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Injection of higher DNA concentration into pronucleus as putative troubleshooting for generating transgenic mice

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Microinjection of DNA into zygote pronucleus is still one of the most common methods to obtain transgenic animals. Pronuclear injection seems to be a standardized method, however there are problematic constructs that researchers struggle with to obtain transgenic animals. Here we present a modification of the standard transgenesis method, which we suggest to use in those cases in which the standard method results unsuccessful. By increasing DNA concentration by one order of magnitude we obtained 4 founder mice out of 64 born mice. Moreover, we did not notice toxic effect on embryos attributable to the higher DNA concentration employed. According to our results, we suggest that increasing DNA concentration in the standard protocol for pronuclear injection might be viable solution when dealing with problematic constructs.

KEY WORDS: DNA concentration / pronucleus injection / transgenesis

Pronuclear injection is still one of the most commonly and extensively used method to obtain transgenic animals, particularly in species for which embryonic stem cells (ESCs) are not available yet. The first transgenic line obtained by this method

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was published by Gordon *et al.* [1980]. Not only was it a novelty as a method for obtaining transgenic animals, but it was also the first published genetic modification of the embryo at the zygote stage. Until then, transgenic animals had been obtained by viral transfection of embryos at the blastocyst stage [Jaenisch and Mintz 1974] and later at the 8-cell stage [Jaenisch 1976]. Consequently, the developing embryos obtained with both methods were chimeras with the transgene integrated at different sites in each blastomere. In order to obtain embryos with the same site of transgene integration and expression pattern in each cell of the embryo and later in the adult individual animal, Gordon *et al*. used the zygote as a recipient for exogenous DNA and developed a method of transgenesis [Gordon and Ruddle 1981], which has now been used for more than 40 years.

Transgenic animals have been used in a variety of research lines, regenerative medicine and in the biotechnology industry for years [Bulfield *et al*. 1984, Edmunds *et al*. 1998, Goldring 1999, Kerkhofs *et al*. 2009, Maga *et al.* 2006, Parker *et al*. 2004, Plettenberg *et al*. 1994, Reitman *et al*. 1999, Rokkones *et al.* 1995, Roy and Matzuk 2006, Strömqvist *et al.* 1997, van Leuven, 2000, Vanhove *et al*. 1998, Wilczynski *et al.* 2008, Zhang *et al*. 2008]. However, the number of pharmaceutical treatments with drugs obtained from transgenic animals approved so far is still very low – there are three products approved so far: ATryn [European Medicines Agency 2018a, Kling 2009], Kanuma [European Medicines Agency 2018b] and Ruconest [Ruconest 2016, European Medicines Agency 2018c, Bork *et al*. 2016, Feussner *et al*. 2014]. For both research and applied usage it is important to obtain transgenic animals with high efficiency.

Factors important for the efficient transgenesis by pronuclear injection were studied already in the ’80s Brinster *et al*. 1985]. For instance, it was shown that the size of the construct might affect the successful integration into genome – smaller constructs integrate more efficiently. Moreover, the efficiency of transgenesis (define as a ratio of transgenic animals obtained to per injected embryo) was shown to be higher for linear than for coiled DNA. An additional factor to be considered is the concentration of EDTA in the buffer in which the construct is dissolved for injection – 0.1mM EDTA was found to be optimal for efficient transgenesis. It is known that, in the mouse, transgenesis is more efficient if the DNA construct is injected into the male pronucleus, likely because of its larger size, which makes it more resistant to an increasing in volume than the female pronucleus. It has also been shown that DNA concentration higher than 10ng/µl turns out to be toxic for embryos since they do not develop until blastocyst stage [Brinster *et al*. 1985], however this phenomenon was never explained at the molecular level. The mouse strain also seems to affect the success rate, in fact transgenesis in hybrids might be more efficient than in some other strains [Auerbach *et al.* 2003]. When performing pronuclear injection there is one factor, integration site, that cannot be accounted in the experimental design. It has been hypothesized that integration of the transgene by this method is due to physical breaks in the chromosomes caused by the injecting pipette. According to this hypothesis, the DNA repair mechanisms accidentally connect the transgene with

the broken chromosome, thus restoring the DNA strand [Yamauchi *et al*. 2007]. Such technique of pronuclear injection for generating transgenesis seems to be very well standardized; however, the question of what the next step should be remains when all requirements described above are met and yet there is no successful transgenesis.

