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GENERATION OF MICE WITH TISSUE SPECIFIC TRANSGENE EXPRESSION USING SPERM MEDIATED GENE TRANSFER

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ABSTRACT

The production of transgenic animals has extended our knowledge of physiology and allowed the generation of animal models for human diseases. Previous work in the laboratory had reported the use of lentiviral vectors to generate transgenic animals using sperm mediated gene transfer (SMGT). To determine if lentivirus mediated SMGT could be used to generate tissue specific transgenic animals, a lentiviral vector containing the K14 promoter driving the expression of turbo RFP was used to generate transgenic animals. The pups were screened for presence of the transgene and for transgene expression for two generations. Transgenic animals were generated at a very high frequency and the transgene was stably inherited in the germline. In addition, the transgene was expressed in the hair follicle as previously reported for K14 driven transgenes suggesting that our SMGT protocol can be used to generate tissue specific transgenic animals.

KEYWORDS: *Transgenic, SMGT, K14 promoter, Turbo RFP.*

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INTRODUCTION

Transgenic animals are important tools to study animal physiology and to generate animal models of human disease, which can lead to the generation of novel therapeutic strategies. Transgenic animals were earlier produced by microinjection of the transgene into two or four cell embryos or by fusion of the blastocyst with modified embryonic stem cells.[1-4] However, these techniques are expensive, require specialized training and equipment and transgene positive pups are obtained at low efficiency. To overcome these drawbacks, techniques involving the *in vivo* and/or *ex vivo* modification of male gametes at different stages of spermatogenesis were developed. These included (i) incubating sperm with circular or linearized DNA fragments,[5] (ii) *in vitro* modification of spermatogonial stem cells using either retroviruses or cDNA constructs followed by introduction of the modified spermatogonial stem cells into the testes of chemically sterilized animals,[6-9] (iii) injecting the transgene into the testes of animals,[10] (iv) injection of the DNA construct into the testes followed by electroporation[11, 12] and (v) retroviral or lentiviral mediated transduction of spermatogonial stem cells *in vivo*.[13, 14] Collectively, these techniques are termed Sperm Mediated Transgenesis or Sperm Mediated Gene Transfer (SMGT). The constitutive expression of a transgene is important while studying the role of a particular gene in the development of an organism or in diseases where global expression of a particular protein is affected and this altered protein expression plays an important role in disease development. Similarly, studying the development of a particular tissue or pathological conditions arising in a specific tissue, requires tissue specific gene manipulation. This tissue specific manipulation can be achieved using tissue specific promoters as described previously.[15] Therefore, transgenic animals with tissue specific expression are equally or more important than transgenic animals with constitutive transgene expression. Previous work from this laboratory has demonstrated that modifying spermatogonial stem cells *in vivo* using lentiviral particles leads to the generation of transgenic animals with constitutive transgene expression and stable transgene integration at very high efficiency.[14] To determine if a similar approach can be used to generate transgenic animals with tissue specific transgene expression, lentiviral particles containing turbo RFP under the control of the K14 promoter were injected into the testes of pre-pubescent male mice. The progeny of these mice generated transgenic animals at high frequency, which showed tissue specific transgene expression.

MATERIALS AND METHODS

Plasmids

pCCLK14 GFP plasmid was a kind gift from Dr. Francesca Miselli (University of Modena and Reggio Emilia, Italy). Turbo RFP cDNA was PCR amplified from pTRIPz vector (Open Biosystems) using primers containing Age I and Sal I restriction sites (Table I). The PCR product was cloned into TA vector (Thermo Scientific) and was further sub-cloned into pCCLK14 GFP plasmid using Age I and Sal I sites to generate pCCLK14 turbo RFP.

Lentivirus Packaging

All cell lines used in this study were cultured in DMEM (Gibco) with 10% FBS (Gibco). 293T cells were co-transfected with pCCLK14 turbo RFP and virapower packaging mix (Invitrogen) according the manufacturer's instructions to generate lentiviral particles containing the transgene. The viral particles were concentrated as described.[16] Viral titer was determined by infecting HaCaT cells with 10 µl of the concentrated viral suspension. 48 hours post transduction, a single cell suspension was analyzed by flow cytometry for the presence of RFP and and viral titer was determined using the formula (number of fluorescent cells x dilution factor) as described.[16]

