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Research article

Maintenance and gene electrotransfer efficiency of antibiotic resistance gene-free plasmids encoding mouse, canine and human interleukin-12 orthologues

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ABSTRACT

Interleukin 12 (IL-12) is a cytokine used as a therapeutic molecule in cancer immunotherapy. Gene electrotransfer mediated delivery of IL-12 gene has reached clinical evaluation in the USA using a plasmid that in addition to IL-12 gene also carry an antibiotic resistance gene needed for its production in bacteria. In Europe however, European Medicines Agency recommends against the use of antibiotics during the production of clinical grade plasmids. We have prepared several antibiotic resistance gene-free plasmids using an antibiotic-free selection strategy called operator-repressor titration, including plasmids encoding mouse, canine and human IL-12 orthologues. The aim of this study was to evaluate the maintenance of these plasmids in bacterial culture and test their transfection efficiency using gene electrotransfer. Plasmid maintenance was evaluated by determining plasmid yields and topologies after subculturing transformed bacteria. Transfection efficiency was evaluated by determining the plasmid copy number, expression and cytotoxicity after gene electrotransfer to mouse, canine and human melanoma cells. The results demonstrated that our IL-12 plasmids without an antibiotic resistance gene are stably maintained in bacteria and provide sufficient IL-12 expression after *in vitro* gene electrotransfer; therefore, they have the potential to proceed to further *in vivo* evaluation studies.

1. Introduction

Interleukin 12 (IL-12) is a cytokine used as a therapeutic molecule in cancer immunotherapy. It boosts the ability of the host immune system to recognize and kill cancer cells [1, 2, 3, 4, 5]. However, IL-12 has a narrow therapeutic index, meaning it has to be released locally, without a systemic spillover [6, 7, 8, 9]. This can be achieved by using gene electrotransfer (GET) mediated delivery of a plasmid vector encoding IL-12 [10, 11]. GET is one of the most efficient non-viral *in vivo* gene therapy approaches [12]. The delivery is enabled by the application of short high energy electric pulses, *i.e.,* electroporation, that transiently increases cell membrane permeability, thus allowing the transfer of otherwise non-permeable molecules, like plasmids, through the cell membrane [13, 14]. Due to its efficacy for localized intratumoral delivery, electrotransfer mediated delivery of plasmids encoding IL-12 is currently approaching clinical use for the treatment of melanoma and other superficial solid tumors in the USA [15, 16, 17].

Active IL-12 cytokine is a heterodimeric protein composed of two subunits; an α- chain known as IL-12 p35 subunit, encoded by IL12A gene, and a β-chain known as IL-12 p40 subunit, encoded by IL12B gene [18, 19]. Since both subunits are encoded by separate genes, IL-12 GET was made possible by cloning of the IL-12 fusion gene in plasmid expression vectors [20, 21]. In addition to IL-12 fusion gene, plasmid used in clinical studies in the USA also carries an antibiotic resistance gene functioning as a selection marker, which simplifies plasmid production in bacteria [17]. Due to safety concerns raised by European Medicines Agency regarding the use of antibiotics during the production of plasmids [22, 23], antibiotic resistance genes should be avoided in

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plasmids intended for clinical use, if possible. As an alternative to antibiotic selection, several antibiotic-free plasmid production techniques have been developed in the last decades [23, 24, 25].

One such strategy is Operator-Repressor Titration (ORT) method developed by CobraBio (Keele, UK) [26]. This method was already employed for preparing the Antiangiogenic Metargidin peptide Encoding Plasmid (AMEP) used in a clinical trial confirming the safety of GET [27], and for preparation of different plasmids used in DNA vaccination studies [28]. In our group, we implemented the ORT method to prepare several antibiotic resistance gene-free plasmids, including plasmids encoding mouse, canine and human IL-12 orthologues [9, 29, 30, 31, 32]. The plasmid encoding the mouse orthologue was prepared to facilitate preclinical evaluation in mouse tumor models since human IL-12 is not fully functional in mice. While canine IL-12 was prepared for cancer treatment of client-owned dogs to replace the previously used feline and human IL-12 [33].

In previous studies, we have shown that plasmids prepared using the ORT® method are functional, but we noticed that isolation yields tend to be lower than with plasmids prepared by conventional methods using antibiotics. Therefore, our aim was to evaluate the yields and maintenance of selected antibiotic resistance gene-free plasmids prepared by ORT® method (ORT plasmids) encoding mouse, canine and human IL-12 in bacterial cultures. Since these plasmids are intended for GET studies, we further tested their transfection efficiency, *i.e.,* plasmid copy number and expression, as well as cytotoxicity, after GET in mouse, human and canine melanoma cells.

2. Materials and methods

2.1. Plasmid construction

Three ORT plasmids encoding mouse (pORF-mIL-12-ORT), canine (pORF-caIL-12-ORT) and human IL-12 (pORF-hIL-12-ORT) were tested in this study. They were prepared using the ORT® method and have the same backbone without an antibiotic resistance gene (Figure 1). A commercially available plasmid encoding a mouse IL-12, *i.e.;* pORF-mIL-12 (p40p35) (Invivogen, Toulouse, France), with an ampicillin resistance gene in the backbone, was used as a control. The pORF-mIL-12 (p40p35) plasmid was transformed in JM109 *E. coli* strain (Thermo Fisher Scientific, Waltham, MA, USA), while the IL-12 ORT plasmids were transformed in DH1-ORT *E. coli* strain (Cobra Biologics, Keele, UK).

The construction of pORF-mIL-12-ORT and pORF-caIL-12-ORT plasmids was described previously [30, 32]. The plasmid encoding the human IL-12 orthologue, pORF-hIL-12-ORT, was constructed using the same methodology. Briefly, the human IL-12 fusion gene was cut from the pORF hIL-12 G2 plasmid (Invivogen) using *Sal*I and *Hpa*I restriction enzymes (New England Biolabs, Ipswich, USA) and ligated in to the pORF-mIL-12-Xmark plasmid [32] using the T4 DNA ligase (Thermo Fisher Scientific). The resulting recombinant plasmid pORF-hIL-12-Xmark, carrying the chloramphenicol resistance gene, was transformed into DH1-PEPA *E. coli* cells (Cobra Biologics, Keele, UK) using the TransformAid Bacterial Transformation kit (Thermo Fisher Scientific). The transformed bacteria were selected from LB agar plates with chloramphenicol (Merck, Kenilworth, NJ, USA). The p21-hIL-12-Xmark plasmid was then transformed into the DH1-ORT *E. coli* cells (Cobra Biologics) in which the antibiotic resistance gene was excised by Xer recombination resulting in antibiotic resistance gene-free pORF-hIL-12-ORT plasmid (Figure 2a).

