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Rederivation of transgenic rodent models expressing disease modified tau protein - a report

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Summary

Transgenic animals are used extensively as *in vivo* experimental systems for modelling human diseases. A prerequisite for obtaining reliable experimental results is that the animal models used are free of defined infectious agents, including those that rarely produce disease, but still can modulate immune responses and alter the phenotype of affected animals. We have recently rederived transgenic rodent models of tauopathy to meet specific-pathogen free health standards for breeding in barrier areas. The surgical implantation of 2- cell embryos in three transgenic rat lines SHR24, SHR72 and W72 resulted in a total of 86 pups, out of which 36 were positive for transgene (42% efficiency). An overall 48 % transgenic efficiency was achieved in two transgenic mice lines R3m/4 and R3m/7 following non-surgical transfer of embryos. Microbiological evaluation, based on sentinel screening and serological examinations, confirmed the SPF status of all rederived strains. Most importantly, all rederived transgenic rodents developed AT8 positive neurofibrillary structures demonstrating high reproducibility of the phenotype. To the best of our knowledge, this report is the first on rederivation of transgenic models of human tauopathy.

Keywords Rederivation; non-surgical embryo transfer; surgical implantation; transgenic model

Introduction

Currently, the vast majority of biomedical studies are performed on specific pathogen free (SPF) animals which need to be free of pathogens that may affect animal welfare and confound the validity of experimental data (Van Keuren and Saunders, 2004; Shek *et al.,* 2008; Amstislavsky *et al.,* 2013; Mähler *et al.,* 2014). Rederivation enables the production of SPF animals from potentially infected rodents. It can be achieved either by hysterectomy of late-term fetuses, cross-fostering of new born pups to clean surrogate mothers or by transfer of preimplantation embryos to recipient females (Artwohl *et al.,* 2008). The technique preferred depends on several factors and is highly influenced by the health condition of ani-

mals, colony size, number of staff involved and their personal skills, as well as financial considerations and barrier facility design (Fray *et al.,* 2008). Although both cross-fostering and hysterectomy are useful to rescue poorly breeding strains, they are not efficient enough against fetoplacental infection caused by pathogens, such as *Listeria monocytogenes*, *Murine norovirus* (MNV) and *Helicobacter hepaticus* (Le Monnier *et al.,* 2006; Yeom *et al.,* 2009). In contrast, rederivation by embryo transfer (ET) provides full protection against pathogens, and thus represents the most secure way of introducing animals into barrier colonies (Suzuki *et al.,* 1996; Morrell, 1999; Van Keuren and Saunders, 2004; Du *et al.,* 2010; Sztein *et al.,* 2011; Raspa *et al.,* 2016). Surgical transfer of rodent embryos, with respect to their developmental stage, can be carried out either by the puncture of the oviductal wall/ uterine horn or through the uterotubal junction (Mclaren and Michie, 1956; Sato *et al.,* 1999; Chin *et al.,* 2001; Sarvari *et al.,* 2013; Bermejo-Alvarez *et al.,* 2014). A non-surgical method using a non-surgical embryo transfer (NSET) device (ParaTechs, Lexington, KY) has recently been developed as a refinement to surgical uterine transfer in mice. This approach is technically based on the insertion of a catheter containing blastocysts through the cervical canal into the lumen of the uterine horn (Green *et al.,* 2009). Recently, we have established a rederivation protocol by means of embryo transfer in order to generate SPF transgenic animals. Here we report the rederivation of transgenic rodent models of human tauopathy and evaluate the neuropathological phenotype of the transgene positive animals.

