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Fertility comparison between wild type and transgenic mice by in vitro fertilization

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Abstract

Transgenic mice are increasingly used as animal models for studies of gene function and regulation of mammalian genes. Although there has been continuous and remarkable progress in the development of transgenic technology over several decades, many aspects of the resulting transgenic model's phenotype cannot be completely predicted. For example, it is well known that as a consequence of the random insertion of the injected DNA construct, several founder mice of the new line need to be analyzed for possible differences in phenotype secondary to different insertion sites. The Knock out technique for transgenic production disrupts a specific gene by insertion or homologous recombination creating a null expression or replacement of the gene with a marker to localize its expression. This modification could result in pleiotropic phenotype if the gene is also expressed in tissues other than the target organs. Although the future breeding performance of the newly created model is critical to many studies, it is rarely anticipated that the new integrations could modify the reproductive profile of the new transgenic line. To date, few studies have demonstrated the difference between the parent strain's reproductive performance and the newly developed transgenic model. This study was designed to determine whether a genetic modification, knock out (KO) or transgenics, not anticipated to affect reproductive performance could affect the resulting reproductive profile of the newly developed transgenic mouse. More specifically, this study is designed to study the impact of the genetic modification on the ability of gametes to be fertilized in vitro. We analyzed the reproductive performance of mice with different background strains: FVB/N, C57BL/6 (129Sv/J × C57BL/6)F1 and outbred CD1[®] and compared them to mice of the same strain carrying a transgene or KO which was not anticipated to affect fertility. In vitro Fertilization was used to analyze the fertility of the mice. Oocytes from superovulated females were inseminated with sperm of same background. Fertility rate was considered as the percentage of two cell embryos scored 24 h after insemination. The data collected from this study shows that the fertilization rate is affected (reduced to half fold) in some of the transgenic mice compared to the respective Wild Type (WT) mice. For the WT the average fertility rate ranged from 80% (C57BL/6), 90% (FVB/N), 45% (129Sv/J × C57BL/6)F1 and 43% (CD1). For transgenic mice it was 52% (C57BL/6), 65% (FVB/N), 22% (129Sv/J × C57BL/6)F1 and 25% (CD1).

Keywords

Transgenic mice; IVF; Superovulation; Cryopreservation

Introduction

The technique of pronuclear microinjection, which allows the introduction of engineered DNA into the pronucleus of a fertilized egg to modify the genetics of a mouse line, was first developed by (Gordon et al. 1980). This powerful tool became a firmly established technique, when optimized protocols for the procedure were published over two decades ago (Brinster et al. 1985; Hogan 1986). Since then, transgenic mice have been the most popular models used for studies of gene function and its regulation as demonstrated by thousands of publications utilizing transgenic animal models for biomedical investigations. The knockout technology where one or more genes have been turned off through homologous recombination or insertion has permitted scientists to determine the role of specific genes in development, physiology, and pathology.

For most studies, the development of a transgenic or knockout animal was only the first step in the process of developing and characterizing a unique line. Same as the natural mating where mice have different indices of fertility (Sztejn et al. 2000; Styrna and Krzanowska 1995; Niwa et al. 1980; Kaleta 1977; Parkening and Chang 1976) the fertility rate of the genetically modified mice is also influenced by the genetic background. Subsequently, genetic differences were also observed in the superovulation rate (Spearow and Barkley 1999), the post thaw survival of cryopreserved spermatozoa (Sztejn et al. 2000, 2001; Thornton et al. 1999) and the rate of embryo development (Scott and Whittingham 1996) in mice of different background strains. Since the genetic background plays a major role on the in vivo reproductive performance of each mouse strain the success of other in vitro Assisted Reproduction Techniques (i.e. in vitro fertilization (IVF), cryopreservation, etc.) may also be linked to the genetic background of the mouse under consideration. In this study we used IVF as a tool to produce fertilized embryos and analyzed the reproductive profile of several transgenic mouse lines with different strain backgrounds and compared the fertility rate of each transgenic line to its wild-type background strain.