Construction of pORF-hIL-12-ORT was confirmed by restriction analysis and full-length plasmid sequencing (Applied Biological Materials Inc., Richmond, Canada) (Figure 2b). For the restriction analysis, the plasmid was cut with different combinations of restriction enzymes (Thermo Fisher Scientific). Cut plasmid was loaded onto a 1% agarose gel in TAE (Tris-acetate EDTA) buffer (Sigma-Aldrich, Saint Louis, MO, USA) pre-stained with 1× SYBR™ Safe (Thermo Fisher Scientific) together with a linear DNA ladder (GeneRuler DNA ladder Mix, Thermo Fisher

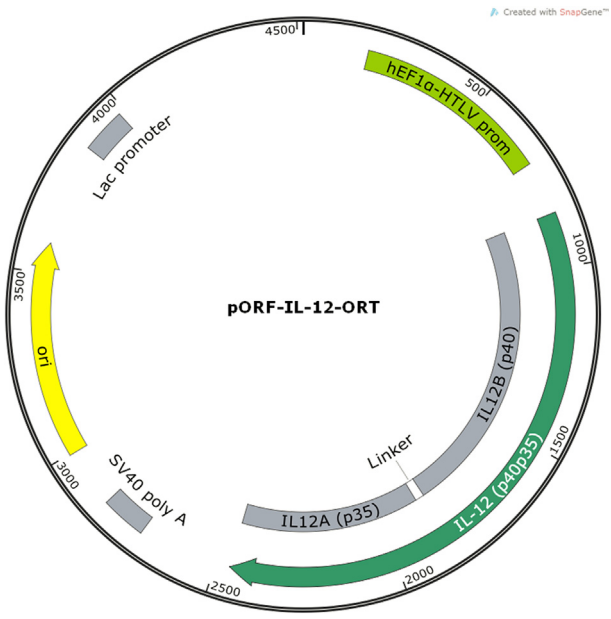

Figure 1. Composition of IL-12 ORT plasmids. All three ORT plasmids (pORF-mIL-12-ORT, pORF-caIL-12-ORT and pORF-hIL-12-ORT) have the same backbone carrying the *E. coli* origin of replication (ori) and lactose (Lac) promoter sequence. The expression cassette consist of a composite EF1a-HTLV promoter comprising the Elongation Factor-1α (EF-1α) core promoter and part of the U5 sequence of the Human T-Cell Leukemia Virus (HTLV) Type 1 Long Terminal Repeat, IL-12 open reading frame (ORF) and Simian Vacuolating virus 40 polyadenylation signal (SV40 polyA). ORFs for mouse, canine and human IL-12 fusion gene consist of relevant orthologue IL12A and IL12B subunits and a linker connecting both subunits.

Scientific). The gel was run at 100 V for 1 h (PowerPac Basic Power Supply, BioRad, Hercules, CA, USA) and imaged by E Box gel documentation system (Vilber, Collégien, France). Plasmid identity was confirmed by positive matching of band patterns on the electrophoretic gel to the expected patterns obtained by a simulation experiment performed by SnapGene software (SnapGene 5.3, GSL Biotech LLC, San Diego, CA, USA) (Figure 2c). For long-term storage, glycerol stocks of overnight culture were prepared in 25% glycerol, snap-frozen in liquid nitrogen, and stored at −80 °C in the laboratory's plasmid bank.

2.2. Maintenance of the plasmids in bacterial culture

To evaluate the stability of ORT plasmids, transformed *E. coli* bacterial cells (DH1-ORT strain transformed with IL-12 ORT plasmids and JM109 strain transformed with the commercial IL-12 plasmid) were subcultured five times on agar plates overnight (16 h) at 37 °C using basic microbiology techniques [34]. At each passage, a single colony was used to inoculate 5 mL of liquid LB Broth (Lennox) (Alfa Aesar, Haverhill, MA, USA; Thermo Fisher Scientific) and cultured overnight at 37 °C and 300 rpm. The next day optical density (OD_{600}) of the bacterial culture was measured spectrophotometrically (Epoch microplate spectrophotometer, BioTek, Bad Friedrichshall, Germany), and plasmid DNA was isolated from 3 mL of overnight culture using a miniprep plasmid isolation kit (GeneJET Plasmid Miniprep Kit, Invitrogen, Thermo Fisher Scientific). Plasmid purity and concentration were determined spectrophotometrically (Epoch Microplate Spectrophotometer, Take3 micro-volume plate, BioTek). The absolute yield was determined based on the volume of the eluate. The procedure was repeated three times.

For the yield visualization, 10 μL of the eluate was linearized with *Not*I restriction enzyme (Thermo Fisher Scientific) and ran on the electrophoretic gel, as described above. To visualize plasmid topologies, uncut plasmids were run (1 h, 100 V, PowerPac Basic Power Supply, BioRad) on un-stained 1% agarose gel together with supercoiled DNA

Ladder (New England Biolabs). After electrophoresis, gels loaded with uncut plasmids were stained in 1x SYBR^{tm} Gold solution (Thermo Fisher Scientific) and imaged as described above. To resolve potential topoisomers visible after electrophoretic separation of uncut pORF-hIL-12-ORT plasmid, increasing concentrations of intercalating dye SYBR^{tm} Safe (0.5×, 1× and 2×) were added to the uncut plasmid before electrophoresis.

2.3. Large scale plasmid isolation

For GET experiments, plasmids (pORF-mIL-12-ORT, ORF-caIL-12-ORT, pORF-hIL-12-ORT and pORF-mIL-12 (p40p35)) were isolated using the EndoFree Plasmid Mega Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and resuspended in endotoxin free water. Plasmid purity and concentration were measured spectrophotometrically (Epoch microplate spectrophotometer, Take3 micro-volume plate, BioTek). Additionally, the concentration and the identity were confirmed by restriction analysis on an electrophoretic gel. The final concentrations were fine-tuned using Qubit 4 Fluorometer (Thermo Fisher Scientific). All plasmids were diluted to a final molar concentration of 335 nmol/L, containing 2.016×10^{11} molecules per µg (Table 1). This concentration was based on the mass concentration standardly used for GET, *i.e.,* 1000 ng/µl. Adjusted equimolar mass concentration of individual plasmids were 1000 ng/µL of pORF-mIL-12 (p40p35), 906 ng/µL of pORF-mIL-12-ORT, 888 ng/µL of pORF-caIL-12-ORT, and 934 ng/µL of pORF-hIL-12-ORT.