Transgenic core facilities often struggle with the situation in which there is a lack of transgenic animals because a particular construct was used. In the present manuscript, such constructs are referred as "difficult". Even though we know the crucial factors that affect efficient transgenesis, there is still lack of knowledge on how such factors influence transgene integration on a molecular level. Despite the fact that the highest standards for transgenesis were followed, we failed to obtain transgenic animals by this standard method. Here we propose a non-standard solutions for such "difficult constructs", which has helped us to obtain transgenic animals after the standard method of pronuclear injection had failed. In particular, increasing the DNA concentration from 2 to 20 ng/μl allowed for the successful integration of the transgene, which resulted in the generation of transgenic mice. Based on our results we propose an innovation for the standard protocol for pronuclear injection when the used construct is found to be problematic.

Material and methods

Animals

F_1 (C57Bl/6xCBA/H) and Swiss mice were used in this study (colonies of both strains were maintained at the Faculty of Biology, University of Warsaw). Animals were kept under a 12-hour light cycle and were given access to food and water *ad libitum*. 8 to 12-week-old female mice were mated with males of the same genetic background. The presence of the vaginal plug indicated pregnancy, defining the first day of embryonic development as embryonic day 0.5 (E0.5). Mouse handling and husbandry practices were performed following established regulations. This study was approved by the Polish Local Ethics Committee for Experimentation on Animals no. 1 in Warsaw, Poland.

Embryo collection

Zygotes were obtained from superovulated females. Superovulation was induced with 10 IU of PMSG (pregnant mare's serum gonadotropin; Follignon, Intervet, Poland) followed by 10 IU of hCG (human chorionic gonadotropin; Chorulon, Intervet, Poland) after 48 hours. Pregnant females were sacrificed by cervical dislocation on the day when the vaginal plug was found. Oviducts were collected into sterilised culture dishes and zygotes were collected from oviducts by disrupting the ampulla in 300μg/ml hyaluronidase solution in homemade M2 around 24 hours after hCG injection. After the zygotes were cleaned off the follicular cells, they were washed in M2 medium and kept for the next steps in drops containing the same culture medium under mineral oil at 37.5°C and in 5% CO_2 atmosphere. Zygotes with both pronuclei were selected for pronuclear injection.

DNA preparation and injection

Linear DNA from VEEAk plasmid made in the lab (Wawrzyniak 2005) was used for pronuclear injection. pVEEAk was digested with KpnI (R0142S, New England BioLabs, USA) and PacI (R0547S, New England BioLabs, USA) (endonuclease digestion was performed at 37°C for 15 hours and the obtained plasmid fragments (3480bp, 2950bp and 1732bp) were separated by electrophoresis in 1.5% agarose gel. The largest fragment (3480 kb) with tetracyclin reversed transactivator under the EF1α promoter was cut out from the gel and cleaned by using the gel extraction kit (28106, Qiagen, USA). DNA was dissolved in TE Embryo buffer (10mM Tris-HCl, 0.1mM EDTA, pH-7.4), aliquoted at a concentration of 2 ng/µl or 20 ng/µl and frozen at -20°C. For each experiment a single aliquot was thawed at RT (room temperature) and centrifuged for 15 minutes at 15 300 x g. The correct sequence of 3480bp DNA fragment was confirmed by sequencing (DNA sequencing and oligonucleotide synthesis Service of the Institute of Biochemistry and Biophysics of Polish Academy of Sciences).

To obtain transgenic mice with tetracycline reversed transactivator under the EF1α promoter integrated into the genome, we used standard methods of pronuclear microinjection of linear DNA. We performed experiments with four different approaches, all of them named as a unique variant of the experiment. To clearly distinguish every variant, we coded all of them according to the mice strain (Swiss or F_1), number of injected pronuclei (1 or 2) and DNA concentration (2ng/µl or 20ng/µl) in each case (Fig.1B).