Animals

Swiss mice Crl:CFW(SW) were bred and maintained in the laboratory animal facility of ACTREC. Maintenance of the animal facility is as per the national guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The animals were housed in a controlled environment with the temperature and relative humidity being maintained at 23 ± 2^0C and 40-70% respectively and a day night cycle of 12 hrs each (7:00 to 19:00 light; 19:00 to 7:00 dark). The animals were received an autoclaved balanced diet prepared in-house as per the standard formula and sterile water *ad libitum*. Mice were housed in the Individually Ventilated Cage (IVC) system (M/S Citizen, India) provided with autoclaved corn cob bedding material (ATNT Laboratories, Mumbai). Protocols for the experiments were approved by the Institutional Animal Ethics Committee (IAEC) of ACTREC. The animal study proposal number is ACTREC IAEC project No. 11/2012. The viral particles (3.5×10^5 TU/µl) were surgically injected into pre-pubescent male mice to generate pre-founder mice as described previously.[14] Five weeks post-surgery, mice were mated with wild type female mice to obtain transgenic pups.

Genotyping of Animals

Tail biopsies (~3mm) from 3 week old pups sired by pre-founder males were lysed for 16h at 50°C in high salt digestion buffer containing 50mM Tris HCl, 1% SDS, 100mM NaCl, 100mM EDTA and 1200µg/ml Proteinase K (Jackson Laboratories). The lysate was processed for isolation of DNA using phenol-chloroform extraction followed by ethanol precipitation.[16] The genomic DNA was subjected for PCR analysis using transgene-specific primers whose sequences are listed in Table I. Every PCR reaction set had three controls. The pCCLK14 turbo RFP plasmid was used as a template for a positive control, genomic DNA obtained from WT mice was used as a negative control and amplification of the endogenous patched (Ptch) gene was used as a loading control. Genomic DNA isolated from the human origin cell line, HCT116, was used as a negative control for the Ptch PCR. To validate the turbo RFP PCR results, two sets of primers were used. First set of PCR primer recognizes a part of turbo RFP sequence

whereas second set of primer recognizes the entire turbo RFP sequence taken from pTRIPz vector. To ensure that the PCR product is indeed turbo RFP sequence, PCR product was cloned into the pTZ57RT/T vector (Thermo Scientific) and was sequenced using Sanger sequencing.

Fluorescence Microscopy

To detect the turbo RFP expression, epidermis was separated from the dermis of the tail sections of transgene positive pups, using 5mM EDTA solution[17] and epidermis was then mounted on a glass slide using Vectashield (Vector) as per the manufacturer's instructions. The epidermis sections were then imaged using Zeiss LSM510 confocal microscope. As a control epidermis section from WT mouse was used.

Table 1
List of oligonucleotides used for genotyping.

Name of oligonucleotides	Sequence
Ptch Forward	CTGCGGCAAGTTTTTGGTTG
Ptch Reverse	AGGGCTTCTCGTTGGCTACAAG
Turbo RFP I Forward	ATGAGCGAGCTGATCAAGG
Turbo RFP I Reverse	TTATCTGTGCCCCAGTTTGC
Turbo RFP II Forward (Age I)	GGACCGGTCGCCACCATGAGC
Turbo RFP II Reverse (Sal I)	GGGTCGACTGGCCGGCCGCATTAGTCT

RESULTS

Generation of pCCLK14 turbo RFP viruses

To generate mice that express the transgene only in the epidermis, we selected the Keratin 14 (K14) promoter because K14 and Keratin 5 (K5) are expressed in the basal layers of epidermis,[18] are specific markers of the basal epithelium and the K14 promoter has been widely used to generate epidermis specific transgenic animals.[19-21] Turbo RFP was cloned downstream of the K14 promoter in pCCLK14 vector and pCCLK14 turbo RFP viral particles were generated using the virapower packaging system. 5-10 µl of concentrated pCCLK14 turbo RFP viruses (3.5 x 10^5 TU/µl) were surgically injected into the testicles of pre-pubescent Swiss mice to generate pre-founder mice. Five weeks post-surgery these mouse were mated with wild type female to obtain transgenic pups.