2.4. GET

Human, mouse and canine malignant melanoma cell lines were used for GET experiments. The human malignant melanoma cell line SK-Mel-28 (HTB-72, American Type Culture Collection (ATCC), Manassas, VA, USA) and mouse melanoma cell line B16-F10 (CRL-6475, ATCC) were cultured in Advanced Minimum Essential Medium (AMEM, Life Technologies, Thermo Fisher Scientific). Canine malignant melanoma CMeC-1 cell line, gift from Nakagawa [35], was cultured in Advanced Dulbecco modified Eagle medium (A-DMEM, Life Technologies). Both media were supplemented with 5% fetal bovine serum (FBS, Life Technologies), 10 mM/µL-glutamine (Life Technologies) and 1% (v/v) Penicillin-Streptomycin (Sigma Aldrich, Merck, Kenilworth, NJ, USA). All cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were continuously tested every 3 months for mycoplasma with MycoAlert™ PLUS Mycoplasma Detection kit (Lonza, Basel, Switzerland) and found negative.

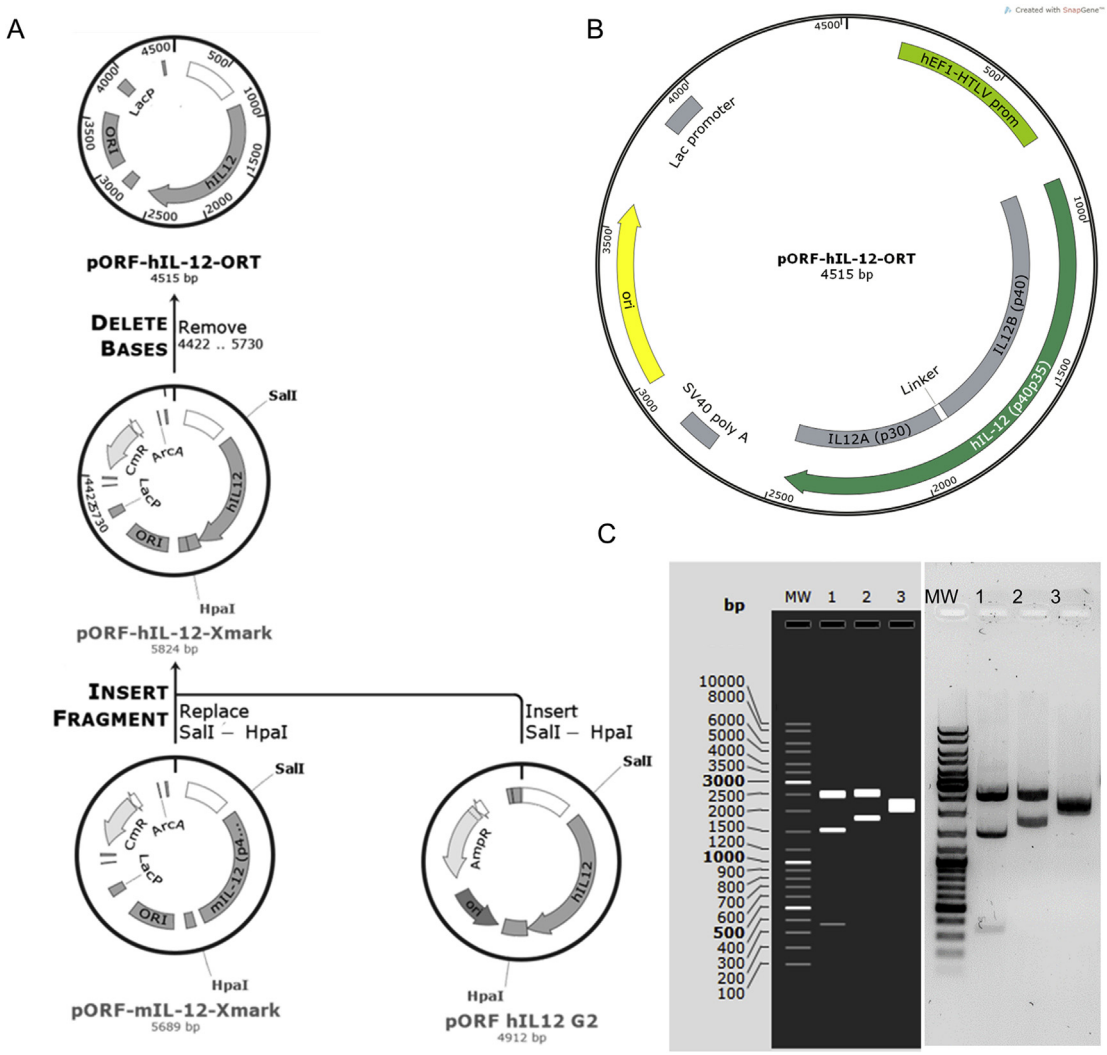

Figure 2. Construction of the pORF-hIL-12-ORT plasmid. (a) Construction plan. (b) Annotated plasmid map confirmed by whole plasmid sequencing. c) Restriction analysis confirming the pORF-hIL-12-ORT plasmid construction: The plasmid was cut with *Nhe*I, *Nco*I and *Hpa*I restriction enzymes and its identity was confirmed by positive matching of the simulated pattern of bands created by SnapGene software (left) to the actual pattern on the electrophoretic gel (right). MW: molecular weight, GeneRuler DNA ladder Mix. Lane 1: *Nhe*I: 2596 bp, 1556 bp, 363 bp; lane 2: *Nco*I + *Hpa*I: 2648 bp, 1857 bp; lane 3: *Kpn*I: 2317 bp, 2198 bp.

Table 1. Equimolar concentrations of plasmids corresponding to 1000 ng/ul of commercial plasmid pORF-mIL-12 (p40p35).

Plasmid	Length (bp)	Molecular weight (g/mol)	Molar concentration (M)	Equimolar concentration (ng/μL)
pORF-mIL-12 (p40p35)	4833	2.99E+06	3.35E-07	1000
pORF-mIL-12-ORT	4380	2.71E+06	3.35E-07	906
pORF-caIL-12-ORT	4293	2.65E+06	3.35E-07	888
pORF-hIL-12-ORT	4515	2.79E+06	3.35E-07	934

Table 2. qRT-PCR oligonucleotides.

