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Citation for published version:

Whitelaw, CBA, Radcliffe, PA, Ritchie, WA, Carlisle, A, Ellard, FM, Pena, RN, Rowe, J, Clark, AJ, King, TJ & Mitrophanous, KA 2004, 'Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector' FEBS Letters, vol 571, no. 1-3, pp. 233-6., 10.1016/j.febslet.2004.06.076

Digital Object Identifier (DOI):

[10.1016/j.febslet.2004.06.076](https://doi.org/10.1016/j.febslet.2004.06.076)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher final version (usually the publisher pdf)

Published In:

FEBS Letters

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Efficient generation of transgenic pigs using equine infectious anaemia virus (EIAV) derived vector

C. Bruce A. Whitelaw^{a,*}, Pippa A. Radcliffe^b, William A. Ritchie^a, Ailsa Carlisle^a,
Fiona M. Ellard^b, Romi N. Pena^c, Jo Rowe^a, A. John Clark^a, Tim J. King^a,
Kyriacos A. Mitrophanous^b

^aDepartment of Gene Function and Development, Roslin Institute, Roslin, Midlothian EH25 9PS, UK

^bOxford BioMedica, The Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA, UK

^cArea de Produccion Animal, UdL-IRTA, 25198 Lleida, Spain

Received 21 June 2004; accepted 23 June 2004

Available online 8 July 2004

Edited by Jesus Avila

Abstract Traditional methods of transgene delivery in livestock are inefficient. Recently, human immunodeficiency virus (HIV-1) based lentiviral vectors have been shown to offer an efficient transgene delivery system. We now extend this method by demonstrating efficient generation of transgenic pigs using an equine infectious anaemia virus derived vector. We used this vector to deliver a green fluorescent protein expressing transgene; 31% of injected/transferred eggs resulted in a transgenic founder animal and 95% of founder animals displayed green fluorescence. This compares favourably with results using HIV-1 based vectors, and is substantially more efficient than the standard pronuclear microinjection method, indicating that lentiviral transgene delivery may be a general tool with which to efficiently generate transgenic mammals.

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Keywords: Equine infectious anaemia virus; Lentivirus; Pig; Livestock; Transgenic; Biotechnology

1. Introduction

The generation of transgenic animals holds considerable promise to advance understanding in biomedical and agricultural systems. Nevertheless, applications in livestock have been restricted by the considerable effort and cost required to generate individual animals. The recent development of lentiviral vectors for transgene delivery [1–4] may overcome some of these limitations.

The first transgenic livestock were generated nearly two decades ago using the pronuclear microinjection technique [5], which involves the direct injection of the transgene DNA into

one of the two pronuclei in the zygote. Although inefficient, with 1% of injected eggs resulting in a transgenic founder animal, the reliability of pronuclear injection has ensured that it remains the most used method [6]. Alternative methods have been developed. The use of transgenic sperm to generate transgenic livestock is attractive due to its simplicity but suffers from apparent variability [7], while nuclear transfer using transgenic cells [8] has the advantage that all founder animals should be transgenic but is currently severely restricted due to very low foetal survival and high neonatal mortality [9].

Recently, transgenic mammals have been generated using a lentiviral vector [1–4]. In contrast to using an oncoretroviral vector [10], lentiviral delivery appears to offer a spectacularly efficient method for the generation of transgenic mammals. The lentiviral vector can be introduced by injection into the perivitelline space of the zygote [2,3] or by co-culture with a zona-free zygote [1]. In mice, 10–30% of lentivirus-infected eggs can give rise to transgenic founder animals with 60–90% of these founders expressing the transgene [1,2]. Similar frequencies have been obtained in pigs [3] and cattle; the latter only successful by infecting oocytes that are subsequently fertilised in vitro [4]. This should be compared to the standard pronuclear injection method where 5% of mouse zygotes and 1% of livestock zygotes result in a transgenic founder animal [6].

All reports on lentiviral transgenic mammals to-date have used an human immunodeficiency virus (HIV-1) based vector [1–4,11–13]. We now show that a vector based on another lentivirus, the equine infectious anaemia virus (EIAV), can also be used to efficiently generate transgenic mammals. This suggests that lentiviral transgene delivery may be a general tool with which to efficiently generate transgenic mammals.

2. Materials and methods

2.1. EIAV vector production

The method for generating EIAV based vectors has been previously described [14]. Briefly, HEK293T cells were seeded in DMEM supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine and 1× MEM non-essential amino acids at a density of 4.4×10^4 cm⁻². The next day, cells were transfected with the pESYNGP (an EIAV codon optimised gag/pol expression construct: Wilkes et al., manuscript in preparation), pRV67 (a VSV-G envelope expression plasmid)

* Corresponding author. Fax: +44-131-440-0434.

