an injectable blastocyst stage, introducing ES cells from another animal into said cultured blastocysts and then implanting the injected blastocysts into a suitable animal for development of chimeric off-spring and then back-crossing a chimeric animal off-spring with the same animal species, or more preferably same animal strain, from which the ES cells were isolated or an F1

hybrid thereof and selecting off-spring exhibiting said introduced genetic trait.

When the species of animal from which the blastocysts and ES cells are obtained is the same species as the implant animal, then the system is referred to as a "homologous" system. When the species of animal is different, then the system is referred to as "heterologous"system. An example of a heterologous system is the use of ovine embryos in goat implant animals. Homologous systems are the preferred systems.

The implant animal can be considered as an "incubator" animal and is generally a pseudopregnant female. Conveniently, the implant animal may also be an F1 hybrid. With respect to mice, for example, a suitable implant animal is a C57BL/6 mouse or the F1 hybrid C57BL/6 x BALB/c mouse.

Chimeric animals are conveniently detected by skin or fur colour. For example, in accordance with the present invention, ES cells from black mice (eg. C57BL/6) are introduced into blastocysts from white mice (eg. BALB/c) and chimeric animals are those which are black and white. The present invention extends, however, to a range of other means for detecting chimeras such as but not limited to eye colour, genetic screening (eg. by PCR, dot blot, etc.), fur length or other traits such as tail length or a combination of phenotypes. Skin or fur colour is, however, up to the present time, the most convenient indication of chimerism.

Preferably, the ES cells carry a modified genomic sequence when injected into the blastocysts. Modifications to a genomic sequence include for example, inserting, deleting and/or substituting a nucleotide sequence. The modification may be, the introduction of an antisense molecule, a sense molecule for co-suppression, a nucleotide sequence encoding a particular protein or a molecule capable of replacing an existing endogenous sequence or introducing a new nucleotide sequence by homologous recombination. In addition, the genetic modification may entail a recombinase system such as but not limited to the cre/*lox* (15, 16) and the flp/*frt* systems (17, 18). Such systems generally involve loci (eg. *lox* or *frt)* for DNA recombination flanking a selected nucleotide sequence in combination with a recombinase (eg. cre or flp). The recombinase specifically recognises the recombinant loci resulting in site-specific recombination

and deletion of the nucleotide sequence.

The genetically modified animals obtained *via* these ES cells are particularly useful models for assessing gene expression and function and their effects on phenotype.

Accordingly, another aspect of the present invention contemplates a method for producing a genetically modified animal, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts, and introducing into said blastocysts, ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant animal for development of genetically modified off-spring.

In a related embodiment, the present invention is directed to a method for producing a genetically modified animal capable of germ-line transmission of a genetic modification, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, ES cells carrying a modified genomic sequence implanting the blastocysts into a suitable implant animal for development of genetically modified off-spring and then back-crossing a genetically modified off-spring with the same species of animal, or more preferably the same strain of animal, from which the ES cells were isolated or an F1 hybrid thereof and selecting an off-spring exhibiting said genetic modification.

The present invention is hereinafter described with reference to the production of genetically modified mice. However, this is done with the understanding that the present invention is applicable to ES cells and blastocysts from all animals including but not limited to humans, primates, livestock animals (eg. sheep, pigs, cows, horses, donkeys, goats), laboratory test animals (eg. rabbits, guinea pigs, rats) companion animals (eg. dogs, cats) and captive wild animals (eg. foxes, kangaroos, deer).

Accordingly, another aspect of the present invention contemplates a method for producing a chimeric mouse said method comprising introducing ES cells from one mouse into blastocysts from another mouse said blastocysts obtained from embryos isolated at an early embryonic stage and cultured to maturity *in vitro,* and then implanting said blastocysts into a suitable mouse for

development of chimeric mice off-spring.

