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Optimization of transgenesis conditions for the generation of CXCL2-luciferase reporter mice line

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Abstract

Background: Transgenesis by microinjection has been widely used for the generation of different mouse models. Different variables of the procedure may critically affect the efficiency of the process. A DNA construction that carries the CXCL2 promoter gene and firefly luciferase has been used to optimize aspects of the procedure. Three different concentrations (0.5, 1.0 and 4.0 ng/µl) of the DNA construction to microinject a total of 1981 zygotes has been tested. Intact/injected embryos, pregnancy and birth rate, survival of pups 7 days after birth, number of transgenic pups and overall transgenic efficiency was registered and analyzed by Z test of proportions for each group.
Results: A total of seven transgenic founders were detected for the three DNA concentrations used, 1 in 46 alive pups in the 0.5 ng/µl group, 5 in 38 alive pups in the 1 ng/µl group and 1 in 21 alive pups in the 4 ng/µl group ($p < 0.1$). The overall transgenic efficiency was higher for the 1 ng/µl concentration, with a transgenic rate of 13.2%.
Conclusions: In conclusion, we have selected the best operative conditions to maximize the transgenesis efficiency. Furthermore, the transgenic lines developed could be used as a reporter model of innate immunity activation with many different applications in the fields of immunology, cancer and neurodegenerative diseases.

Keywords: DNA concentration; microinjection; reporter systems.

INTRODUCTION

Chemokines are a special type of cytokines released by many different cells after activation of inducible innate response (Vinader and Afarinkia, 2012a). They can be released after activation of pattern recognition receptors (PRR) such as toll like receptors (TLR) that recognized structural motifs present in different classes of microorganisms. Pro-inflammatory cytokines like TNFα, IL-1, IL-18 and IFNγ may also activate chemokine production (Kobayashi, 2006; Kobayashi, 2008). Chemokines participate in several physiopathological processes, such as inflammatory bowel diseases suggesting they can act as potential therapeutic targets through the development of antagonists of chemokine functions (Papadakis, 2004). The role of these proteins in the clearance of hepatitis C virus has also been described (Heydtmann and Adams, 2009), although its persistent expression can lead to chronic hepatic inflammation. A recent study with chemokine receptors in cancer suggested that several chemokine receptors such as CXCR4, CCR4, CCR7, and CCR10 play different roles in tumor growth, metastasis, angiogenesis and microenvironment composition (Wu et al. 2009; Vinader and Afarinkia, 2012b).

CXCL2 is a potent chemotactic agent for polymorphonuclear leukocytes and haematopoietic stem cells, released by macrophages and epithelial cells in response to endotoxins and other microbial ligands (Wolpe et al. 1989). In mice, it is one of the main chemokines responsible for the recruitment of neutrophils to the site of infection (Roche et al. 2007; Day and Link, 2012). The expression of this chemokine increases several orders of magnitude after activation of the innate immune response; therefore CXCL2 constitutes a good candidate for the establishment of reporter systems to study these events.

It is well known that pronuclear microinjection technique used to generate transgenic animals has a very low efficiency, with around 10% of the animals born being positive for the transgene. Several attempts have been made in order to improve the yield, taking into account DNA purification, injection buffer, pronuclei visualization, time of microinjection, size of DNA fragment and number of copies. DNA concentration seems to affect embryo viability also, and determining optimal concentration is crucial for the success of the project.

The objective of the present study was to optimize the working conditions in order to generate a transgenic reporter model of innate immunity activation. To this aim, we have analyzed the impact of the concentration of DNA construction in order to optimize the efficiency of transgenesis with this construct.

MATERIALS AND METHODS

Animals

A total of 140 B6SJL hybrid F1 female and 20 male mice were used as embryo donors for DNA microinjection, and a total of 51 Swiss and B6D2 hybrid F1 female mice were used as embryo recipients. Animals were bred at the SPF animal facility of the Transgenic and Experimental Animal Unit of Institut Pasteur de Montevideo. They were housed on individual ventilated cages (Sealsafe rack, Tecniplast, Milano, Italy), in controlled environment at 20 ± 1ºC with a relative humidity of 40-60%, in a 14/10 light-dark cycle. Food and water were administered *ad libitum*. All experimental animal procedures have been approved by IPMon Animal Care Committee and were in accordance with national and international guidelines of FELASA regarding laboratory animal's protocols.