Materials and methods

Animals

Transgenic and KO male and female mice from different background strains such as inbred: FVB/NCrI, C57BL/6NCrI, outbred CrI:CD1(ICR; Charles River) and [129Sv/J × C57Bl/6]F1 (Lu et al. 1998)] strains were maintained in a breeding program (brother × sister mating) under a centralized breeding program in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). The genetically modified animals used in the study are described in Table 1. None of the strains selected has a genetic modification expected to alter the breeding performance. The animal housing included a controlled light and dark cycle (12 h:12 h), ad libitum food, Purina Labdiet 502, 9%—Fat Breeding chow and ultra filtered water, ventilated caging systems and standardized environmental enrichment. Male mice were housed individually for at least 5 days prior to the collection of spermatozoa. This study was carried out with mouse strains sent for cryopreservation to our unit. Although CD1 and (129Sv/J × C57Bl/6)F1 have only one transgenic strain each, they were included in the study with the rationale of having different transgenics backgrounds.

In vitro fertilization

In vitro fertilization was performed using a modified version of the method described by Szejn et al. 2000. In vitro fertilization culture medium, Mouse Vitro Fert (Cook V-MVFE-50, Australia), was used for sperm incubation, IVF and zygotes culture.

Sperm was collected from the caudae epididymides of 3–5 month old male mouse as described in Szein et al. 2000. After euthanasia with CO₂ both caudae epididymides and vas deferentia were removed aseptically and placed in 300 µl of IVF media (Cook V-MVFE-50, Australia) covered with embryo tested mineral oil (Sigma, St. Louis, MO) in a 35-mm dish (Falcon 1008, Becton–Dickinson, NJ). The tissue was incised 4–5 times with the edge of a 30G injection needle to allow the sperm to “swim out” and disperse for 20–30 min at 37°C under 5% CO₂ in the humidified incubator. Sperm motility and concentration was confirmed by visual observation.

Five to ten 21–35 day old female mice were superovulated by intraperitoneal injection (IP) of 5 international units (IU) of Gonadotropin from Pregnant Mare Serum (PMSG; Sigma, St. Louis, MO). Forty-six to fifty hours post PMSG were injected with 5 IU of Human Chorionic Gonadotropin (HCG; Sigma, St. Louis, MO) intraperitoneally. Thirteen to fifteen hours following HCG administration, the female mice were euthanized with CO₂; their oviducts were aseptically removed and dissected free from surrounding tissues and placed in a culture dish containing 2 ml of M2 medium (Sigma, St. Louis, MO) warmed at 37°C. The cumulus oocytes complexes were removed from the ampullae using micro dissecting forceps and subsequently used for the IVF procedure.

Ten micro liters of the sperm suspension was added using a wide-bore pipette tip to a 250 µl IVF media drop covered with embryo tested mineral oil (Sigma). The isolated oocytes complexes were then transferred to the drop of media containing the sperm and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 5 h. After that time the eggs were washed to eliminate excess sperm and cultured overnight in a 250-µl drop of IVF medium under the same conditions. The following day the number of two-cell embryos were counted and prepared for either transfer to recipient females or for cryopreservation. The oocytes from the superovulated females were pooled for each IVF and the experiment was done 3 or more times for each line.

Statistical analysis

Fertility was considered as the percentage of two cell stage embryos scored 24 h after insemination. For the statistical analysis the percentages were transformed into arcsine values and then evaluated by one-way ANOVA and by unpaired *t* test (two tailed). For the C57BL6 and FVB/N background mice the Dunnett’s multiple-comparison test was used to compare all transgenic mice against the WT mice. Differences were considered to be significant when a *P* value of <0.05 was obtained. Error bars on figures represent the standard error of the mean (SEM) of the in vitro fertilization rate. The data was analyzed using the Graph Pad Prism version 5.0 computer program (Graph Pad Software, San Diego, CA).