In a related embodiment, the present invention provides a method for producing a chimeric mouse capable of germ-line transmission of an introduced genetic trait said method comprising isolating embryos from a mouse at an early embryonic stage, culturing said embryos *in vitro* to an injectable blastocyst stage, introducing ES cells from another mouse to said cultured blastocysts, implanting the injected blastocysts into a suitable mouse for development of chimeric off-spring and then back-crossing a chimeric mouse off-spring with the same mouse species, or more preferably the same mouse strain, from which the ES cells were isolated or an F1 hybrid thereof and selecting off-spring exhibiting said introduced genetic trait.

Yet another aspect of the present invention provides a method for producing a genetically modified mouse, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts, and introducing into said blastocysts, ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant mouse for development of genetically modified off-spring.

In a related embodiment, the present invention is directed to a method for producing a genetically modified mouse, said method comprising isolating embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts, and introducing into said blastocysts, ES cells carrying a modified genomic sequence, implanting the blastocysts into a suitable implant mouse for development of genetically modified off-spring and then back-crossing a genetically modified off-spring with the same species, of more preferably strain, of mouse from which the ES cells were isolated or an F1 hybrid thereof and selecting an off-spring exhibiting said genetic modification.

The present invention is particularly useful for establishing blastocysts from animals exhibiting delayed embryonic development and/or from animals which generally yield low numbers of mature blastocysts after superovulation procedures or following natural mating. For example, BALB/c mice generally exhibit a low yield of embryos per mouse from naturally mated animals and a poor yield of blastocysts. Accordingly, the present invention is, in its most preferred form,

directed to mice strains exhibiting delayed embryonic development such as but not limited to BALB/c mice.

In BALB/c mice, embryos are preferably isolated early on day 2, more preferably early on day 3 and still more preferably before approximately day 3½, where day 0.5 is considered to be the time of conception after time 0 of mating. After this time, the yield of healthy embryos is greatly reduced. Accordingly, reference to an early embryo stage in BALB/c mice means embryos up to about day 2-3½ post conception, and these general concepts apply to other mouse strains and other animals. Early embryos may also include compacted morulae. The embryos are cultured *in vitro* generally at 37°C in a CO_2 atmosphere. Generally, within 12 to 48 hours, and more preferably within about 24 hours, a substantial number of the cultured embryos develop into fully expanded blastocysts. The embryos, blastocysts and ES cells may also be cultured in the presence of one or more active ingredients such as but not limited to one or more cytokines. A particularly useful cytokine is leukemia inhibitory factor. See, for example, International Patent Application No. PCT/AU91/00307.

The ability to produce blastocysts from recipient animals having delayed embryonic development enables such animals to be used in chimeric animal production which increases the number of species or strains of animals that may be produced with well defined genetic backgrounds. In particular, BALB/c embryos are, following the advent of the present invention, now easily accessible for the production of chimeras.

In a particularly preferred embodiment, the methodology is employed to make mutations in particular genes in mice. These are referred to as gene knockout mice. Such mutant mice are particularly useful in studying the physiological effects of the mutations or for developing mice with a propensity to develop certain disease conditions such as cancer, diabetes, autoimmune disease or a range of other diseases.

Accordingly, another aspect of the present invention provides a genetically modified animal produced by isolating animal embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, animal ES cells carrying a

modified genomic sequence and then implanting the blastocysts into a suitable implant animal for development of genetically modified offspring.

In a related embodiment, there is provided a genetically modified animal produced by isolating animal embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, animal ES cells carrying a modified genomic sequence, implanting the blastocysts into a suitable implant animal for development of genetically modified offspring and then back-crossing a genetically modified off-spring with the same species, and preferably same strain, of animal from which the ES cells were isolated or an F1 hybrid thereof and then selecting an off-spring exhibiting said modification.

A particular aspect of the present invention is directed to a genetically modified mouse produced by isolating mouse embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, mouse ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant mouse for development of genetically modified offspring.