Cloning of mouse CXCL2 promoter fragment

Upstream primer (5'-GGGTGCATTTCAAAGATAAGAAGC-3') and downstream primer (5'-GGAGTGCCCGAGGAAGCT-3') were designed to clone the mouse (m) CXCL2 promoter fragment. Platinum® Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA) with proofreading activity was used to amplify a 1403 base pairs (bp) fragment upstream the ATG of the CXCL2 mouse promoter. PCR reaction was performed in 50 µl volume, using 0.1 mM of each primer, 2.5 µl enhancer solution, 1 µl MgSO4, and approximately 20 ng of genomic DNA. Genomic mouse DNA was isolated from homogenized spleen by using Wizard® genomic DNA purification kit (Promega, Madison, WI, USA). PCR cycling protocol consisted in: 94ºC for 2 min; 35 cycles of 94ºC for 15 sec, 55ºC for 30 sec, and 68ºC for 150 sec; 68ºC for 10 min. The specific primers generated a 1403 bp fragment, which was directly ligated into the pCR® -Blunt II- TOPO® vector from the Zero Blunt® TOPO® PCR Cloning kit following the manufacturer's protocol (Invitrogen). The insert contained at the 5' end a KpnI and at the 3' end an XhoI site. After digestion with the KpnI and XhoI restriction enzymes (both from Promega) and DNA extraction from agarose gel, the 1503 bp fragment was subcloned into the pGL3-firefly luciferase basic vector (Sigma, St. Louis, MO, USA) using compatible sites. Fragment was verified by sequencing.

Functional tests of vectors

Construct pGL3-mCXCL2 promoter fragment luciferase, was tested *in vitro* for its functionality by measuring the luciferase activity in cell extracts using Dual Luciferase™ assay system (Promega) as follows:

Cell culture. All cell culture products were from Gibco BRL (Rockville, USA). The human intestinal epithelial-like cells Caco-2 cells (clone 1), human embryonic kidney HEK-293 and human airways epithelial-like cells A549 were maintained as previously described (Rumbo et al. 2004). Briefly, A549 and HEK cells were grown in 50% DMEM, 50% Ham's F12 medium, 5% fetal calf serum (FCS) and 2 mM L-glutamine. Caco-2 cells were cultured in DMEM with glutamax, 10% FCS, 1% non-essential amino acids and 4 µg/ml transferrine. Endotoxin-free flagellin (FliC) was prepared from *Salmonella enterica* Serovar Typhimurium ATCC 14028 as previously described (Sierro et al. 2001).

***In vitro* functional assay.** Epithelial cells were transfected for 12 hrs with the CXCL2 reporter plasmid and the normalizing pRL-TK plasmid coding *Renilla* luciferase (Promega) using Lipofectin® (Invitrogen). Fresh cultured medium was added for 48 hrs and the cells were stimulated for 6 to 9 hrs with flagellin-containing culture medium at 1 ug/ml final concentration. HEK cells were stimulated with recombinant human interleukin 1 (R&D systems, USA). Firefly luciferase activity was measured and normalized to *Renilla* luciferase activity using the Dual luciferase® assay (Promega) following manufacturer's instructions. Relative luminescence (RLU) was normalized as luciferase RLU (RLU-L) with Renilla RLU (RLU-R) and the variation in luciferase activity was calculated as follows [(RLU-Ltreated/RLU-Rtreated)/(RLU-Lmock/RLU-Rmock)] using not activated cells transfected with full length promoter fusion as mock condition.

Purification of pGL3-mCXCL2 construction for embryo microinjection

Once the sequence and functionality of the construct was verified, several hundred micrograms of endotoxin-free plasmid were produced using Endo-Free Gigaprep Kit (Macherey-Nagel, Düren, Germany). Subsequently BamHI/BglII double digestion was performed using 50 µg of the plasmid and the 3457 bp fragment containing the CXCL2-luciferase construct was purified from agarose gel electrophoresis using a SV gel cleanup purification kit (Promega). Purified fragment was eluted in water at 100 ng/µl and subsequently used for microinjections.