Results

The results of the in vitro fertilization procedure are summarized in the tables and figures displaying the average fertilization rate (percentage of zygotes which progressed to the two-cell stage) obtained from a minimum of three IVF tests. Fertility was scored at the two-cell stage embryos after overnight culture in IVF medium (MVFE-50) at 37°C under 5% CO₂ in humidified environment. The fertility rate was calculated by dividing the number of 2 cells by total number of oocytes exposed. The results show that the fertility rate varied between the WT and transgenic line of same background strain.

The average fertilization for WT C57BL/6 mice as shown in Table 2 and Fig. 1 was $80 \pm 5\%$ and the average fertilization for all four C57BL/6 transgenic mice was $48 \pm 7\%$. Although the fertility rate between the wt and the transgenic mice turned out to be not significant

different after the arcsine transformation, in two groups the rates obtained have 50% difference.

Table 3 and Fig. 2 show the average fertilization for the WT FVB/N mice was $90 \pm 5\%$ and the average fertilization for all the transgenic mice was $65 \pm 8\%$. One of the FVB/N transgenic lines had a significantly reduced fertility rate ($P < 0.05$) $50 \pm 8\%$ compared to the WT mice $90 \pm 5\%$.

For the WT (129Sv/J $\times$ C57Bl/6)F1 the fertilization rate was $45 \pm 4\%$ and the transgenic mice was significantly lower ($P < 0.05$) $22 \pm 3\%$. For the CD1 WT it was $43 \pm 8\%$ and the transgenic mice was $25 \pm 5\%$. This result is not significantly different ($P < 0.05$). Both results are shown in Table 4 and Fig. 3.

Discussion

Archiving mouse strains by embryo cryopreservation has great potential because it is simple, rapid, and inexpensive. The use of IVF for producing embryos for rederivation and/or embryo banking, even under some difficult circumstances can speed up and in some cases improve the process of cryopreservation. However, the success of the cryopreservation is influenced by the genetic background and the reproductive performance of the mice used (Choi et al. 2000; Fraser 1977; Parkening and Chang 1976). A Number of differences exists between the mouse strains commonly used in the research field and in transgenic technology (e.g., B6; 129 $\times$ 1/SvJ, FVB/N and BALB/c). Reproductive traits such as litter size, sperm production and hormone induced ovulation rate differ between mouse strains (Bouxsein et al. 2005; Spearow and Barkley 1999; Silver 1995). Also the in vitro fertilization in mice varies depending on genotype and the conditions used for capacitation and fertilization (Sztejn et al. 2000; Choi et al. 2000; Fraser 1977; Parkening and Chang 1976; Fraser and Drury 1976; Iwamatsu and Chang 1971). Further, inbred mouse strains have defined reproductive parameters which are so distinctive that they are considered to be a characteristic of the strain (Byers et al. 2006; Silver 1995). Inbred strains also differ in their capacity for embryo development in culture (Scott and Whittingham 1996; Roudebush and Duralia 1996; Dandekar and Glass 1987) and in survival after embryo cryopreservation (Dinnyes et al. 1995; Pomp and Eisen 1990; Schmidt et al. 1985).

During the transgenic mice production, the genetic background characteristic of the newly established strain can be serendipitously modified by the insertion of DNA construct or by the process of knocking out a gene which can impact the mice response to superovulation and its fertility. The integration of the exogenous DNA occurs at apparently random locations within the mouse genome. Occasionally the integration of the transgene can fortuitously interrupts an intrinsic mouse gene responsible for some interesting aspect of mouse development and causes a special class of mutations referred as “insertional mutations” (Palmiter and Brinster 1986). Insertional mutations may lead to a variety of phenotypes such as embryonic lethalties (Covarrubias et al. 1987; Soriano et al. 1987; Mark et al. 1985; Wagner et al. 1983), morphological, neurological disorders (Zeller et al. 1989; Krulowski et al. 1989; McNeish et al. 1988; Kothary et al. 1988; Overbeek et al. 1986) and abnormal sperm functions (Palmiter et al. 1984). Magram and Bishop (1991) showed that male hemizygous transgenic mice carrying HCK protooncogene were sterile due to a defect in spermatogenesis. They found that the sterile males mated normally but failed to impregnate the females due to the presence of a variety of abnormally shaped nuclei during spermatogenesis.