Material and methods

Transgenic animals

The following transgenic models of tauopathy expressing human truncated tau aa151-391 have been rederived: models with 3 microtubule-binding repeats (3R), i.e. SHR24 rat (Filipcik *et al.,* 2012) as well as R3m/7 and R3m/4 mice lines (Zimova *et al.,* 2016), and models with 4 microtubule-binding repeats (4R) i.e. SHR72 (Zilka *et al.,* 2006) and W72 rats (Stozicka *et al.,* 2010). These animals were used as donors of the embryos. The generation and characterization of most of these transgenic lines have been previously described (Zilka *et al.,* 2006; Koson *et al.,* 2008; Stozicka *et al.,* 2010; Filipcik *et al.,* 2012; Zimova *et al.,* 2016). The characterization of R3m/7 is currently underway. All these transgenic lines are hemizygous for the transgene and express human truncated tau under Thy-1 promoter. Briefly, the transgene construct was prepared by ligation of a cDNA coding for human tau protein truncated at amino acid positions 151-391 into the mouse Thy-1 gene downstream of the brain promoter/enhancer sequence. The part of original Thy-1 gene sequence coding for exons II-IV together with the thymus enhancer sequence was replaced by the cDNA. The transgenic DNA was linearized by cleavage with EcoRI and all vector sequences were removed prior to microinjection. Transgenic rats were generated by pronuclear microinjection into 1-day old SHR embryos and similarly, the transgenic mice by microinjection into 1-day old C57BL/6NCrl embryos. Founders were double screened by polymerase chain reaction (PCR) using Thy-1-specific and human tau specific primers amplifying START codon (forward: 5′-GTGGATCTCAAGCCCTCAAG-3′, reverse: 5′-CCTGATTTTGGAGGT-3′) and STOP codon (forward: 5′- CCTGATTTTGGAGGT-3′, reverse: 5′-TATGCATGGAGGGAGAAG-3′) flanking sequences (Zilka *et al.,* 2006). Phenotypically, the rodents develop cardinal features of tauopathies, such as progressive age dependent tau hyperphosphorylation, formation of neurofibrillary tangles and ultimately reduced lifespan (Zilka *et al.,* 2006; Filipcik *et al.,* 2012; Zimova *et al.,* 2016).

Recipient animals

For surgical implantation of transgenic rat embryos, Crl: CD (SD) (Velaz, Prague, Czech Republic) rats in the SPF state were used as recipients.

Outbred Crl: CD1 (ICR) (CD-1) mice were first purchased from Charles River Laboratories (Wilmington, MA, USA) and then bred under strictly regulated conditions of the barrier facility. These mice, notable for good maternal instincts, were used as recipients for non-surgical transfer of transgenic mouse embryos.

Animal husbandry

Transgenic rodent models were housed conventionally prior to the rederivation. These animals received a non-sterilized diet (LASQCdiet® Rod16-H, LASvendi, Germany) and untreated water *ad libitum*. Water bottles and cages were changed once per week.

SPF animals were fed certified rodent diet (LASQCdiet® Rod18-H, LASvendi, Germany) and autoclaved water was available *ad libitum*. In the barrier facility, all cages, cage tops, bedding and foodstuff went through peroxidase sterilization (Bioquell Z- 2, Bioquell, UK) before use. Staff members entering the quarantine suite followed all necessary pre-

cautions according to established SPF guidelines and protocols.

Both conventional (embryo donors and breeding males) and SPF animals (recipient females and vasectomized males) were maintained under controlled environmental conditions according to good laboratory practice requirements: at 22±2˚C with 55±10% relative humidity and a 12 h light/dark cycle (with the light phase starting at 7 am). Vasectomized and breeding males were housed individually, donor and recipient females were housed five per cage before being mated.

Ethical statement: This project was approved by the State Veterinary and Food Committee of Slovak Republic (Ro 4429/16-221h; Ro 4429/16-221g; Ro 4429/16-221a) and by the Ethics Committee of the Institute of Neuroimmunology at Slovak Academy of Sciences. All procedures involving animals and their care were in accordance with the approved guidelines conforming to international standards.