E-mail address: bruce.whitelaw@bbsrc.ac.uk (C.B.A. Whitelaw).

Abbreviations: CMV, cytomegalovirus; GFP, green fluorescent protein; EIAV, equine infectious anaemia virus; HIV, human immunodeficiency virus

and pONY8.7NCG, an EIAV vector genome expressing enhanced green fluorescent protein (GFP) under the control of the human cytomegalovirus immediate-early enhancer/promoter (CMV) (Wilkes et al., manuscript in preparation), using Fugene-6 (Roche). Sixteen hours after transfection, the cells were treated with 10 mM sodium butyrate for 6 h and then the culture medium was replaced with butyrate-deficient medium. At 40 h post transfection, the culture medium was collected, centrifuged at $1000 \times g$ for 5 min and filtered through a 0.45 μm filter unit. The vector was concentrated by low speed centrifugation ($6000 \times g$, for 16 h at 4 °C) followed by ultracentrifugation ($50\,000 \times g$, for 90 min at 4 °C). The vector was resuspended in TSSM buffer consisting of sodium chloride (100 mM), Tris, pH 7.3 (20 mM), sucrose (10 mg/ml) and mannitol (10 mg/ml), aliquoted and stored at -80 °C. Vector titres on D17 cells were 1.6×10^9 TU/ml.

2.2. Zygote collection and infection

Embryos were obtained from Large-White gilts that were approximately 9 months of age and weighed at least 120 kg at the time of use. Super-ovulation was achieved by feeding 20 mg altrenogest (Regumate, Hoechst Roussel Vet. Ltd.) once daily for 4 days, between day 11 and 15 following an observed oestrus, and twice on the 5th day. On the 6th day, 1500 IU of equine chorionic gonadotropin (pregnant mare's serum gonadotropin, Intervet UK Ltd.) was injected at 20:00 h. Eighty three hours later, 750 IU human chorionic gonadotropin (Chorulon, Intervet UK Ltd.) was injected. Donors gilts were mated twice every 6 h apart from a Large White boar after exhibiting heat generation following super-ovulation. Embryos were surgically recovered from mated donors by mid-line laparotomy under general anaesthesia on day 1 following oestrus (heat = oestrus Day 0).

Zygotes were recovered by flushing the oviducts of five gilts with warm phosphate buffered saline with the addition of 1% foetal calf serum. They were removed from the PBS and stored in HEPES North Carolina State University 23 medium (HNCSU 23 medium) at 38 °C with the addition of 10% FCS. 70–80 pl of virus suspension was injected into the peri-vitelline space of the zygotes, using a "WPI PV820 Pico Pump". A few zygotes were kept from each donor animal and the remainder of the zygotes was mixed and transferred back into synchronous recipient animals.

Recipient females were treated identically to donor gilts but remained un-mated. After treatment, fertilised embryos were transferred to recipient gilts following a mid-line laparotomy under general anaesthesia. During surgery, the reproductive tract was exposed and embryos were transferred into the oviduct of recipients using a Drummond positive displacement micropipette.

Zygotes to be maintained *in vitro* to assess GFP activity were transferred to micro drops of HNCSU 23 medium and cultured at 38 °C in a 5% CO_2 atmosphere.

2.3. Southern blot

Lentiviral transgene integration number was determined by Southern blot analysis of DNA from an ear biopsy. 10 μg of DNA was digested with *ScaI*, separated on a 0.5% agarose gel, blotted to a nylon membrane and probed with a 570 bp-long PCR amplified fragment from the pEGFP-N1 plasmid (Clontech) using primers GFP-forward 5'-CGAGGGCGATGCCACCTAC-3' and GFP-reverse 5'-CTCCAGCAGGACCATGTGATC-3'. For complete restriction and loading control, we stripped the blots in boiling 0.5% SDS, $0.1 \times \text{SSC}$ and reprobed them with a 600 bp-long PCR amplified fragment from the porcine hepatic nuclear factor-1 (HNF1) gene using primers TCF1/F 5'-CCCAGCAGATCCTGTTC-3' and TCF1/R 5'-CTCCGTGACA-AGGTTGGAG-3'.

2.4. Fluorescence imaging

GFP auto-fluorescence was detected using blue light illumination (GFP excitation frequency 455–495 nm) with a barrier filter cut off below 500 nm. For animal and wet tissue, this was with a GFSP-5 headset (Biological Laboratory Instruments, Budapest). Photomicrographs of embryos were taken using a Zeiss Axiovert 25 Microscope equipped with an Axiocam colour Digital camera and using Axiovision software. The images were taken at either 200 or 400 magnifications and UV images were taken using a GFP filter. Photomicrographs of organs were taken on a Leica MZ 111 Zoom dissecting microscope fitted with UV and a GFP filter and a Leica digital camera.