Generation of transgenic founders

A total of 25 microinjection sessions were performed using five to seven donor female (3-4-week-old) per session, that were induced to superovulate with an i.p. injection of 5 IU of equine chorionic gonadotrophin (eCG, Novormon, Syntex, Buenos Aires, Argentina) and 5 IU of human chorionic gonadotrophin (hCG, Ovusyn, Syntex) 46 hrs later. Immediately, each female was mated with one fertile B6SJL hybrid F1 male (Day 0). Females were checked for vaginal plug and sacrificed by cervical dislocation on the morning of Day 1, oviducts were excised and one-cell embryos were collected from the swollen *ampulla* in M2 medium (Sigma) containing 0.3 mg/ml hyaluronidase (Sigma) to remove cumulus cells. Collected embryos were rinsed in several drops of M2 and cultured in 100 µl drops of M16 medium (Sigma) under mineral oil (Sigma), in 5% CO_2 in air at 37ºC (Nagy et al. 2003).

Pronuclear microinjection was performed using an inverted microscope (TE 2000, Nikon, NY, USA) and mechanical micromanipulators (Transferman NK2, Eppendorf, Hamburg, Germany) using embryo holding pipettes (35º bent angle, opening diameter of 20-25 µm, Biomedical Instruments, Zöllnitz, Germany) and home-made microinjection pipettes. Three different concentrations of linearized DNA vector were used; 0.5, 1.0 and 4.0 ng/µl, diluted in microinjection buffer (5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) (Nagy et al. 2003). For each microinjection session, 100-150 zygotes were loaded in groups of 30 in glass depression slides containing M2 medium under mineral oil. DNA solution was injected into the male or female pronucleus, until the pronuclear membrane visibly swelled. After microinjection, embryos were maintained in the aforementioned culture conditions for thirty minutes to evaluate survival rate before being transferred to foster mothers. Surviving zygotes were defined according to standard criteria (Nagy et al. 2003).

Embryo transfer

The pseudopregnancy of the Swiss and B6D2 F1 recipient females was induced by mating them with vasectomized males with proven sterility (Day 0). Vaginal plugs were checked 12 hrs later on Day 1. Thirty minutes after the microinjection, females were weighted and anesthetized by an i.p. injection of a mixture of ketamine (100 mg/kg; Seton, Lab Calier, Barcelona, Spain) and xylazine (10 mg/kg; Vetanarcol, König Lab, Buenos Aires, Argentina) (Bagis et al. 2004). Surviving DNA-injected zygotes

were loaded into a pulled glass pipette and transferred into both oviducts of recipients through the *infundibulum* (20 to 25 zygotes per recipient). After the surgery, females were injected with 1 mg/kg s.c. of tolfenamic acid (Tolfedine, Vetoquinol, France) (Schlapp et al. 2011) and left to recover from anaesthesia over heating pads (Nagy et al. 2003).

Genotyping of transgenic offspring

Screening of possible transgenic founders was performed by end-point PCR. DNA samples were obtained from tail biopsies of weaned pups (3-week-old). PCR was performed using Lucif Fw (5'TCAAAGAGGCGAACTGTGTG3') and Lucif Rev (5'TCGCGGTTGTTACTTGACTG3') primers, which amplify a luciferase gene region. As positive control, a transgenic murine DNA containing luciferase gene was used. When PCR using these primers was positive, a second DNA amplification round was performed using another pair of primers (Fwd 5'ACTTCAGCGCAGACATCACTTCCT3', Rev 3'CTTCTGCCAACCGAACGGACATTT5') which amplify both the CXCL2 promoter and luciferase genes regions. Primers for the gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal control of DNA quality in both rounds of PCR. Transgenic lines were established by backcrossing detected CXCL2-luc founders to non-transgenic BALB/cJ mice.