Embryo collection, culture and transfer

Mature wild-type females (2-4 months old) were naturally mated with heterozygous transgenic males. Plug-positive dams were sacrificed by cervical dislocation the following morning to obtain 1-cell embryos (surgical method) or at Day 4 to obtain blastocysts (NSET method). For surgical implantation in rats, 1-cell embryos were recovered from the excised oviduct into M2 medium containing 0.1% hyaluronidase to remove surrounding cumulus cells. After repeated washing (5 times) in sterile media (M2), embryos were placed into M16 medium and cultured overnight in an incubator (at 37˚C in 5% CO2) to reach 2-cell stage. The following morning, the resulting embryos were washed again 5x in M2 media before being transferred unilaterally into the oviduct. Surgical transfer was performed under general anesthesia using Zoletil 100® (1:1 tiletamine-zolazepam mixture, 50mg/ml + 50mg/ml; Virbac, Carros, France) in combination with Xylariem® (20mg/ml; Ecuphar N.V., Oostkamp, Belgium) administered intraperitoneally. At the end of surgery, buprenorphine (0.1 mg/kg; Buprex; Reckitt Benckiser Pharmaceuticals Ltd, Berkshire, UK) was administered intraperitoneally to prevent postoperative pain. Following surgery, the animals were placed inside clean cages warmed to 37°C until they regained consciousness. The recipients were monitored regularly during next 2 days and received analgesics if required.

For non-surgical transfer in mice, embryos at blastocyst stage were collected by flushing the uterine horns of donor females at 3.5 days post coitum (dpc) and microscopically evaluated (Nikon SMZ645 stereoscopic Zoom Microscope, Prague, Czech Republic). Only morphologically normal blastocysts with intact zona pellucida were selected for subsequent transfer. Apart from repeated washing (5x) of embryos in sterile media by using the new capillary for each washing drop, no other pretreatment was used to reduce the concentration of pathogens. Before each transfer, 6–8-week-old nulliparous CD-1 mice (SPF animals) at proestrus were mated with vasectomized males to induce pseudo-pregnancy. Plug-positive females were used as recipients on 2.5 dpc. Briefly, each pseudopregnant mouse was placed on a wire-top cage and allowed to grip the bars. A small speculum (ParaTechs, Kentucky, USA) was inserted inside the vagina to open and expose the cervix. The catheter then was inserted through the speculum, past the cervical opening, and into the lumen of uterine horn (Ali *et al.,* 2014).

Embryo transfer procedures were repeated until a sufficient number of transgenic founders was obtained. To evaluate the effectiveness of the ET, the following parameters were analyzed: 1) pregnancy rate (% of recipients becoming pregnant after ET); 2) birth rate (% of pups born per embryos transferred); 3) transgenic rate (% of pups carrying transgene per pups born). The success of the entire rederivation procedure was determined based on criteria proposed by Van Keuren and Saunders (2004) for transgenic lines.

Rederivation protocol

Two separate working groups were involved in the rederivation program. Personnel at the conventional unit were responsible for embryo isolation and initial washing with sterile M2 media (Sigma-Aldrich, Bratislava, Slovakia) prior to delivery. For short-term transportation (5-10 mins), donor embryos were placed into M2 medium in NUNC 4well dish (Thermo Fischer scientific, Bratislava, Slovak Republic) at room temperature and delivered directly to the SPF unit. Staff members at the receiving SPF area repeated washing before transferring embryos to clean surrogate mothers. All embryo transfers were performed within 30 min after embryo collection.

Genotyping and health monitoring

The offspring resulting from embryo transfers were tail tipped between 10-15 days of age. Genomic DNA samples from tail biopsies were analyzed for the transgene expression by PCR assay using transgene-specific primers mentioned earlier. Confirmed

transgenic founders were used to establish a clean breeding core of the respective strains under SPF conditions. The next generation was obtained by natural mating of a heterozygous transgenic male with a wild- type (WT) female. The efficacy of the rederivation protocol was tested using sentinel animals to monitor the microbiological state of an existing colony (Reuter *et al.,* 2003). Sentinels with the proven SPF state were exposed to soiled bedding material from the cages of rederived animals for at least 5 weeks. After this period, samples from the sentinels were collected and outsourced to IDEXX BioAnalytics (Germany) for microbiological testing.