3. Results

3.1. Generation of transgenic pigs

Approximately 70 pl of a high-titre (1.6×10^9 TU/ml) preparation of an EIAV vector containing the CMV-GFP expression cassette (Fig. 1) was injected into the perivitelline space of fertilised porcine zygotes. Of 147 zygotes collected from five donor animals, all were injected and 120 zygotes subsequently transferred into five recipient gilts. Visualisation of non-transferred embryos clearly showed GFP-positive 4/5-cell stage embryos (Fig. 2A). Four pregnancies were carried to term and 40 piglets born. Of the 40 piglets born, 37 (92%) were transgenic by Southern blot and/or polymerase chain reaction assay. Of these 37 animals, 34 piglets expressed GFP as visualised by direct fluorescence in comparison to non-transgenic piglets (Fig. 2B). A further animal displayed mosaic GFP expression at postmortem; a total of 35 out of 37 transgenic founder piglets expressed GFP (95%).

3.2. EIAV-delivered transgene expression

Lentiviral vectors integrate as a single-copy, however, animals can carry more than one lentivector. We have determined

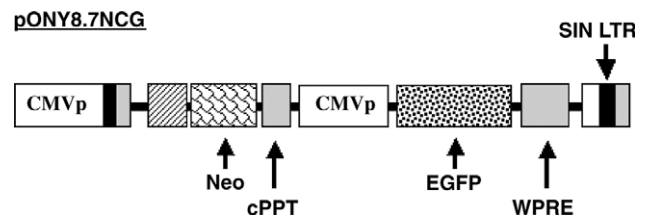


Fig. 1. EIAV vector. Schematic representation of the EIAV (pONY8.7NCG) vector used in this study. The vector contains a self-inactivating long terminal repeat (SIN LTR), Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and central polypurine tract (cPPT). Expression of EGFP transgene is under the control of the hCMV promoter. Expression of the Neo gene is minimal in transduced cells due to the SIN LTR.

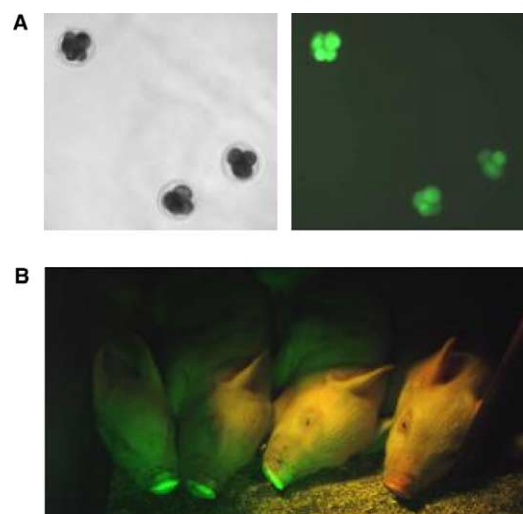


Fig. 2. EIAV transduced pig embryos and piglets. (A) Transduction of porcine preimplantation embryos with EIAV lentiviral vector. GFP expression in early morula derived from zygotes treated by perivitelline injection of pONY8.7NCG. (B) Ten day old founder pigs #112, #113, #114 and non-transgenic piglet #20.

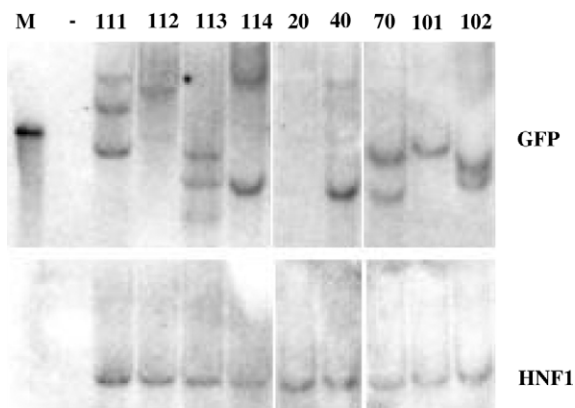


Fig. 3. Founder pigs carry different transgene copy-numbers. Representative Southern blot of *ScaI* digested ear biopsy DNA (individual piglets identified by number); M, GFP-transgenic mouse DNA. Blot hybridised with probe against GFP, then stripped and re-probed with porcine HNF1 to confirm DNA digestion and estimate loading. Piglet # 20 was non-transgenic (see Fig. 2).

transgene-copy number for 33 transgenic founder animals (five animals were still born and not analysed for transgene integration; all five were visibly expressing GFP) by Southern blot analysis of ear biopsy DNA (Fig. 3). In these founder animals, the number of lentiviral vectors present ranged from 1 to 5 copies, which is lower than the 1–20 copies (mean = 4.6) reported for HIV-1 based vectors of similar titre [3].