Oligonucleotide	Specificity	Sequence
mACTB primer pair	Mouse Beta-actin	Forward: CTGTGCTGT CCCTGTATGC
		Reverse: GGCACAGTGTGGGTGAC
hGUSB primer pair	Human Glucuronidase Beta	Forward: AGGTGATGGAAGAAGTGGTG
		Reverse: AGGATTTGGTGTGAGCGATC
caHPRT primer pair	Canine Hypoxanthine Phosphoribosyltransferase 1	Forward: GCTCGAGATGTGATGA AGGAAA
		Reverse: TAATCCAGCAGGTCAGCAAAG
hIL12 primer pair	Linker region in human IL-12 fusion gene	Forward: TAGCTCATCTTGGAGCGAATG
		Reverse: GGTGAAGGCATGGGAACA
mIL12 primer pair	Linker region in mouse IL-12 fusion gene	Forward: GCAAGCTCAGGATCGCTATTA
		Reverse: GGTCCAGAGACCGGTATGA
caIL12 primer pair	Linker region in canine IL-12 fusion gene	Forward: CGACTGGGCATCTGTGTC
		Reverse: GTGGTTGAGGCACTGGAATA
hgBlocks	Linker region in human IL-12 fusion gene	GCCCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTGCCCTGC AGTGTTCCTGGAGTAGGGGTACCTGGGGTGGGCGCCAGAAACCTCCCCGTGGCCACTCCAGACCC AGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGCTGAGGGCC
mgBlocks	Linker region in mouse IL-12 fusion gene	ATGCAAAGGCGGGAATGTCTGCGTGCAAGCTCAGGATCGCTATTACAATTCCTCATGCAGCAAGTGGGCATGTGTT CCCTGCAGAGTCCGATCGGTTCCTGGAGT AGGGGTACCTGGAGTGGGCAGGGT CATACCGGTCTCTGGACCTGCCAGGTGTCTTAGCCAGTCCCGAA
cagBlocks	Linker region in canine IL-12 fusion gene	TACTATAGTTCGTCC TGGAGCGACTGGGCATCTGTGTCCTGCAGTGGTGGCGGTGGCGGCGGATCTAGGAGCCTCCCCACAGCCTCACCGAGCCCAG GAATATTCCAGTGCCTCAACCACTCCCAAAACCTGCTGAGAGCCGTCAGCAAC

For GET, cells in exponential growth phase were trypsinized and prepared in ice-cold electroporation buffer (125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, and 2 mM $MgCl_2$) to a concentration of 2.5×10^7 cells/mL. Cell suspension was mixed with plasmid solution at a 4:1 ratio and 50 μL of the mixture (containing 10 μL or 2.016×10^{12} copies of plasmids DNA and 1×10^6 cells) was pipetted into a 2-mm gap between two parallel stainless-steel electrodes. GET of plasmids was performed using a clinically relevant electric pulse protocol [27, 36, 37] with eight square wave electric pulses with voltage to distance ratio of 1300 V/cm, pulse duration of 100 μs, and at a frequency of 5 kHz, generated by the electric pulse generator Cliniporator (IGEA, Carpi, Italy). These pulse parameter were selected to be comparable to a parallel study confirming the suitability of IL-12 ORT plasmids for first-in-human clinical trial [38]. After GET, cells were incubated for 5 min. Then, 1 mL of appropriate cell culture medium was added and the resulting suspension of cells was plated for further assays. All experiments were performed in three biological replicates.

2.5. Total DNA isolation

Total DNA (tDNA) was isolated from transfected cells 2 days after GET. Cells were trypsinized, counted and resuspended in 2 mL of Hank's Balanced Salt solution with calcium and magnesium (HBSS, Gibco, Thermo Fisher Scientific) containing 200 U/μL DNase I (Invitrogen, Thermo Fisher Scientific) to digest any plasmid DNA associated with the plasma membrane [39, 40, 41]. After a 10 min incubation at 37 °C and 300 rpm (ThermoMixer C, Eppendorf, Hamburg, Germany), 0.5 M EDTA (Abcam, Cambridge, UK) was added to inactivate DNase I. Thereafter, tDNA isolation was performed using the DNeasy® Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. The quantity and purity of the isolated tDNA was determined using Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific) and tDNA was stored at −80 °C for further analysis.

2.6. Total RNA isolation and complementary DNA preparation

Total RNA (tRNA) was isolated from transfected cells 2 days after GET. Isolation was performed using the peqGOLD Total RNA Kit (VWR, Radnor. PA, USA) according to manufacturer's instructions including a DNA digestion step to remove any remaining DNA. The quantity of the isolated RNA was determined by Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific). SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific) was used to synthesize complementary DNA (cDNA) from 1000 ng of isolated total RNA using a thermal cycler (Primus 25 advanced Thermocycler, VWR) under following conditions: 10 min incubation at 25 °C, 60 min incubation at 42 °C and 5 min at 85 °C for reaction termination. The isolated undiluted cDNA was stored at −80 °C for further analysis.

2.7. Plasmid copy number and expression

Isolated tDNA and cDNA from isolated tRNA were used as templates for determining plasmid copies inside transfected cells and IL-12 transgene expression by quantitative real-time PCR (qRT-PCR), respectively. Primer pairs were designed for the linker region between p40 and p35 IL-12 subunits of all three IL-12 fusion genes, to avoid detection of any