Histological examination of brains

Animals from the subsequent generation of rederived lines (n=5/line) were deeply anaesthetized with a mixture of Zoletil (30mg/kg) and Xylazine and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline, pH 7.2, using a mini peristaltic pump (Harvard apparatus, USA). The tissues were post-fixed in 4 % PFA after perfusion and then embedded in paraffin and cut on a microtome. 8µm paraffin embedded sections were immunostained with AT8 antibody (epitope pS202/ T205; Thermo Scientific, Illinois, USA). Following washing, sections were stained with biotinylated secondary antibody and developed using Vectastain ABC Kit (Vector Laboratories, CA, USA). After mounting, sections were evaluated using an Olympus BX51 light microscope equipped with an Olympus DP27 digital camera (Olympus, Prague, Czech Republic). Stained sections were examined for presence of AT8 positive structures.

Results

Rederivation of transgenic rats inside the SPF facility

We initiated our rederivation program in transgenic rat colonies in which antibodies to *Clostridium piliforme* (formerly *Bacillus piliformis*) were detected by MFI (Multiplex Fluorescent Immunoassay) and confirmed by the indirect fluorescent antibody (IFA*)* test. Since these transgenic animal models are not available commercially, it was necessary to rederive them to safeguard their unique genotypes. The well-established surgical ET method was employed for this purpose. A total of 289 embryos were transferred into 24 pseudopregnant recipients with an average (all embryos/ all recipients) of 12 embryos transferred per unilateral oviduct. Of all recipients, 18 delivered a total of 86 pups, out of which 36 were transgenic, with an overall rate of 42% (Table 1).

Import of transgenic mouse lines from conventional into a barrier facility

The next step was to import transgenic mouse models from conventional animal house into a specific pathogen free unit while simultaneously eliminating murine pathogens: *Mycoplasma pulmonis, Klebsiella oxytoca* and *Morganella morganii* from breeding colonies. To accomplish this goal, the non-surgical ET technique using the non-surgical embryo transfer (NSET) device was selected. The reproductive data of transgenic mouse lines following the NSET procedure are presented in Table 2. A total of 109 *in vivo*-derived blastocysts were transferred to 11 pseudopregnant CD1 recipients with an average of about 10 blastocysts received per female. Of all recipients, 7 became pregnant and gave birth to a total of 27 pups with an overall 48 % transgenic positive rate.

Table 1. Results from the surgical implantations of transgenic rat lines

Transgenic rat line	Number of recipients	Number of pregnancies	Number of embryos transferred	Number of pups born	Number of transgenic pups
SHR72	6	5 (83%)	77	17 (22%)	6 (35%)
SHR24	9	5 (56%)	108	30 (28%)	14 (47%)
W72	9	8 (89%)	104	39 (38%)	16 (41%)
Combined Total	24	18 (75%)	289	86 (30%)	36 (42%)

Table 2. Results from nonsurgical transfer of transgenic mice lines

Transgenic mouse line	Number of blastocysts collected	Number of blastocysts transferred	Number of recipients	Number of pregnancies	Number of pups born	Number of transgenic pups
R3m/4	78	70 (89.7%)	7	4 (57%)	11 (15.7%)	5 (45%)
R3m/7	46	39 (84.8%)	4	3 (75%)	16 (41.0%)	8 (50%)
Combined Total	124	109 (87.9%)	11	7 (64%)	27 (24.8%)	13 (48%)

Health monitoring of rederived colonies

A soiled bedding sentinel program was utilized to verify the health status of the animals after rederivation. In this study, C57BL/6NCrl or Crl: CD1 (ICR) mice (depending on their availability) and Crl: CD (SD) rats were employed as sentinels. These animals were not screened individually for infection prior to usage, but the health reports declared that the source colonies were free of infection. For each room, two sentinel cages (each stocked with two 4- to 6-week-old females of a particular strain) were placed on the top or the bottom of the rack to maximize the exposure to potential pathogens present in the colony. Bedding samples from several randomly selected cages were placed into each sentinel cage during weekly cage changing. After at least 5 weeks, samples from sentinel animals were collected. These included blood for serology testing, fecal samples for endoparasitology and PCR testing, swabs from the respiratory tract as well as a fur swabs for an ectoparasitology examination. As a result, the success of the rederivation procedure was confirmed by the absence of all pathogens listed in Tables 3 and 4 of FELASA recommendations (Mähler *et al.,* 2014).