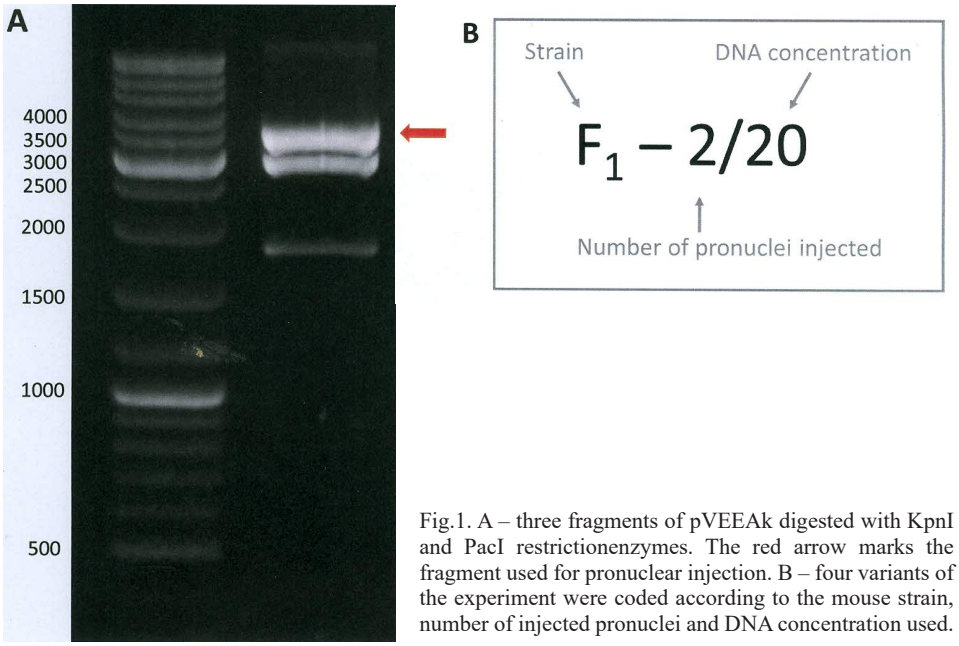
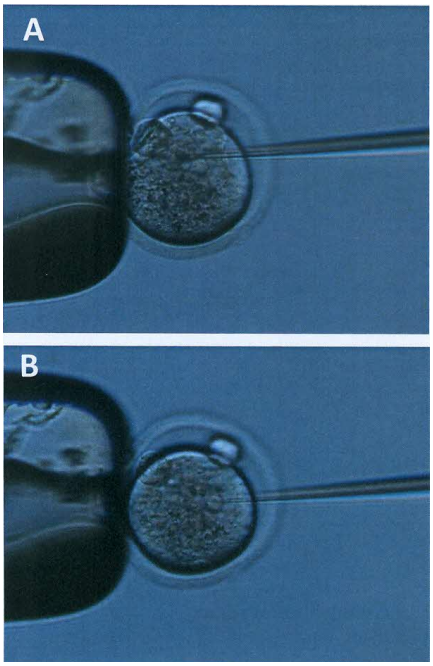


Fig.1. A – three fragments of pVEEAk digested with KpnI and PacI restrictionenzymes. The red arrow marks the fragment used for pronuclear injection. B – four variants of the experiment were coded according to the mouse strain, number of injected pronuclei and DNA concentration used.

DNA was injected into the male pronucleus, except for variant SW – 2/2, in which both pronuclei were injected. For this purpose, the injection was performed with the injection pipette going first through the male pronucleus – given its larger size – and into the female one. Once the tip of the pipette was in the female pronucleus, pressure was applied to deliver the DNA solution containing the transgene (Fig.2A). As the needle was being removed and its tip was positioned in the male pronucleus, the second dose of the DNA solution was released into it (Fig.2B). Injections were performed under an Axiovert 200 (Zeiss) microscope with FemtoJet micromanipulators and FemtoJet microinjection pump. Microinjection pipettes were made on premises from borosilicate glass capillaries with filament OD: 1.0m, ID: 0.78mm [Harvard Apparatus, Cambridge, Massachusetts, USA].



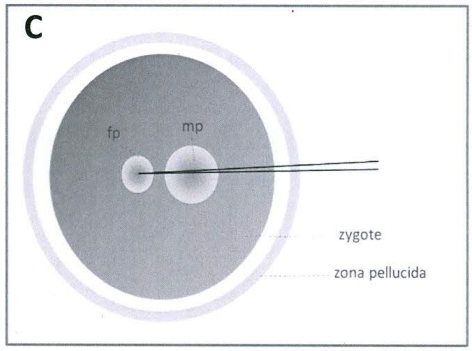


Fig.2. Photographs and graphical representation of the injection of both pronuclei in the SW-2/2 variant of the experiment. A – injection of female pronucleus – the pipette goes through the male pronucleus. B – injection of male pronucleus. C – schematic representation of the pipette position in the zygote during both pronuclei injection. fp – female pronucleus, mp – male pronucleus.

Embryo transfer

After injection, live embryos were transferred into the oviduct of the recipient females plugged during the previous night by vasectomized males. When possible, embryo transfer was performed on the same day; however, due to the shortage of recipients, 2-cell stage embryos were transferred during the following day in some cases (Tab. S). Transfers were made under anaesthesia as described previously [Tarkowski *et al*. 2005].