In a related embodiment, the present invention relates to a genetically modified mouse produced by isolating mouse embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, mouse ES cells carrying a modified genomic sequence, then implanting the blastocysts into a suitable implant mouse for development of genetically modified offspring and then back-crossing a genetically modified off-spring with the same species of mouse, and preferably the same strain of mouse, from which the ES cells were isolated or an F1 hybrid thereof and then selecting an off-spring exhibiting said genetic modification.

Preferably, the ES cells genome are manipulated such that a particular gene or genes is/are targeted to remove or modify expression of the gene. Recombinase systems are also useful in this respect. In a particularly preferred embodiment, the gene is a cytokine gene such as but not limited to tumour necrosis factor (TNF), an interferon (INF), a tyrosine kinase, leukaemic inhibitory factor (LIF), a colony simulating factor (CSF), lymphotoxin (LT), an interleukin (IL),

a hormone and/or a neurological factor. Multiple, such as double or triple, mutations are also contemplated by the present invention such as but not limited to TNF/LT double mutations.

Preferably, the recipient blastocysts for ES cell injection are from BALB/c mice or related to similar mice.

The present invention further contemplates gene-targeted mice produced using the method herein described. Preferably, the gene-targeted mice are mice such as but not limited to those incapable of producing TNF and/or LT-α or LT-β directly in the C57BL/6 mouse strain using ES cells derived from C57BL/6 mice. The genetically modified mice (or other animals) of the present invention are particularly useful as *in vivo* animal models for assessing genetic effects on phenotype including the effects of gene function, expression and regulation on phenotype (7). They are also useful *in vivo* models, for example, in natural product and/or small molecule screening for agents which can alter, reverse or modify a particular engineered phenotype. For example, a genetically altered mouse with a propensity for developing cancer may be injected or contacted with a range of isolated products or synthesised chemical agents to screen for agents which prevent or delay onset of cancer.

In yet another aspect of the present invention, there is provided a gene knock-out animal wherein said animal is produced by isolating animal embryos at an early embryonic stage, culturing said embryos *in vitro* until maturation to the blastocyst stage and introducing into said blastocysts, ES cells genetically modified by insertion, deletion and/or substitution of a genetic sequence and then implanting the blastocysts into a suitable implant animal for development of said gene knock-out animal.

In a preferred embodiment the present invention is directed to a gene knock-out mouse wherein said mouse is produced by isolating mouse embryos at an early embryonic stage, culturing said embryos *in vitro* until maturation to the blastocyst stage and introducing into said blastocysts, ES cells genetically modified by insertion, deletion and/or substitution of a genetic sequence and then implanting the blastocysts into a suitable implant mouse for development of said gene knock-out mouse.

Still a further aspect of the present invention provides a genetically modified mouse produced by isolating embryos from BALB/c or related mice at an early embryonic stage, culturing said embryos *in vitro* until maturation to the blastocyst stage, introducing into said blastocysts, ES cells isolated from C57BL/6 or related mice wherein said ES cells are genetically modified by insertion, deletion and/or substitution of a genetic sequence and then implanting said blastocysts into an implant mouse such as but not limited to C57BL/6 or an F1 hybrid thereof.

In a related aspect, the present invention is related to a genetically modified mouse produced by isolating embryos from BALB/c or related mice at an early embryonic stage, culturing said embryos *in vitro* until maturation of said blastocysts, introducing into said blastocysts, ES cells isolated from C57BL/6 or related mice wherein said ES cells are genetically modified by insertion, deletion and/or substitution of a genetic sequence, implanting said blastocysts into an implant mouse such as but not limited to C57BL/6 or an F1 hybrid thereof and then back-crossing a chimeric mouse produced by said method with a C57BL/6 mouse or a C57BL/6 x BALB/c F1 hybrid mouse.

Another aspect of the present invention contemplates the method for isolating embryos from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric animals, said method comprising isolating said embryos from an animal at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

More particularly, the present invention contemplates the method for isolating embryos from mice exhibiting delayed embryonic development or from mice which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric mice, said method comprising isolating said embryos from a mouse at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

Still a further aspect of the present invention contemplates a method of isolating embryos for use

in the production of chimeric animals.