This study was conducted to determine whether there is a difference in the reproductive performance between transgenic mice and wild-type (WT) mice of the same background

strain. Reproductive performance was analyzed by the number of oocytes fertilized by IVF. Fertility was scored at the two cell stage-stage embryos. From the results we observed that the reproductive performance of the WT mice and the transgenic mice of same background strain was different. This statistical difference of the normalized distribution is not significant for the C57BL/6 and CD1 strains, however, it was for FVB/N and (129Sv/J × C57BL/6)F1 lines ($P < 0.05$) used in this study. The FVB/N mice have a better IVF outcome compared to some other inbred strains. In this study one of the FVB/N transgenic mice lines had a low fertility rate of 50% compared to the WT fertility rate of 90%. This study clearly demonstrates a direct effect of the man made genetic modification on the gametes of the resulting transgenic line. The variation in fertility between the WT mice and the transgenic mice of same background strain could be perhaps, due to the consequence caused by the transgene insertion resulting in disruption of a gene (or genes) important, or the insertion causing the knock out of a gene which could be linked to gametogenesis resulting in abnormal sperm production, oocyte genesis or others factors regulating fertility. It has been described that knockout mice that have a silenced functional gene expressing in tissues which are not directly involved in reproduction may show a pleiotropic phenotype with reduced fertility (Baker et al. 1995). Toshimori et al. (2004) described various gene KO models where the decrease in sperm production could be induced as a secondary effect of gene knock out. Alternatively, the result could be a direct effect on gametogenesis. It should be noted that only two of the transgenic mice used in the study carries a marker or neutral gene and all the others have active expressing genes, although none of the transgenic mice used in this study had any gene modification related to neither reproduction nor an expected phenotype that could affect fertility. Therefore, the fertility variation observed in this study could be a possible effect of the modification caused by the integration of a transgene or KO of a gene.

Although the statistical analysis of some of the lines showed no significant difference between wt and transgenic mice, in the practice, harvesting 50% less number of embryos implicates the use of more females, longer time to complete the cryopreservation of projected number, more time for housing the colony and therefore, total more expenses. Then, the variations in the fertility rate between the WT mice and the transgenic mice of same background strain described in this study should be taken under consideration while planning any assisted reproduction procedure (i.e. embryo rederivation, cryopreservation, colony expansion, etc.), especially in relation to the time it takes to complete the embryo banking or rederivation by means of IVF. For transgenic lines which are difficult to breed or collect embryos for archiving, other options may of benefit such as using WT females with the Tg males for IVF or for breeding to preserve the valuable genotype.

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Fig. 1.

In vitro fertilization rate of WT and transgenic mice of C57BL/6 strain. Fertility was scored at two cell stage after overnight culture in IVF medium. Each *bar* represents the mean IVF rate from a total of three or more IVF for each line

Fig. 2.

In vitro fertilization rate of WT and transgenic mice of FVB/N strain. Fertility was considered as the percentage of two cell stage embryos after overnight culture in IVF medium. Each *bar* represents the mean IVF rate from a total of three or more IVF for each line

Fig. 3.