Statistical analysis

Survival of embryos to microinjection, pregnancy and birth rates, survival of pups at day 7 after birth, number of positive transgenic founders by PCR and overall transgenic efficiency were recorded for each DNA concentration. Data were analyzed by Z test of proportions for independent groups.

RESULTS

Functional test of mouse CXCL2 promoter construct

In order to test functionality of the generated construct, epithelial cell lines were transfected using the reporter vector mCXCL2 and TK-renilla as normalizer. We used flagellin or interleukin-1 as stimulant, that was previously shown to be able to activate the selected cell lines (Rumbo et al. 2004; Sirard et al. 2009). Stimulation produced a significant upregulation of relative luminescence units of 4.3 fold increase in HEK cell line (Figure 1). Similar results were observed in the other cell lines (not shown).

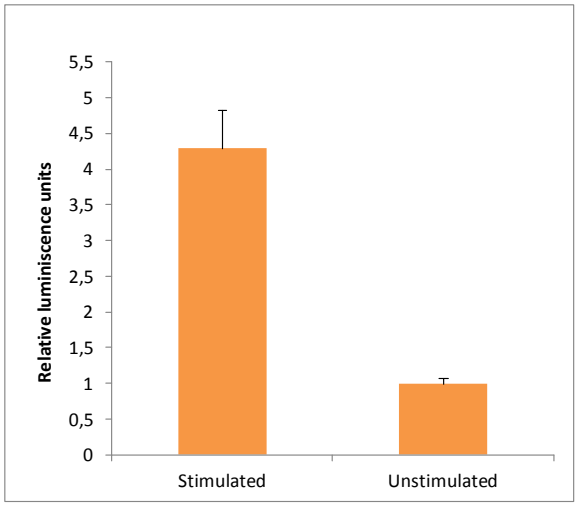

Fig. 1 CXCL2-luciferase functionality is confirmed *in vitro*. HEK-293 cells were transfected with a plasmid containing CXCL2-firefly luciferase construct and a normalizing plasmid coding *Renilla* luciferase. Cells were stimulated 48 hrs after transfection with IL-1 100 ng/ml. Luciferase activity is expressed relative to the reporter fusion in unstimulated conditions. *Renilla* luciferase was used to normalize the transfection efficiency. Results are the average of at least three biological determinations. Similar results were obtained in two independent experiments.

Generation and screening of transgenic mouse lines

The construct was microinjected in several sessions using different concentrations of DNA. Upon embryo transfer, pregnancy and successful delivery and weaning, the offspring was screened by PCR using primers detecting the luciferase gene (Figure 2). Positive samples were confirmed by a second PCR using primers that amplify the CXCL2-luciferase construction (Figure 3). Founders that were positive in both reactions were then mated to BALB/cJ background and transmission of the transgene to the progeny was evaluated (Figure 4).

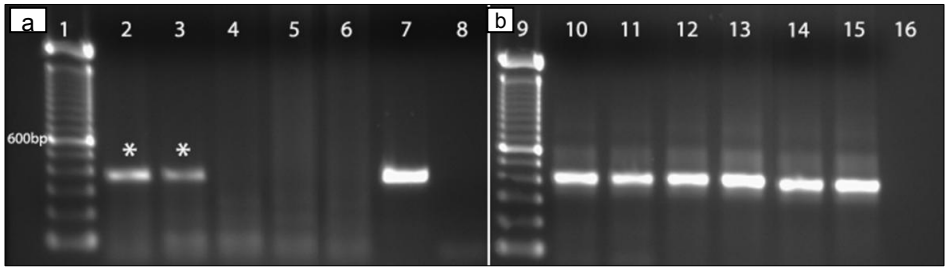

Fig. 2 Genotyping of founder animals. (a) PCR amplification of DNA samples obtained from weaned pups, using primers for luciferase gene (lanes 2 to 8, amplification product 380 bp) and (b) internal control primers for GAPDH gene (lanes 10 to 16, amplification product 410 bp). DNA from a luciferase transgenic mouse and from a wild-type C57BL/6 mouse was used as template for positive controls (lane 7 and 15). Lanes 8 and 16 are negative controls (no DNA). Lane 1: 100 bp ladder (Invitrogen).