Preferably, the animals are mice or other laboratory test animals such as but not limited to BALB/c mice or related mice.

A further embodiment is directed to the use of embryos isolated from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, in the manufacture of blastocysts by isolating said embryos at an early embryonic stage from said animals and culturing said embryos *in vitro* until production of mature blastocysts for use in production of chimeric animals.

Preferably, the animals are mice such as but not limited to BALB/c mice.

Preferably, ES cells from a strain of mouse different to the BALB/c mouse are introduced into the BALB/c blastocysts and introduced into an implant mouse prior to production of chimeric off-spring.

Preferably, to maintain genetic purity the chimeric off-spring are back-crossed to the same species and more preferably same strain from which the ES cells are isolated or from a F1 hybrid thereof.

Still a further aspect of the present invention contemplates a composition comprising animal embryos isolated at an early embryonic stage from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating. Generally, the composition would be in a form suitable for long term storage such as in a frozen form. Preferably, the animal embryos are mice embryos.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Isolation of embryos from BALB/c mice

Embryos were harvested from BALB/c mice early (7am to 8am) on the third day (d3) after natural mating (d0). The timing is important as later on d3, the yield of healthy embryos was substantially reduced. The uterus was removed and the two uterine horns were flushed as described (8, 9). Flushing of the uterus and embryo collection was performed with embryo-tested M2 medium (Sigma, St Louis, USA). Recovered embryos exhibited a stage of development varying between early and compacted morulae, with a few (~2%) of recovered cells being early blastocysts (Table 1).

EXAMPLE 2

In vitro culture of embryos

Embryos from Example 1 were transferred to M16 medium at 37°C (Sigma, St Louis, USA) then incubated at 37°C in a 5% v/v CO_2 atmosphere under embryo-tested mineral oil (Sigma, St Louis, USA). Within 24 hours, 70-80% of the cultured embryos were found to develop into fully expanded blastocysts. A further 10-20% developed into early blastocysts (Table 1) which were injected with ES cells last or reimplanted uninjected, together with injected blastocysts, to supplement the numbers of the latter and increase litter sizes (Table 2).

EXAMPLE 3

Introduction of ES cells

Transfected and G418-selected ES cells were thawed from stocks three days before injection and cultured basically as described (10) on neomycin-resistant foetal fibroblasts (11) in DMEM (high glucose and sodium pyruvate; Gibco BRL, UK) supplemented with 10% v/v FCS (pre-tested for ES cell culture as described (12)), glutamine (2mM; Gibco BRL, UK), NEAA's (0.1mM; Gibco BRL, UK), 0.1 mM β-mercaptoethanol (Sigma, St Louis, USA) and 200 U/ml leukaemia inhibitory factor (LIF) (ESGRO, Amrad, Australia). The ES cells were passaged on the day

before injection to disperse larger cell clumps and increase the growth rate of the culture. On the day of injection, ES cells were harvested and plated twice on gelatine-coated (0.1% w/v) petri dishes to remove fibroblasts and resuspended in DMEM-5% v/v FCS - 50 U/ml DNase (Sigma, St Louis, USA). Twenty four hour cultured BALB/c blastocysts were micro-injected with ES cells using a Zeiss stereo-microscope at room temperature. Each blastocyst received between 8-12 ES cells.

EXAMPLE 4

Implantation

Injected blastocysts were reimplanted into pseudopregnant (d2.5) Foster mothers (implant animals). Eight blastocysts were implanted in one uterine horn and usually both uterine horns received blastocysts.

EXAMPLE 5

Generation of knock-out mice

ES cells from C57BL/6 mice were subjected to genetic manipulation to inactivate the genetic loci for tumour necrosis factor (TNF) and/or lymphotoxin (LT)-α or LT-β (13). Genes for TNF, LT-α and LT-β reside on the same chromosome as the major histocompatibility complex (MHC) and in fact reside within the MHC.