Neuropathological phenotype of rederived lines

The subsequent generation from rederived lines was obtained by breeding (natural reproduction) under SPF animal facility conditions. Interestingly, it has been observed that reproducibility of the phenotype may be altered, in some cases, by relocating the animals (Masopust *et al.,* 2017). Therefore, in order to evaluate if the rederived lines retained their phenotypes, we performed histochemical staining of brainstem and cortex (SHR24) using phospho-tau antibody AT8 (marker of neurofibrillary pathology). We confirmed the presence of AT8 positive structures in transgenic mouse (Fig.1 A-B) and rat (Fig. 1 C-E) models as previously reported (Zilka *et al.,* 2006; Stozicka *et al.,* 2010; Filipcik *et al.,* 2012; Zimova *et al.,* 2016).

Discussion

Transgenic rodents are used extensively to replicate different aspects of human diseases including critical aspects of the pathology of Alzheimer's disease (Spires and Hyman, 2005; Zilka *et al.,* 2006; Filip-

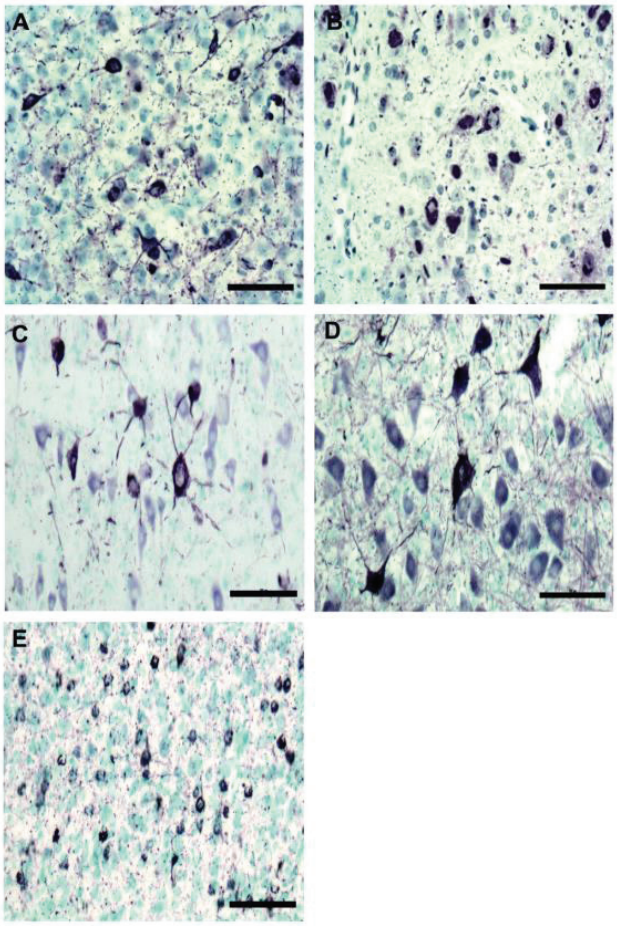

Figure 1. Histological staining for neurofibrillary pathology in rederived transgenic lines.
Immunohistochemical analysis using AT8 antibody revealed the presence of neurofibrillary structures in (**A**) R3m/7, (**B**) R3m/4, (**C**) W72, (**D**) SHR72 and (**E**) SHR24 transgenic lines. Brain areas-**A-D**: Brainstem; **E**: Cortex. Scalebar: 100µm.

cik *et al.,* 2012; LaFerla and Green, 2012; Zimova *et al.,* 2016). However, most of these valuable rodent models are still being bred in conventional animal facilities. It is therefore essential to ensure that these animals are free of pathogens which may alter their unique phenotypes, and thus confound the interpretation of study results (Franklin, 2006; Treuting *et al.,* 2011).