Since recipient females were not always available at the day of the experiment, some transfers were performed the next day with 2-cell stage embryos (Tab. S). For

simplicity, data on embryo transfer and transgenesis efficiency are presented together for both zygotes and 2-cell stage embryos; however, detailed data are available in the supplementary section (Tab. S).

Genotyping

For genotyping, 1mm-length of tissue was collected from the tip of the tail from each animal at 3 weeks of age. DNA was isolated using the Genomic Mini kit (new name of the kit QIAamp DNA kit, Qiagen 56304, USA). To test the presence of the transgene, a PCR reaction was performed with the following primers: rtTA fwd: 5' AAGGTTTAACAACCCGTAAAC 3' and rtTA rev: 5'ACAAACAGTTCTGAGACCGTTCTT3' (DNA sequencing and oligonucleotide synthesis Service of the Institute of Biochemistry and Biophysics of Polish Academy of Sciences). PCR was performed in 30 cycles (94°C for 0:45 min, 52°C for 1:00 min, 72°C for 1:00 min) using the PCR kit (Qiagen, 201203, USA) according to the manufacturer manual. For each PCR round positive (plasmid) and negative (RNAse/DNAse free water added instead of DNA solution) controls were also included. The presence of the desired amplicon (1018 bp) was validated by its size on the agarose gel.

Statistics

Because of the different conditions defining the experiment variants, only one-to-one comparisons were possible. Therefore, we decided to use a Z-test (95% confidence) for comparison of population proportions to assess differences in two variants simultaneously (multiple comparisons were carried out).

Results and discussion

First, the efficiency of pronuclear injection was tested by using dextran 500 kDA conjugated with FITC. Out of 20 zygotes that survived, 19 were FITC-positive, meaning the injection efficiency to deliver liquid into the embryo pronucleus was 95% (data not shown). This step verified injection efficiency.

For the first variant of experiments (SW-1/2, Tab. 1) a total of 1647 zygotes were injected, out of which 1128 survived (68%). From these, 964 embryos were transferred into 60 recipient females, resulting in 85 pups born (9%, Tab. 2). None of them was transgenic (Tab. 3), meaning that details of the procedure must be revised.

Table 1. Description of four variants which were performed to obtain transgenic mice

Variant of experiment	Females		Number of injected pronuclei	DNA concentration (ng/µl)
	donors	recipient		
SW-1/2	Swiss	F_1 (C57Bl/6xCBA/H)	1	2
SW-2/2	Swiss	F_1 (C57Bl/6xCBA/H)	2	2
F_1-1/2	F_1 (C57Bl/6xCBA/H)	Swiss	1	2
F_1-1/20	F_1 (C57Bl/6xCBA/H)	Swiss	1	20

Table 2. Embryo survival after microinjection in four experimental variants

Variant of experiment	Number of zygotes		Zygote survival rate after injection (%)
	injected	survived injection	
SW-1/2	1647	1128	68.49[a]
SW-2/2	135	80	59.26
F_1-1/2	533	365	68.48[a]
F_1-1/20	487	363	74.54

a – no statistical difference (Z-test for population proportions).

Table 3. Efficiency of embryo transfer and transgenesis

Variant of experiment	Number of transplanted embryos	Number of born pups		Transfer efficiency (%)	Transgenesis efficiency (%)*
		total	transgenic		
SW-1/2	964	85	0	8.8[a]	0
SW-2/2	80	3	0	3.75	0
F_1-1/2	333	30	0	9.0[a]	0
F_1-1/20	331	64	4	19.3	6.25

*Calculated as a ratio of transgenic pups/number of pups born.

With the aim of increasing the chances of transgene integration in the second approach, we tried to inject both pronuclei in the same attempt (variant SW-2/2, Tab. 1). A total of 135 embryos were injected following this method, 80 of which survived (59%) and were transferred into 11 recipients. However, only 3 pups were born from these experiments (Tab. 2), none of which was transgenic (Tab. 3). It is worth noting that the survival rate and transfer efficiency of these embryos was significantly decreased comparing to other variants, meaning their developmental rate was very low. According to this conclusion we decided not to continue with this injection protocol.

Having been so far unsuccessful with obtaining transgenic mice, we decided to use a different mouse strain. Swiss strain was our first choice as it matched the other transgenic strain available at the facility, which was supposed to be crossed with for further experiments. Because embryos from hybrid mice had been previously described to show a higher rate of efficient transgenesis [Auerbach *et al*. 2003, Brinster *et al*. 1985], we chose to proceed on this type of mice for our study (F_1-1/2, Tab. 1).