The generation of gene knockout mice in the C57BL/6 mouse strain using two separately-derived C57BL/6 ES cell lines and eight different cell clones from six transfections is shown in Table 2. Injection of C57BL/6 ES cells into BALB/c blastocysts produced black and white tissue mosaic offspring of varying host (white) and donor (black) genetic contribution. When backcrossed to C57BL/6, the offspring exhibited genetic segregation back to the original genotypes and where the germline of the chimeric mouse was of BALB/c origin (host) the offspring were of agoutic coat colour (C57BL/6 x BALB/c) Fl. If germline transmission occurred the offspring were black. All injected ES cell clones resulted in the birth of chimeric mice with the expected bias to the male sex. With clones 1-6 each chimeric mouse, irrespective

of the level of chimerism, was mated, and two litters were analysed. With the exception of clone #2, all ES cell clones transmitted through the germline. Increased success with rates of germ-line transmission and performance of individual clones is probably due to experience in handling of the clones during and after transfection and in the period before injection. Clones are numbered in the order produced and represent a period of five months. The outcomes in terms of rates of germline-transmission chimeras per implanted blastocyst were not significantly different to that reported for 129 ES cells (14).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

TABLE 1

BALB/c blastocyst recoveries after 24h *in vitro* culture

	Embryos recovered[1]	Early Blastocysts	Viable embryos recovered at 24h[2]	Blastocysts recovered at 24h[2,3]
Total	410	8 (2%)	365 (89%)	294 (72%)[4]
per mouse[5]	4.9	0.1	4.3	3.5

Two-three female BALB/c mice were naturally mated with one stud male. All female showing vaginal mucus plugs on early morning examination (d0) were sacrificed at d3 of gestation. The light-cycle of the holding room was 0600-1800.

[1] Embryos collected over seven injection days

[2] After 24h *in vitro* culture

[3] Fully expanded and injectable blastocysts

[4] The remaining 28% of cells was composed of compacted morulae, incompletely-expanded blastocysts and some non-viable embryos

[5] Mean embryo/blastocyst recovery. The average figure is reduced by inclusion in this data of the 15-20% of plugged mice that remained unfertilised and thus produced no viable embryos. Of those mice plugged and fertilised, six-eight embryos per mouse were recovered routinely.

TABLE 2

Rates of germline transmission with C57BL/6 ES cells

ES cell line	Clone number	Cell passage at transfection	Blastocysts reimplanted[1]	Pups born[1]	Pups chimeric[2]	Pups transmitting germline[3]
BL6/III[6]	1	22	124	28(22.5%)[4]	9(7.3%)[5]	1(0.8%)[5]
BL6/III	2	22	97	35(36%)	5(5.1%)	-
BL6/III	3	21	96	21(28.1%)	6(6.3%)	1(1%)[8]
Bruce4[7]	4	18	48	27(56.3%)	8(16.7%)	1(2.1%)
Bruce4	5	18	58	35(60.3%)	6(10.3%)	2(3.4%)[8]
BL6/III	6	>30	88	40(45.5%)	27(30.7%)	4(4.5%)[8]
Bruce4	7	20	45	15(33.3%)	4(8.9%)	2(4.5%)[9]
Bruce4	8	20	151	40(26.5%)	12(8%)	4(2.6%)[10]

1 Injected blastocysts were often supplemented for reimplantation with uninjected blastocysts to improve litter size. Thus, figures shown include ES cell-injected plus uninjected blastocysts reimplanted and total pups born from these reimplantations. Overall, uninjected blastocysts represented ~15% of total reimplanted blastocysts

2 All levels of chimerism are included

3 After two litters

4 Percent of total implanted blastocysts

5 Number of chimeric mice or germline-transmitting mice as a percent of total implanted blastocysts. Thus, these figures are probably slight underestimates as a proportion of the implanted blastocysts were not injected with ES cells and could never have generated chimeras