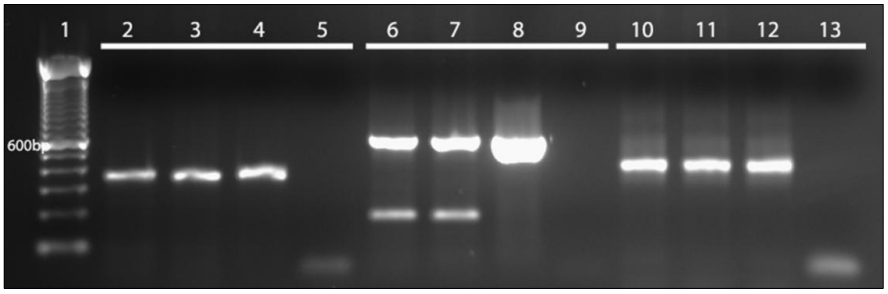

Fig. 3 Confirmation of transgenic founders. Samples that resulted positive at the first round of PCR with luciferase primers, were confirmed with a second round using the same luciferase primers (lanes 2 to 5), a set of primers that amplify a fragment between the 3' end of the mCXCL2 promoter and the 5' end of luciferase (lanes 6 to 9, amplification product 581 bp, confirming the integrity of the transgene) and primers for GAPDH (lanes 10 to 13). Positive controls for each set of primers are showed in lanes 4, 8 and 12, and negative controls in lanes 5, 9 and 13 (no DNA). Lane 1: 100 bp ladder (Invitrogen).

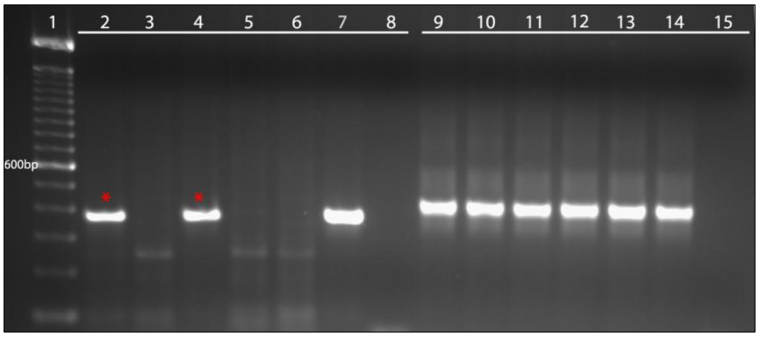

Fig. 4 Detection of the transgene in the progeny. DNA from the progeny of founders was used as template to perform PCR using luciferase (lanes 2 to 8) and GAPDH (lanes 9 to 15) primers. Positive controls are showed in lanes 7 and 14, and negative controls in lanes 8 and 15, for both set of primers. Transmission of the transgene was below 50%, probably due to mosaicism of the transgenic founder. Lane 1: 100 bp ladder (Invitrogen).

Effect of different DNA concentrations on transgenic efficiency

The effect of DNA concentration in the generation of transgenic mice is summarized in Table 1. Intact/injected embryos, pregnancy and birth rate, survival of pups 7 days after birth, number of transgenic pups assessed by standard PCR and overall transgenic efficiency was registered for each group. Statistical differences ($p < 0.05$) were found for survival rate, as well as birth rate and survival at day 7, indicating lower performance of the use of 4.0 ng/µl, probably due to DNA toxicity.

A total of seven transgenic founders were detected for the three DNA concentrations, one founder for 4 ng/µl, five founders for 1 ng/µl and one founder for the concentration of 0.5 ng/µl. The overall transgenic efficiency was higher when using a concentration of 1 ng/µl, with a transgenic rate of 13.2%, indicating a better performance of this concentration, although with low statistical significance ($p < 0.1$).

Furthermore, the different founders were used to generate transgenic lines. Overall in these crossings, the transgene was transmitted to the progeny in a proportion of 13/60 (21.6%).

Table 1. Effect of DNA concentrations in the generation of CXCL2-luc transgenic mice.