Our first challenge was to improve the health status of transgenic rats and to generate colonies free of *Clostridium piliforme* (formerly *Bacillus piliformis*) in a fully occupied and endemically infected breeding facility. For this purpose, we initiated the rederivation program through embryo transfer using the standard surgical procedure. Since *Clostridium piliforme* is an endospore-forming bacterium, special efforts, including peroxidase sterilization and disinfection, were made to remove spores from the environment. This procedure in combination with successful embryo transfer resulted in the elimination of the undesirable pathogen in rats. More importantly, we were able to continuously use the facility without any emergency shutdown or major renovation. We conclude that embryo transfer rederivation coupled with the environmental decontamination can eliminate latent infection caused by *Clostridium piliforme* from transgenic rat colonies.

We then proceeded to import novel transgenic mouse models for tauopathy from a conventional animal house into a barrier unit. The introduction of new lines into SPF conditions demands strict precautions in order to avoid entry of pathogens into the receiving facility. Washing of embryos prior to transfer prevents the transmission of the vast majority of prevalent pathogens, and thus represents the most secure way to introduce potentially contaminated animals into a particular hygienically defined area (Morell, 1999; Van Keuren and Saunders, 2004; Fray *et al.,* 2008; Amstislavsky *et al.,* 2013). Although there is a standardized washing protocol for the embryos of livestock by passing them through 10 drops of sterile media (Springfellow and Seidel, 1998), there are none for rodent embryos (Mahabir *et al.,* 2008). In the present study, the *in vivo* derived blastocysts were washed five times and only zona intact embryos were non-surgically transferred to SPF recipients. None of the infectious diseases were transmitted from infected donors to recipients via embryos, thus providing the evidence that the washing procedures were done properly. Regarding the NSET technique itself, we did not use large specula, as suggested by NSET protocol, and used only smaller specula. A similar refinement to the original protocol was made by Ali et al. (2014). On average about 10 blastocysts, obtained from naturally mated donor females with heterozygous transgenic males, were transferred to each recipient.

Using the NSET method, we set up the rederivation protocol which fulfils all criteria of the 3R concept (Reduction, Refinement and Replacement). The NSET method provided the ability to quickly generate live born offspring which substantially reduced the number of donors and fosters needed. With the repeated use of the surrogate mothers (Kolbe *et al.,* 2012) together with the reduction of embryos transferred, we could achieve the balance between effort and yield as described by González-Jara *et al.* (2017). As a refinement procedure, it allowed smooth atraumatic transfer of embryos that avoided the trauma suffered by the recipient mice from surgical intervention (Steele et al., 2013). Finally, the transport of mouse lines as embryos, instead of living animals, is considered as a partial replacement in the use of animals (Kelley, 2010).

We have previously reported that the expression of truncated tau induced extensive neurofibrillary degeneration in the brainstem and spinal cord of SHR72 and W72 rats (Zilka *et al.,* 2006; Stozicka *et al.,* 2010), and in the cortex of SHR24 rats. Similarly, R3m/4 mice developed tau pathology predominantly located in the brainstem (Zimova *et al.,* 2016). Interestingly, we have observed that despite retaining the desired genotype, some animal models did not develop robust pathology in the SPF barrier, when compared to their conventionally housed counterparts (S. Jadhav, unpublished observations). Furthermore, it was shown, that changes in housing conditions, even within the same institution, can be accompanied by loss of specific disease phenotype (Masopust *et al.,* 2017). Therefore we investigated whether the animal models retained their neuropathological phenotypes following the entire rederivation procedure. Our results confirmed that all rederived animal models developed progressive neurofibrillary degeneration in the examined brain area as previously reported (Koson *et al.,* 2008; Stozicka *et al.,* 2010; Filipcik *et al.,* 2012; Zimova *et al.,* 2016). The presence of pathogens in donor animals, nor the movement of mouse embryos to another facility, had a negative impact on their specific phenotype. We conclude that all transgenic lines retained the neuropathological phenotype following rederivation. Finally, our report, to the best of our knowledge, is the first on rederivation of transgenic models of human tauopathy.

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Conflict of interest

BV, VC, IU, JH and SJ are employees of Axon Neuroscience R&D Services SE. VB have no conflict of interest to declare.

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