6 Reference 5

7 Reference 6

8 One or more of the chimeras were 100% transmitters where all progeny, on crossing with C57BL/6 mice, were black

9 Of the 4 chimeric pups born, only the 2 most chimeric were chosen for breeding, and both transmitted through the germline

10 Of the 12 chimeric pups born, only the 6 most chimeric were chosen for breeding, and of these 4 transmitted through the germline

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CLAIMS:

1 A method for producing a chimeric animal, said method comprising isolating embryos from an animal at an early embryonic stage, culturing said embryos *in vitro* to an injectable blastocyst stage, introducing embryonic stem (ES) cells from another animal to said culture blastocysts and then implanting the blastocysts into a suitable animal for development of chimeric off-spring.

2. A method according to claim 1 further comprising the step of back-crossing a chimeric off-spring with the same species of animal from which the ES cells were isolated or an F1 hybrid thereof and selecting an off-spring expressing the chimera.

3. A method according to claim 2 wherein the animal species is the same animal strain from which the ES cells were isolated or an F1 hybrid thereof.

4. A method according to claim 2 or 3 wherein the ES cells' genomes are genetically modified prior to injection into the blastocysts.

5. A method according to claim 1 or 2 or 3 or 4 wherein the ES cells and embryos are isolated from primates, livestock animals, laboratory test animals, companion animals or a captive wild animals.

6. A method according to claim 5 wherein the ES cells and embryos are isolated from a laboratory test animal.

7. A method according to claim 6 wherein the ES cells and embryos are isolated from a murine species.

8. A method according to claim 7 wherein the ES cells are isolated from a C57BL/6 mouse or related mouse.

9. A method according to claim 7 wherein embryos are isolated from a BALB/c mouse or related mouse.

10. A method according to claim 1 wherein the embryos are isolated from about day 2 to about day 4 wherein day 0.5 is the time of conception after time 0 of mating or functionally equivalent other time.

11. A method according to claim 10 wherein the embryos are isolated early from about 3-3½ days post conception.

12. A method according to claim 10 or 11 wherein the embryos are cultured for from about 12 to about 48 hours under conditions sufficient to develop as fully expanded blastocysts.

13. A method according to claim 1 or 7 wherein said chimeric animal exhibits an introduced genetic trait.

14. A method according to claim 13 wherein the genetic trait results in a defective ability to produce tumour necrosis factor (TNF), lymphotoxin (LT)-α, LT-β, an interferon, a tyrosine kinase, an interleukin, a hormone, a neurological factor and/or a colony stimulating factor.

15. A method according to claim 13 wherein the genetic trait results in a defective ability to produce both TNF and LT-α and/or LTβ.

16. A genetically modified animal produced by isolating animal embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, animal ES cells carrying a modified genomic sequence and then implanting the blastocysts into a suitable implant animal for development of genetically modified offspring.

17. A genetically modified mouse produced by isolating mouse embryos at an early embryonic stage, culturing said embryos *in vitro* until mature blastocysts and introducing into said blastocysts, mouse ES cells carrying a modified genetic sequence and then implanting the

blastocysts into a suitable implant animal for development of genetically modified offspring.

18. A genetically modified animal according to claim 16 or 17 comprising the further step of back-crossing said genetically modified off-spring with the same species from which the ES cells were isolated or an F1 hybrid thereof and selecting an off-spring exhibiting said genetic modification.

19. A genetically modified animal according to claim 18 wherein the same species is the same strain or an F1 hybrid thereof.

20. A genetically modified animal according to claim 17 wherein the embryos are from a BALB/c mouse.

21. A genetically modified animal according to claim 17 wherein the ES cells are from a C57BL/6 mouse.

22. A genetically modified animal according to claim 16 or 17 wherein the animal is defective in its ability to produce TNF, LT-α, LT-β, an interferon, a tyrosine kinase, an interleukin, a hormone, a neurological factor and/or a colony stimulating factor.