Screening of pups by PCR amplification of turbo RFP

The pups were screened for the presence of the turbo RFP transgene by performing PCR reactions using the genomic DNA of pups as a template. As shown in Figure 1I, almost all the pups showed the presence of the turbo RFP transgene while genomic DNA from WT mice did not show the presence of the transgene. A PCR reaction for the mouse Patched (Ptch) gene was performed as a loading control and no signal was observed for Ptch in genomic DNA purified from the human cell line, HCT116 (Figure 1A-C). Since a very high number of the pups scored as transgene positive, we were concerned that this could be an artifact of the PCR reaction. Therefore, we used another set of oligonucleotide primers which amplifies the entire turbo RFP transgene. Similar results were obtained using this primer set (Figure 1A-C), suggesting that these pups were indeed positive for the transgene. To further confirm these results, the PCR products were cloned into the pTZ57RT/T vector system and the PCR products sequenced. The sequencing reactions confirmed that the amplified product was indeed the turbo RFP transgene (Figure 1D). We did not perform Western blots to demonstrate the presence of the RFP protein in the epidermis due to the non-availability of a good antibody to RFP.

Epidermis specific turbo RFP expression

To determine whether RFP was expressed in these animals, tail snips were prepared by separating the epidermis from the muscle and cartilage and the epidermis was examined by confocal microscopy. As shown in figure 2, mice containing the turbo RFP transgene showed red fluorescence in the bulb region of hair follicle, the root sheath of the hair follicle and the sebaceous glands. The arrangement of hair follicles correlated with that given in literature.[22] The pattern of turbo RFP expression was similar to that reported for keratin14[17, 23] suggesting that we achieved tissue specific gene expression. Further, expression of turbo RFP was observed in first generation mice (1230) as well as second generation mice (83), suggesting that the inheritance of the transgene was stable and that expression was maintained over both the generations. The intensity of the turbo RFP fluorescence is greater in the second generation as compared to the first generation. Some auto fluorescence was observed in the hair shaft of WT mice, however, this does not overlap with the regions in which keratin 14 is expressed.[17, 23] These results suggest that the transgenic mice expressed turbo RFP specifically in the epidermis

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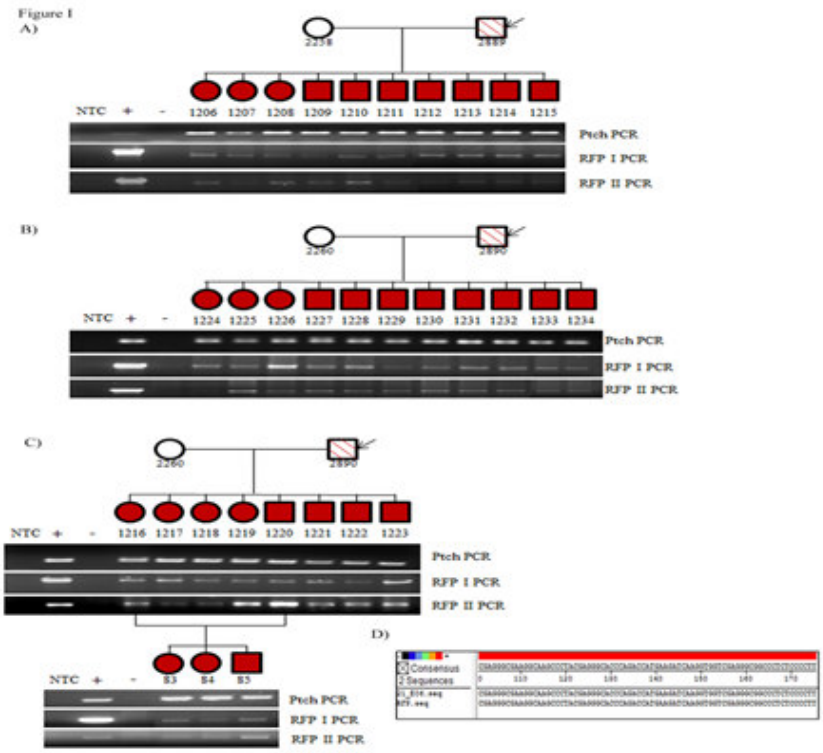

Figure 1

Stable inheritance of the RFP transgene in mice injected with the pCCLK14 turbo RFP construct. A-C. A pedigree analysis for pre-founder mice (indicated with an arrow) 2889 (A) and 2890 (B-C), showing germline transmission of the transgene. Individual mice were assigned numbers for further experiments. Genomic DNA amplification using primers for RFP (RFPI and RFPII) or Patched (Ptch) as a loading control are shown for each animal. The filled squares and circles indicate transgene positive animals. NTC is the no template control , + is a positive control where the template is the pCCLK14 plasmid and – represents the negative control, which is genomic DNA isolated from WT mice for the RFP PCR reactions and genomic DNA from HCT116 cells for the Ptch PCR reactions. D) RFP II PCR product obtained from genomic DNA of mouse 1221 was cloned into pTZ57RT/T vector and sequenced. Note that the sequence shows an exact match to the turbo RFP sequence