Further analysis was carried out on founder animals sacrificed at two months of age. GFP expression was observed in all tissues tested (Fig. 4). Expression was observed in tissues derived from each of the three embryonic lineages, e.g., skin from

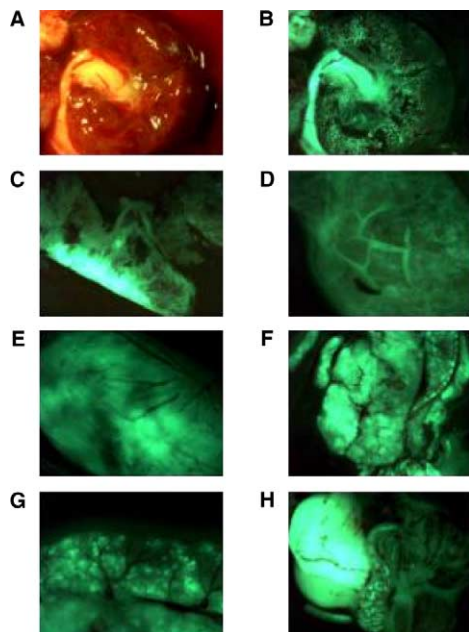


Fig. 4. Lentiviral transgene displays widespread expression. Whole tissue images of representative 4 month old pig (pig # 30; male; three transgene copies). Bright field (A) and fluorescent (B–H) images of kidney (A,B), liver (C), lung (D), heart (E), pancreas (F), gut (G) and testis (H).

ectoderm (Fig. 2B), pancreas from endoderm and kidney from mesoderm (Fig. 4).

4. Discussion

Lentiviral vectors enable the efficient delivery of transgenes into the germline of mammals. To-date, all reports on lentiviral transgenic mammals have used an HIV-1 based vector [1–4, 11–13]. We now show that a vector based on another lentivirus, the EIAV, can also be used to efficiently generate transgenic mammals.

In our study, 37 transgenic piglets were produced from 120 injected and transferred porcine zygotes. Thus, 31% of injected eggs resulted in a transgenic founder piglet, which is dramatically greater than the 1% usually attributed to other methods of generating transgenic livestock [6]. This compares favourably with HIV-1 based vector studies in pigs, where an efficiency of 13% (32 from 244 zygotes) was achieved [3]. Both vectors are associated with an extremely high expression frequency in the founder animals; 93% for HIV-1 based vector [1,3] and 95% for the EIAV-based vector.

Lentiviral vector transgene delivery demonstrates increased efficiency over pronuclear injection at several stages. First, this study was characterised by a high pregnancy rate (four of five recipient animals carried their pregnancy to term), presumably facilitated by the reduced physical intervention that perivitel-line space injection offers over standard pronuclear injection. Second, nearly all founder animals are transgenic and third, most of these founder animals express the transgene. These last two properties are in stark contrast to that obtained by pronuclear injection and nuclear transfer [6]. In this study, we transferred 24 embryos per female pig recipient, which is more than a gilt that could normally take to term. Given the very high transgenesis rate, it is likely that many of the embryos that did not develop to term were also transgenic. It is possible, therefore, that if fewer embryos were transferred per recipient female then an even greater overall efficiency may be achieved. Furthermore, VSV-G pseudotyped lentivectors offer the possibility to perform gene transfer studies in a wide range of species, including those currently not suited to standard transgenic methods, as well as less typical inbred strains of mice. In addition, there is the exciting option of sequential addition of transgenes to established transgenic lines.

To balance these positive aspects, there are some limitations to lentivectors. First, lentivectors have an upper limit on the cloning capacity of about 8 kb. This will limit the type of gene construct that can be studied. Currently, there is no control over the number or location of integration events. In our study, we detected between one and five integrated copies, while in the previous HIV-based study in pigs up to 20 copies were reported [3]. This presents a breeding issue, as each integration event will segregate independently in subsequent generations. We are maintaining some animals to allow transmission and segregation issues to be assessed and will report these data at a later date.

In conclusion, we have demonstrated that EIAV-based lentiviral vectors, in a similar manner to HIV-1 based vectors [1–4, 11–13], represent an extremely efficient transgene delivering method. This suggests that lentiviral transgene delivery may be a general tool with which to efficiently generate transgenic mammals offering exciting opportunities in livestock [6].

Acknowledgements: Thanks to all farm staff especially Marjorie Ritchie, June Bowering, John Bracken, Dougie McGavin, David Chisholm and Chic Sloan; to Ricardo Ribas for embryology assistance and to Norrie Russell for photography. This work was supported by the BBSRC (UK).

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