23. A genetically modified animal according to claim 22 wherein the animal is defective in its ability to produce TNF and LT-α and/or LT-β.

24. A genetically modified mouse produced by isolating embryos from BALB/c or related mice at an early embryonic stage, culturing said embryos *in vitro* until maturation to blastocysts stage , introducing into said blastocysts, ES cells isolated from C57BL/6 or related mice wherein said ES cells carry a modified genomic sequence and then implanting said blastocysts into an implant mouse such as but not limited to C57BL/6 or an F1 hybrid thereof and then selecting an off-spring exhibiting said genetic modification.

25. A genetically modified mouse produced by isolating embryos from BALB/c or related

mice at an early embryonic stage, culturing said embryos *in vitro* until maturation of said blastocysts, introducing into said blastocysts, ES cells isolated from C57BL/6 or related mice wherein said ES cells are genetically modified by insertion, deletion and/or substitution of a genetic sequence and then implanting said blastocysts into an implant mouse such as but not limited to C57BL/6 or an F1 hybrid thereof and then back-crossing a chimeric mouse produced by said method with a C57BL/6 mouse or a C57BL/6 x BALB/c F1 hybrid mouse and selecting an off-spring exhibiting said genetic modification.

26. A method for isolating embryos from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric animals, said method comprising isolating said embryos from an animal at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

27. A method for isolating embryos from mice exhibiting delayed embryonic development or from mice which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, said embryos useful in the production of chimeric mice, said method comprising isolating said embryos from a mouse at an early embryonic stage and then culturing said embryos *in vitro* until maturation into blastocysts.

28. A composition comprising animal embryos isolated at an early embryonic stage from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating.

29. Use of embryos isolated from animals exhibiting delayed embryonic development or from animals which generally yield low levels of mature blastocysts after superovulation procedures or following natural mating, in the manufacture of blastocysts by isolating said embryos at an early embryonic stage from said animals and culturing said embryos *in vitro* until production of mature blastocysts for use in production of chimeric animals.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00817

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A01K 067/027, C12N 005/06, 005/10

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)
WPAT, Chem Abs see below

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: (A01K-67/027/IC OR C12N-005/06/IC) AND (EMBRYONIC()STEM()CELL OR CHIMERA# OR CHIMERIC# OR CHIMAERA# OR CHIMAERIC# OR ES()CELL# OR BLASTOCYST#) cont.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Methods Mol. Biol. (Transgenesis Techniques) 18, pages 217-37 (1993) Wells, D., "Production of chimeras derived from murine embryonic stem cells" see 2.1, 2.2, 3.2.1 and Notes	1-11, 13-27
X	Methods in Enzymology 225, pages 823-55 (1993) Stewart, C.L. "Production of chimeras between embryonic stem cells and embryos" see pages 838-9.	1-11, 13-27
X	Laboratory Animal Science 43(1) pages 91-3 (1993) Kovacs, M.S. et al "Use of Superovulated Mice as Embryo Donors for ES Cell Injection Chimeras".	

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

[ ] See patent family annex

* Special categories of cited documents:

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Date of the actual completion of the international search	Date of mailing of the international search report
	23 DEC 1997

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00817

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P X	Nuc. Acids. Res 25(4) pages 917-8 (1997) Lemckert, F.A. "Gene Targeting In C57BLI6 ES cells. Successful germ line transmission using recipient BALB/c blastocysts developmentally matured in vitro".	

Box B49 cont.

MEDLINE: BLASTOCYST/CT AND EMBRYO TRANDFER/CT AND EMBRYONIC STEM CELL AND (CHIMERA OR CHIMERIC OR CHIMAERA OR CHIMAERIC)

CHEMABS: EMBRYO/CT AND BLASTOCYST AND EMBRYONIC STEM CELL AND (CHIMERA OR CHIMERIC OR CHIMAERA OR CHIMAERIC)