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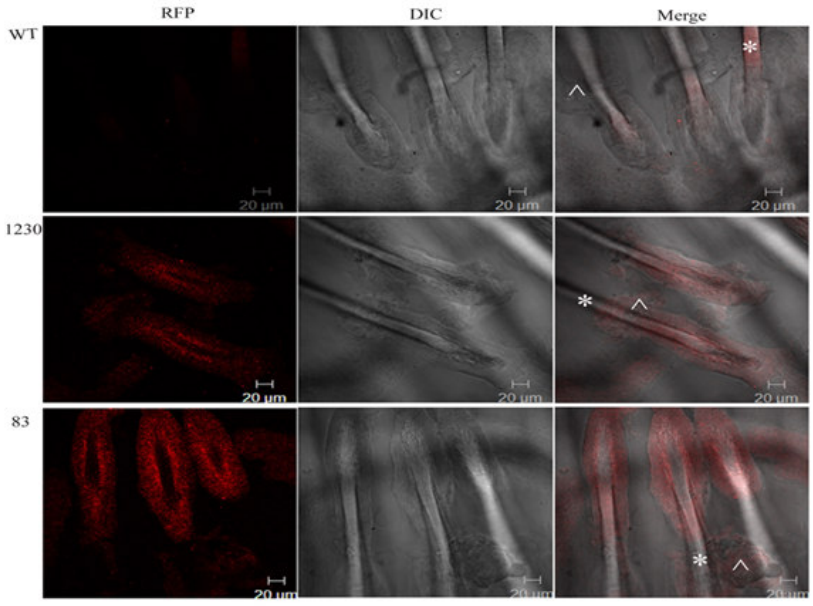

Figure 2

Turbo RFP is expressed in tissues that normally express keratin 14. Epidermis whole mounts from wild type (WT) or transgenic animals (1230 and 83) were examined by fluorescence microscopy (left panel). A DIC image (middle panel) and a merged image (right panel) are also shown. Note that the turbo RFP fluorescence is more intense in the second generation. The fluorescence in the transgenic animals is observed in the hair follicle and sebaceous glands as previously reported for keratin 14. * indicates Hair Shaft, ^ indicates Sebaceous glands.

DISCUSSION

Transgenesis revolutionized the study of mammalian biology by allowing studies on developmental biology, study of genetically inherited diseases and cancer. There are various transgenic mouse models which mimic human diseases and syndromes and hence are used for testing and developing new therapeutic strategies. The methods to achieve transgenesis have also developed considerably to make transgenesis efficient, quick and cost effective. These methods needs to be validated for different types of promoter systems before generating important mouse models or before making transgenic models in larger animals. The present study wished to validate the lentiviral mediated SMGT for tissue specific transgene expression so that epidermis specific gene up-regulation or down-regulation can be achieved. The present study once again demonstrated the efficiency of viral mediated SMGT for generating transgenic animals. The vector system used in this study can be modified to induce expression of the transgene in different tissue types by using the appropriate tissue specific promoter. Alternatively, temporal induction of the transgene can be achieved by driving expression from inducible promoters, such as those containing the Tetracycline operator.[15] These mice can be crossed to mice expressing the Tetracycline repressor under a constitutive promoter and expression induced by addition of Tetracycline in the drinking water. With the advent of CRISPR Cas9 technology,[24] already existing lentiviral systems used for SMGT can also be modified so that Cas9 expression is driven by tissue specific or inducible promoters and the guide RNA expression driven by a pol III based promoter to generate inducible or tissue specific knockout mouse. This will further simplify the generation of knockout mouse models leading to a greater understanding of mammalian physiology and the generation of better mouse models of human disease.

CONCLUSION

The generation of transgenic animals using traditional methods is very cumbersome and all the drawbacks of traditional methods can be avoided by using SMGT. SMGT technique using lentiviral particles allows transduction of quiescent cells and stable transgene integration, which can be inherited by the progeny. The results in this report demonstrate that SMGT can be used to generate transgenic mice with tissue specific expression and this system can be modified to study the effect of over expression or down regulation of particular gene products in skin development.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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