Similarly to what has been previously described, we injected 533 zygotes with the same construct, 365 of which (68%) survived the injection. A total of 333 of such embryos were transferred into 15 recipient females, which resulted in 30 pups born from this variant of the experiment (Tab. 2). Again, no transgenic mice were obtain by this method (Tab. 3). However, we confirm with that in our case there is no difference in survival rate and transfer efficiency between strains we used, as there is no significant difference in variants SW – 1/2 and F_1-1/2 for these factors. Because the employment of F_1 hybrid mice as zygote donors instead of the Swiss strain did not

lead to the generation of transgenic mice, we incorporated one additional modification to our protocol.

For the last variant of the experiment we increased the DNA concentration from 2 to 20 ng/µl (variant F_1-1/20, Tab. 1). We injected 487 zygotes with 10x more concentrated solution of DNA, out of which 363 survived the procedure (survival rate 75%). 331 embryos were transferred into pseudopregnant females and 64 mice were born (19%, Tab. 2). Among born pups we obtained 4 transgenic mice, meaning the transgenesis efficiency was 6.25% (Tab. 3). Moreover, we crossed founder mice to wild type animals and half of their progeny was transgenic (data not shown), which proved successful transgenesis. Thus, because we managed to obtain transgenic mice by increasing the amount of DNA delivered into the pronucleus, we suggest this method as a possible solution when similar difficulties are faced in transgenic facilities or other laboratories. Due to technical reason we were not able to repeat experiments with increased DNA concentration on zygotes from the initially used Swiss strain. Validating results on the Swiss strain, including survival rate and transgenesis efficiency, will prove valuable for a deeper assessment of this study.

Pronuclear injection is a standard method to obtain transgenic animals, however we presented here that some modifications might be helpful in particular cases. The efficiency of pronuclear injection was studied by Brinster *et al*. [1985], who regarded DNA concentration as one of the main contributing factors. It was shown that embryos after pronuclear injection with DNA concentration of more than 10 ng/µl are not developing efficiently untill blastocyst stage (referred to as toxic concentration for embryos). However, results shown in this manuscript are in opposition to such conclusion. From four different approaches only increasing DNA concentration to 20 ng/µl allowed us to generate transgenic mice, probably because of delivery more copies of the transgene into pronucleus. Moreover, we did not notice a toxic influence of the higher DNA concentration as the survival rate and full development percentage of embryos in the variant F_1-1/20 was not lower than in the other experimental groups (Tab. 2 and 3).

In the presented manuscript we also described a method for injecting both male and female pronuclei at the same time. The purpose of this modification was to increase the chance of transgenesis in every single embryo. Since the number of pups born was extremely low, we hypothesize that embryos that survived this procedure did not develop after transfer into the recipient female. To confirm this hypothesis, observation of the preimplantation development of injected embryos will be needed, for example using the time-lapse approach. Recently, double-pronucleus injection has been successfully performed by using a different approach – pronuclei were injected separately, instead of first puncturing the male pronucleus to get to the female one [Abe *et al.* 2020]. Biallelic transgenic animals were obtained by this method (transgenesis efficiency 16.9-34%), meaning the experimental approach was successful [Abe *et al.* 2020].

According to current knowledge, pronuclear injection leads to random integration of the injected construct. It has been demonstrated that insertion of the injection pipette

and the injection of the DNA solution may result in chromosome breakage [Folger *et al.* 1982, Yamauchi *et al*. 2007], and has been hypothesized that repair mechanisms are integrating the injected construct into the genome, frequently in tandem [Rülicke and Hübscher 2000]. However, there are still questions to answer to fully understand how different factors might affect the efficiency of transgenesis in the context of different constructs. According to our results, the increase of DNA concentration for pronuclear injection might be helpful when the standard protocol for this method does not lead to successful transgene integration. We hope this modification of the protocol will be useful for transgenic core facilities and other laboratories looking forward to generate transgenic animals.

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Supplementary material

Table S. Transfer efficiency when zygotes or 2-cell stage embryos were transferred into recipient females

Variant of experiment	Number of zygotes			Number of 2-cell stage embryos		
	transferred/ recipient no.	fully developed (born)	fully developed (born),%	transferred/ recipient no.	fully developed (born)	fully developed (born), %
SW-1/2	658	54	8.21	306	31	10.13
SW-2/2	80	3	3.75	-	-	-
F_1-1/2	210	16	7.62	123	1	11.38
F_1-1/20	219	41	18.72	541	23	20.54

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