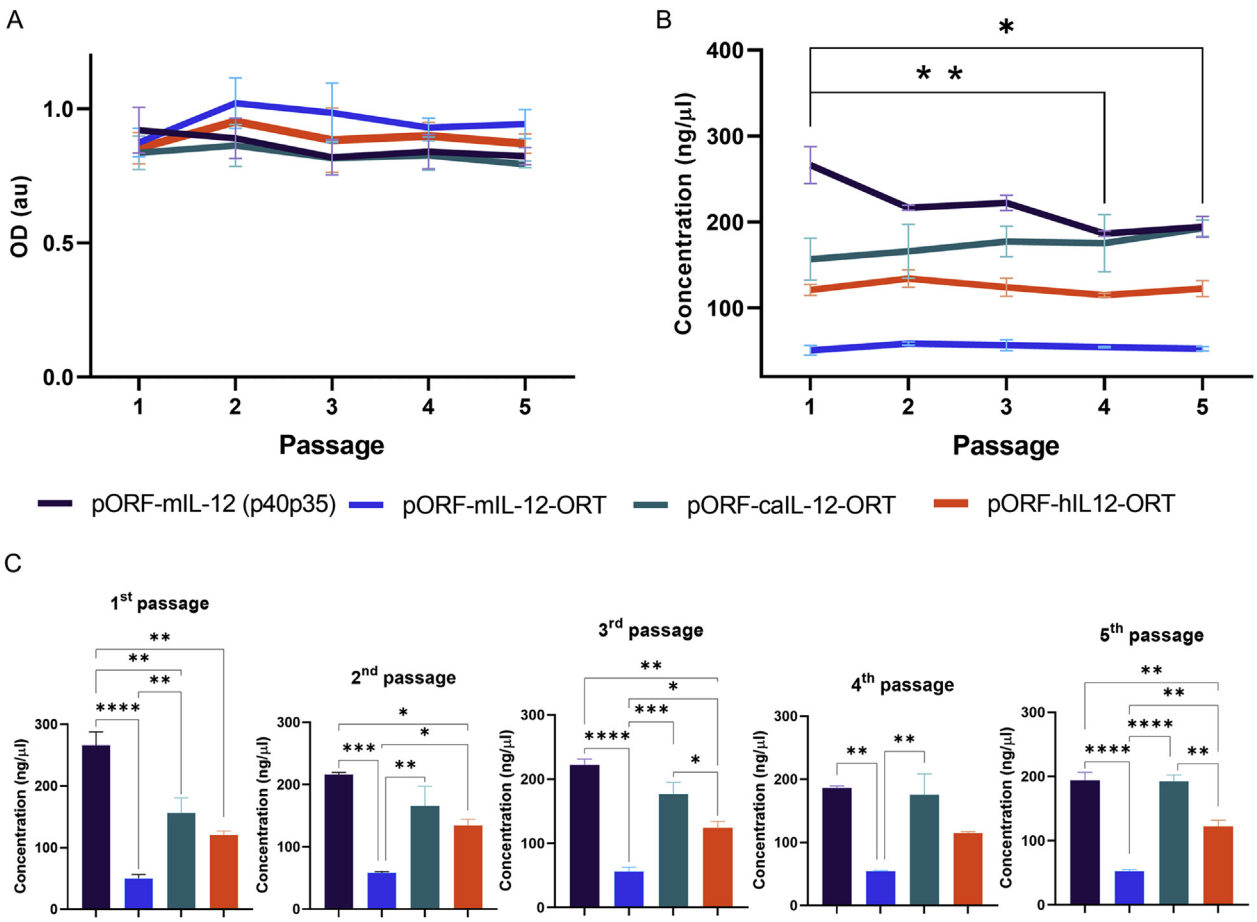

Figure 3. Maintenance of ORT plasmids in bacterial culture: Transformed bacteria were subcultured for 5 days and plasmid DNA was isolated from 3 mL of overnight culture at each passage. (a) Average optical densities of overnight cultures. (b) Average plasmid concentrations after miniprep isolations. (c) Comparison of mean plasmid concentration at each passage. Results are presented as mean with standard error of the mean. The experiments were repeated 3 times for each plasmid. One-way ANOVA: (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, (****) $P \leq 0.0001$.

endogenously produced IL-12 (Table 2). Since this is a plasmid specific sequence, the same primers were also used to determine the plasmid copy number after GET. Primers were designed and ordered using online tools provided by Integrated DNA technologies (Coralville, IA, USA). Every primer set was validated *in silico* for specificity and the presence of secondary structures using the IDT OligoAnalyzer™ Tool and Ensembl BLAST/BLAT (Ensembl Genome Browser release 99) [42], and empirically for melting temperature and amplification efficiency.

Relative quantification was used to determine transgene expression after GET. Primers for Mouse Beta-actin gene, Human Glucuronidase Beta gene, and Canine Hypoxanthine Phosphoribosyltransferase 1 gene were used as internal controls for samples isolated from mouse, human and canine cells, respectively (Table 2). Absolute quantification was used to determine the plasmid copy number in the cells after GET. Synthetic dsDNA gBlocks™ (Integrated DNA technologies) were designed for each amplicon (Table 2). Serial dilutions of gBlocks™ were used to create standard curves and extrapolate the plasmid copy number. Number of copies of gBlocks™ molecules in each dilution was calculated based on the molecular weight of each gBlocks™ fragment according to their sequence, using an on-line DNA calculator [43].

The qRT-PCR was performed in duplicates including No Template Control (NTC). The total volume of a reaction was 20 µL and contained 10 ng of cDNA or tDNA, 200 nM of forward and reverse primers in SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific). Reactions were run in 96-well PCR plates on a QuantStudio 3 (Applied Biosystems, Thermo Fisher Scientific). Cycling conditions for the IL-12 expression determination were: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and for melting curve determination: 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C. Cycling conditions for plasmid copy number determination were: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C, and for melting curve determination: 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C. Analysis Software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific) was used for qRT-PCR analysis.

Transgene expression was expressed as the relative quantity of the transgene to the quantity of the internal control housekeeping gene using the 2-ΔCt method [44]. Copy number was extrapolated from the standard curve. Due to the use of cancer cell lines that are aneuploid [45], normalization to the internal control was not possible, therefore copy number was expressed as a number of plasmid copies per cell using the following formula (Eq. (1)).

$$\text{Plasmid copy number} = \frac{\text{Extrapolated copy number} \times \frac{\text{isolated DNA mass}}{10\ \text{ng}}}{\text{Number of cells from which DNA was isolated}} \quad (1)$$

2.8. Cytotoxicity

Cell viability was quantified using Presto Blue viability reagent (Thermo Fisher Scientific) as a measure of GET cytotoxicity comparable to a congenic assay [46]. Low numbers of cells from each experimental group (200 B16-F10 cells, 150 CMeC-1 cells or 500 SK-MEL-28 cells per well) were plated in 100 µL of cell culture medium in 96-well plates (Corning Incorporated, Corning, NY, USA) and incubated for 7 days at 37 °C in a humidified atmosphere with 5% CO_2 without replacing the cell culture medium. After incubation, 10 µL of Presto Blue™ was added to the