In vitro fertilization rate of WT and transgenic mice of (129Sv/J $\times$ C57Bl/6)F1 and CD1 strain. Fertility was considered as the percentage of two cell stage embryos after overnight culture in IVF medium. Each *bar* represents the mean IVF rate from a total of three or more IVF for each line

Table 1

List of transgenic mice used in this study

Name	Promoter	Gene	Target	Zygosity	Reference
IRBP KO ¹		IRBP coding region	Inter-photo-receptor retinoid binding protein gene	Homo	Cortes et al. (2008)
IL18 KO ²		IL18	IL18	Homo	Hochholzer et al. (2000)
Ig-NRG1 KO ³		NRG1	Ig-like domain	Hetero	Kramer et al. (1996)
ErbB4 ⁴	Actin	ErbB4	Heart/mammary gland	Homo	Tidcombe et al. (2003)
MyoCre ⁵	Myogenin	Cre recombinase	Muscle fiber	Homo	<i>a</i>
PRL1 ⁶	Alpha-A-crystalline	Hen egg lysozyme	Eye	Homo	Lai et al. (1998)
PRL2 ⁷					
MCP-1cel2 KO ⁸		MCP-1	White cells	Hetero	Lu et al. (1998)
RPE65-GFP-RAS ⁹	RPE	GFP	RPE membrane	Hetero	Redmond et al. (1998)

List of transgenic lines used in the study describing the strains, type of transgene and target organ. The homozygosity: *homo* homozygous, *hetero* heterozygous

The superscript numerals 1, 2, 3, 4 indicate strain C57BL/6; 5, 6, 7 indicate FVB/N; 8 indicates (129SvJ × C57BL/6)F1 and 9 indicates CD1

The superscript letter a means no publication

Summary of in vitro fertilization (IVF) results of the WT and the transgenic mice of C57BL/6 strain

Table 2

Strain	Total # of females	Total # of oocytes	Average # of oocytes per female	Total # of 2 cells	IVF % (mean ± SEM)
WT	15	474	32	378	80 ± 5 ^a
KO1	27	344	13	88	40 ± 11 ^a
KO2	15	353	24	229	64 ± 10 ^a
KO3	22	710	32	235	33 ± 1 ^a
T _g 1	24	594	25	324	69 ± 23 ^a

The data shown in the table represent cumulative results of all IVF experiments. Average fertilization for each IVF was calculated by dividing the number of two cell embryos by the number of total oocytes. The IVF was performed three times for the WT and on an average of 4 times for the transgenic mice. The percentage rate transformed by arcsine was used for the Dunnett's test to compare all the transgenic mice against the WT mice.

Superscript letters represent no significant differences ($P < 0.05$) among the groups

Table 3

Summary of in vitro fertilization (IVF) results of the WT and the transgenic mice of FVB/N strain

Strain	Total # of females	Total # of oocytes	Average # of oocytes per female	Total # of 2 cells	IVF % (mean $\pm$ S.E.M)
WT	15	181	12	164	90 $\pm$ 5 ^a
Tg1	15	305	20	249	80 $\pm$ 8 ^a
Tg2	54	890	16	444	50 $\pm$ 8 ^b
Tg4	42	782	19	526	65 $\pm$ 12 ^a

Within a column, superscript letters a, b represent significant differences ($P < 0.05$)

Dunnett's multiple-comparison test using the percentages transformed by arcsine was applied to compare all the transgenic mice against WT mice. The IVF was performed three times for WT, on an average of four times for transgenic mice

Summary of in vitro fertilization (IVF) results of the WT and the transgenic mice of (129Sv/J × C57Bl/6)F1 (in italic) and CD1 (in bold) strain

Table 4

Strain	Total # of females	Total # of oocytes	Average # of oocytes per female	Total # of 2 cells	IVF % (mean ± SEM)
WT	30	504	17	223	45 ± 4 ^a
KO	95	1,576	17	309	22 ± 3 ^b
WT	15	313	21	152	43 ± 8 ^a
Tg	59	1,265	21	264	25 ± 5 ^a

For each strain superscript letters a, b represent significant differences ($P < 0.05$).

Unpaired *t* test (two tailed) was used to compare the transgenic mice with the WT mice. IVF was performed three times for WT and on an average of 4 times for genetically modified mice