	0.5 ng/µl	1.0 ng/µl	4.0 ng/µl	*p value Z test*
Number of injected embryos	783	556	642	
Intact/injected embryos (%)	487/783 (61.7)	333/556 (59.9)	455/642 (70.9)	$p < 0.05$
Pregnant fosters/transferred (%)	7/18 (38.9)	7/16 (43.7)	9/17 (52.9)	$p > 0.1$
Pups born/embryos transferred (%)	48/131 (36.6)	43/132 (32.6)	29/181 (16.0)	$p < 0.05$
Pups alive 7 days after birth/pups born (%)	46/48 (95.8)	38/43 (88.4)	21/29 (72.4)	$p < 0.05$
Transgenic pups/alive pups (%)	1/46 (2.2)	5/38 (13.2)	1/21 (4.8)	$p < 0.1$
Transgenic pups/injected embryos (%)	1/783 (0.13)	5/556 (0.90)	1/642 (0.16)	$p < 0.1$

DISCUSSION

A DNA construction using firefly luciferase gene under the control of the murine CXCL2 promoter was designed and used in order to optimize the transgenesis generation process by microinjection.

This reporter construction was tested *in vitro* to evaluate its functionality before microinjection, and the results were as expected, with a high rate of the expression of luciferase. The relative increase observed in the *in vitro* transfection showed similar results in all the cell lines tested. With that test the functionality of the construct was confirmed and so it could be used to proceed with the microinjection procedure.

As reported, several parameters affect the efficiency of the microinjection technique, like DNA purity and concentration, injection buffer, pronuclei visualization, time of microinjection, size of DNA fragment and number of copies, among others (Wall, 2001; Filipiak and Saunders, 2006). Identification of a DNA concentration that provides good post-microinjection survival rates, birth rates, and transgenic rates is a relevant factor for this technology. An ideal DNA concentration should have a balance between birth and transgenic rates.

For the generation of this particular transgenic line, three different concentrations of DNA (0.5, 1.0 and 4.0 ng/µl) were used in order to establish which one was the most efficient in term of transgenic founders born. As presented in the results section, the birth rate increased as the DNA concentration decreased, suggesting that somehow the DNA resulted toxic for the embryos and they died some days later *in utero*, although the survival rate 30 min after the microinjection and pregnancy rate was higher for the 4 ng/µl group. Some works in transgenic mouse strains have reported early postimplantation embryo lethality due to DNA rearrangements (Covarrubias et al. 1986). It has been described that the insertion of a foreign DNA can disrupt an endogenous gene and produces a marked effect on development in approximately 10% of the transgenic lines. This can be due to gross chromosomal

imbalance after chromosomal rearrangements or mutations in ubiquitously expressed genes essential for cell growth and viability, attributing the survival of embryos until the time of implantation to the persistence of maternally encoded products contributed by the egg (Pravtcheva and Wise, 1995; Pravtcheva and Wise, 2003).

For this particular DNA construct, we have detected that 1.0 ng/µl has a satisfactory transgenic rate (13.2%), in comparison with the data published for other DNA constructions (Auerbach et al. 2003; Fielder et al. 2010; Fielder and Montoliu, 2011). These results are in agreement with the work of Brinster et al. 1985, where they found that DNA injection using a concentration of 1 ng/µl resulted in a higher embryo survival than 10 ng/µl, and resulted in a higher transgene integration frequency than 0.1 ng/µl concentration (Brinster et al. 1985). They concluded that the increase in the number of transgene copies injected augments the integration efficiency but has a limitation due to the toxic effect of DNA in embryo survival.

A murine transgenic model that expresses luciferase under the control of the CXCL2 promoter would allow us to have an efficient and simple way to monitor innate immunity activation and inflammatory processes on different biological scenarios (Doyle et al. 2004). Characterization of the phenotype of the different lines generated is in progress.

In conclusion, we have optimized the transgenesis process and developed a transgenic murine model with an acceptable efficiency rate, to be used as a reporter of innate immunity activation. The use of 1 ng/µl concentration for the microinjection of zygotes allowed the best efficacy in transgenic founder's generation.

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