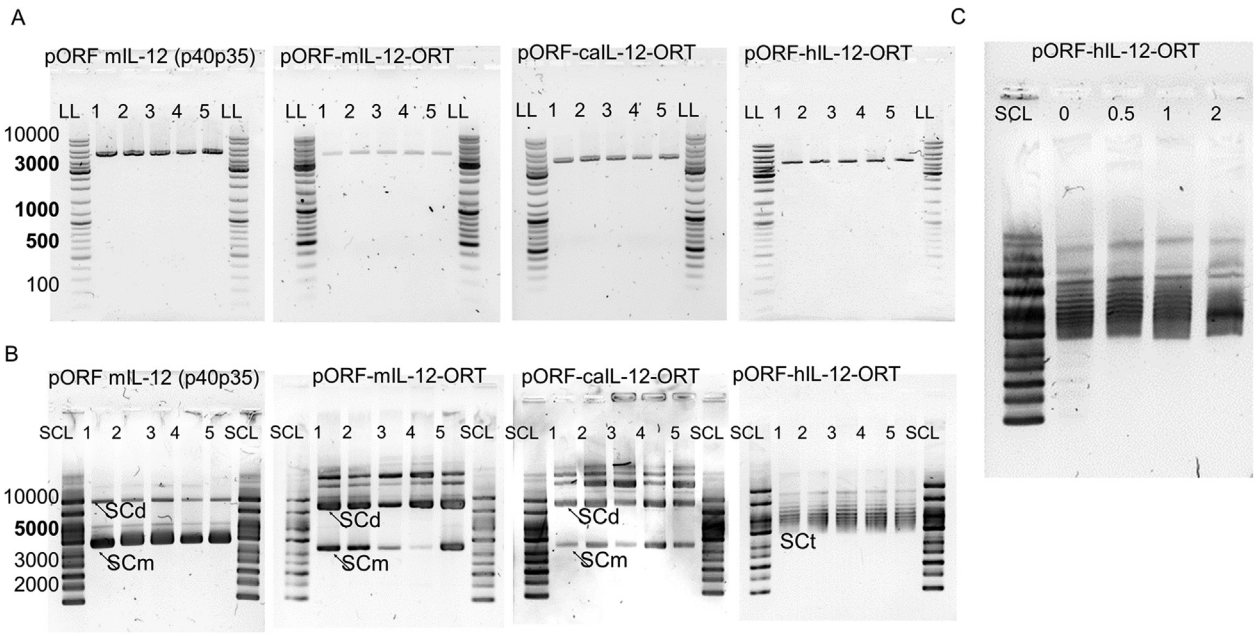

Figure 4. Electrophoretic evaluation of plasmids. (a) Electrophoresis of 10 μL of minipep eluates from first to fifth passage linearized with *Not*I restriction enzyme. Plasmid lengths: pORF-mIL-12 (p40p35) - 4833 bp, pORF-mIL-12-ORT - 4380 bp, pORF-caIL-12-ORT - 4293 bp, pORF-hIL-12-ORT - 4515 bp. 1% agarose pre-stained with 1× SYBR^{tm} Safe, 100 V, 1 h, LL, linear ladder: GeneRuler DNA ladder mix. Lanes 1–5, passages. (b) Electrophoresis of 200 ng of uncut plasmids isolated from first to fifth passage. 1% agarose, 100 V, 1 h, stained in 1× SYBR^{tm} Gold. SCL, supercoiled ladder: Supercoiled DNA Ladder. SCm, supercoiled monomer. SCd, supercoiled dimer, SCt, supercoiled topoisomers. Lanes 1–5, passages (c) pORF-hIL-12-ORT plasmid incubated with increasing concentrations of SYBR^{tm} Safe (0, 0.5×, 1× and 2×). SCL, supercoiled ladder: Supercoiled DNA Ladder.

wells and one hour later fluorescence intensity was quantified utilizing a microplate reader Cytation 1 (BioTek). Cell survival after GET was normalized to the electroporated cells.

2.9. Statistical analysis

GraphPad Prism 9 (GraphPad software, San Diego, CA, USA) was used for statistical analysis. Significance was determined by two-tailed t-test or a one-way ANOVA test. A P value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Plasmid maintenance

Our fist aim was to evaluate the maintenance of ORT plasmids encoding mouse, canine and human IL-12. To test if ORT plasmids are lost during subculturing, plasmid DNA was isolated from 5 consecutive passages on LB agar plates and plasmid yield and electrophoretic patterns were compared to the commercial IL-12 plasmid pORF-mIL-12 (p40p35). After the first passage, the plasmid yields varied significantly among the plasmids, while the culture ODs were similar (Figure 3a, b and c). Commercial plasmid gave a significantly higher yield in the first passage compared to all ORT plasmids, while in the following passages its yields were similar to that of the pORF-caIL-12-ORT plasmid (Figure 3b and d). The lowest yields were obtained with pORF-mIL-12-ORT plasmid. After passaging for five days, yields of the commercial plasmid decreased and were significantly lower in fourth and fifth passage compared to the first passage. Conversely, yields of all three ORT plasmid remained constant throughout subculturing, confirming that ORT plasmids are stably maintained in transformed bacteria during cell division, reducing the probability of plasmid loss over generations.

Consistent yields during subculturing were also evident from the band intensity of linearized plasmids on the electrophoretic gel (Figure 4a). Electrophoresis of uncut plasmids, however, showed that ORT plasmids are prone to form dimers and even trimers, while only a small fraction of plasmid was in supercoiled form (Figure 4b). Furthermore, in ORT plasmids, the fraction of supercoiled form was not consistent after subculturing compared to the commercial IL-12 plasmid, where the fractions were stable over the passages. Additionally, in pORF-hIL-12-ORT plasmid, supercoiled form split to multiple bands. After the addition of increasing concentrations of intercalating dye SYBT®Safe, multiple bands resolved back into one supercoiled band, most likely indicating that these were supercoiled topoisomers that are known to relax in the presence of intercalating agents [47].

We speculate that the reasons for the presence of dimers and further concatemers could lie in the nature of DH1-PEPA strain used during the cloning process. This strain was designed to ease the preparation of selectable marker free plasmids using highly efficient antibiotic selection [48]. It is a mutant for Xer recombination, which enables generation and stable replication of plasmids with its antibiotic resistance gene flanked by Xer recombination psi sites [49]. Transformation of such plasmids into any other strain with functional Xer recombination deletes this gene at psi sites. However, Xer recombination is also responsible for plasmid multimer resolution, allowing efficient segregation of monomers to daughter cells during cell division in *E. coli* [25, 50, 51]. Therefore, malfunction of Xer recombination in DH1-PEPA strain could be the reason for presence of unresolved plasmid concatemers.

The presence of dimers and other unwanted topoisomers and a low percentage of supercoiled form could present an issue for clinical grade plasmid production that necessitates a well-defined, stable product. High yield and high supercoiled fraction are required for the application of plasmid as biotherapeutics, as supercoiled plasmids have a higher biological activity than other plasmid forms [52, 53, 54]. To solve these bioprocess challenges, modification of fermentation temperature and culture media have been studied in the past, in addition to engineering special high producing *E. coli* strains and plasmids [53, 55, 56]. We have recently addressed this issue in collaboration with industry with the goal to implement an antibiotic resistance gene-free IL-12 plasmid in the first human clinical trial for the treatment of cutaneous basal cell carcinoma

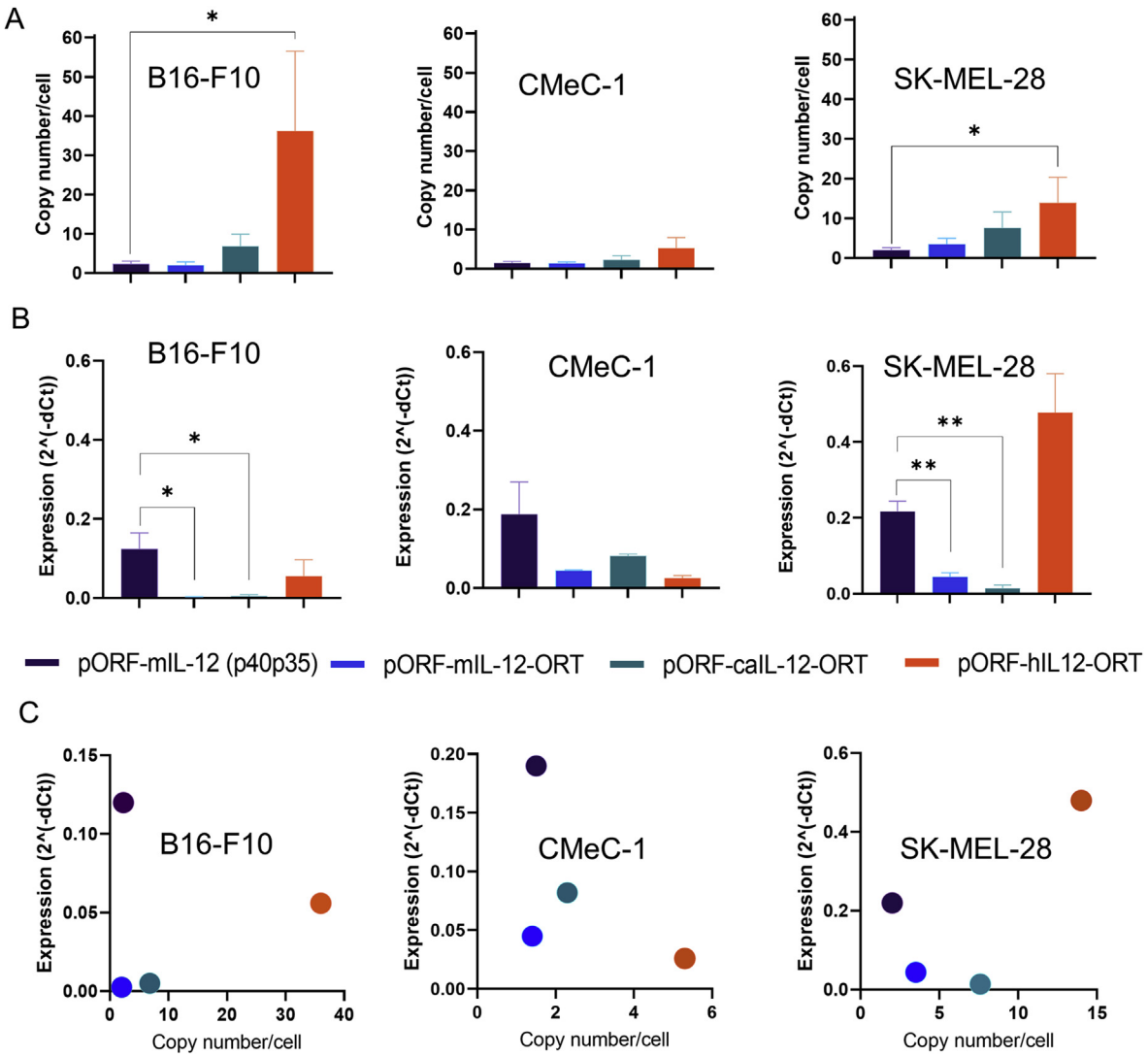

Figure 5. Transection efficiency. (a) Plasmid copy numbers after GET of pORF-mIL-12 (p40p35), pORF-mIL-12-ORT, pORF-caIL-12-ORT and pORF-hIL-12-ORT plasmids to B16-F10, CMeC-1 and SK-MEL-28 melanoma cells. (b) Plasmids expression after GET to B16-F10, CMeC-1 and SK-MEL-28 melanoma cells. Results are presented as mean with standard error of the mean. T test vs. pORF-mIL-12 (p40p35), (*) $P \leq 0.05$, (**) $P \leq 0.01$. (c) Correlation between expression and copy number. Pearson correlation coefficients (r): B16-F10, 0.06258; CMeC-1, -0.5622; SK-MEL,-28, 0.6634.

tumors in the head and neck region [38]. In the mentioned study, the required clinical grade plasmid quality parameters could be reached with ORT plasmids using two-step purifications on anion exchange and hydrophobic interaction chromatography columns.

3.2. Transfection efficiency: plasmid copy number and expression

Since the constructed plasmids are intended for GET studies, our second aim was to test their transfection efficiency after GET to mouse, human and canine melanoma cells. Transfection efficiency was determined by analyzing the plasmid copy number and expression 2 days after GET. Comparable to several GET studies, we confirmed that only a small fraction of plasmids successfully enters the cells, while the rest are trapped extracellularly [37, 38, 39, 58, 59]. We used 10 μL of plasmid DNA per 1×10^6 of electroporated cells, *i.e.,* 2.016×10^6 plasmid copies per cell. From this, only around 20 copies per cell were detected in cells 2 days after GET. This means that only one out of 10,000 plasmids successfully entered the cells.

The highest plasmid copy number per cell was achieved with pORF-hIL-12-ORT plasmid in B16-F10 cells, *i.e.,* 36 copies per cell, which was significantly higher than with the commercial plasmid, where only 3 copies per cell were detected (Figure 5a). Similar pattern was observed in SK-MEL-28 cells, although with lower copy numbers, *i.e.;* 15 copies after GET of pORF-hIL-12-ORT to 2 copies after GET of the commercial plasmid. The lowest copy numbers were detected in CMeC-1 cells, *i.e.*, 2 copies after GET of the commercial and pORF-mIL-12-ORT, however this should still be sufficient for transgene expression in mammalian cells. Theoretically, only one copy per cell should suffice, as was also confirmed in viral transduction or plasmid DNA microinjection studies [57, 58, 59]. However, for non-viral methods, reported numbers were mostly much higher, in the range of 10–50,000 [39,40,60–62]. Based on the recently published statistical prediction, the minimal number of plasmid DNA copies required to be electroporated into a cell for transgene expression is in the order of 10 [40].

Regardless of low copy numbers inside the cells, IL-12 expression from all four plasmids was detected in all three cell lines, confirming that even 2 copies per cell are sufficient for transgene expression. The expression was the highest in human SK-MEL-28 cells, specifically expression from the pORF-hIL-12-ORT plasmid (Figure 5b), while the lowest expression was achieved with pORF-mIL-12-ORT plasmid in all three cell lines. Although we have previously confirmed that transfection of IL-12 plasmids prepared by ORT technology leads to expression of a functional IL-12 protein [38,

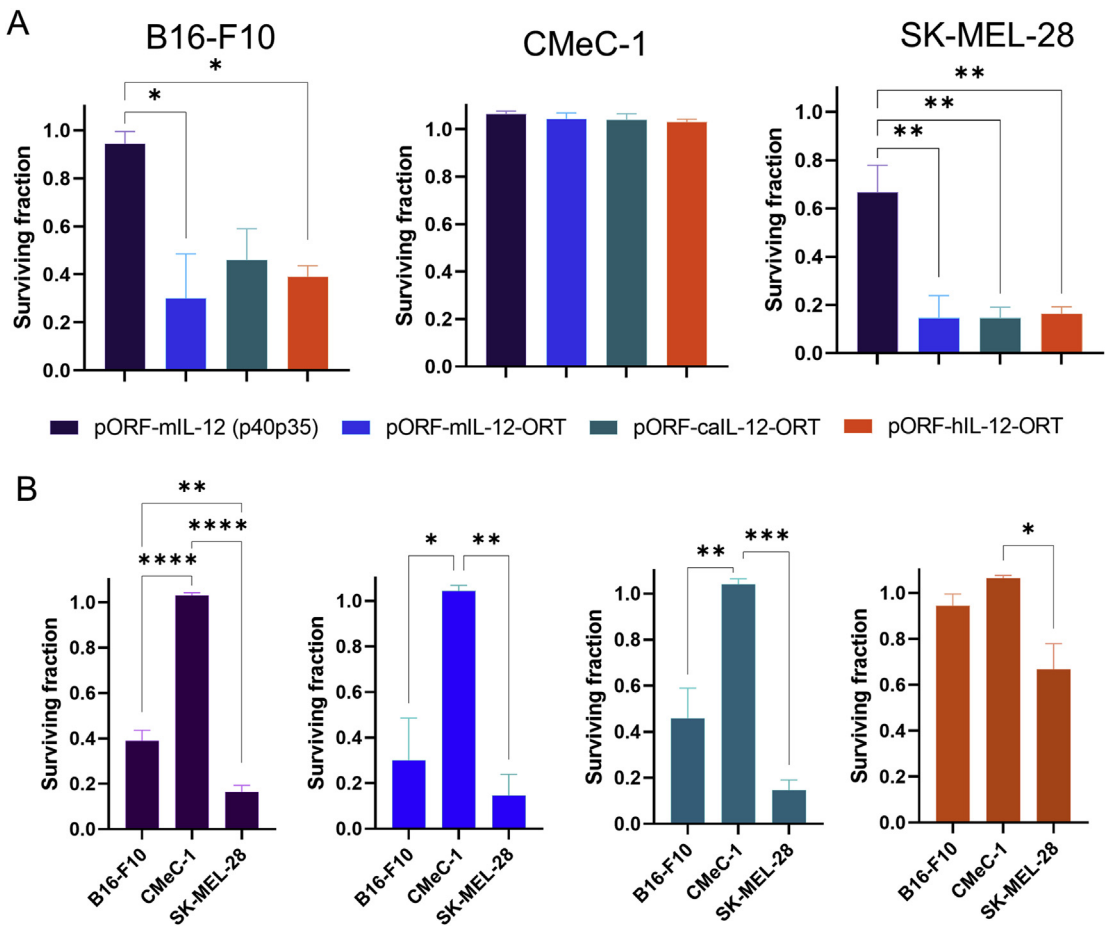

Figure 6. Cytotoxicity. (a) Surviving fraction after GET of pORF-mIL-12 (p40p35), pORF-mIL-12-ORT, pORF-caIL-12-ORT and pORF-hIL-12-ORT plasmids to B16-F10, CMeC-1 and SK-MEL-28 melanoma cells. (b) Comparison of surviving fraction after GET of each plasmid in separate cell lines. Results are presented as mean with standard error of the mean. One-way ANOVA: (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, (****) $P \leq 0.0001$.

63], it would be interesting to see, how much of the transcribed IL-12 actually translates to IL-12 protein. However, determination of the correlation between IL-12 mRNA and IL-12 protein was omitted in the current study, because it is not expected to be straightforward, as any endogenously produced IL-12, induced by introduction of foreign DNA, would also be detected along with the IL-12 expressed form transfected plasmid. On the mRNA level, however, expression of IL-12 could be distinguished from naturally produced IL-12 by designing specific primers, since all three IL-12 ORT plasmids encode an intronless p40/p35 IL-12 constructs that are transcribed to one single IL-12 mRNA, while endogenous IL-12 is transcribed to two separate mRNAs.

Interestingly, the IL-12 mRNA expression levels did not correlate with the plasmid copy number (Figure 5c). Similar results were obtained in the already mentioned study determining the minimal copy number required for transgene expression after GET [40]. Using flow cytometry to assess the transfection efficiency of fluorescent reporter genes, Wang and colleagues were able to confirmed correlation between copy number and the percentage of transfected cells. However, they were unable to confirm correlation when median fluorescence intensity was used as measure of transfection efficiency, which is comparable to average qRT-PCR expression level used in our study. Similarly, no correlation was detected in the study by Maucksch *et al* [54]. The main reason for observed discrepancy between the copy number and expression is that not all the plasmids that enter the cell, also enter the cell's nucleus and are successfully transcribed to mRNA [39, 40, 41, 64, 65]. Expression additionally depends on the presence of transcription factors for a given promoter. In the current study, we used a promoter of a housekeeping gene EF-1α that is known to support high ubiquitous and constitutive expression; however, even expression of housekeeping genes can vary between cell lines [66, 67, 68].

3.3. Cytotoxicity

Finally, we also tested the cytotoxicity after GET. In B16-F10 and SK-MEL-28 cells, cell survival was significantly lower after transfection of ORT plasmids compared to the commercial plasmid, while in CMeC-1 cells, transfection of either of the plasmids did not affect cell survival (Figure 6a and b). Similarly inconsistent results were also demonstrated in our previous studies, where different ORT plasmids were found either equally [30], more [30] or less cytotoxic [9] than non-ORT plasmids in different cell lines.

It is worth mentioning that the IL-12 protein is not cytotoxic *per se*; GET, on the other hand, is. Partially due to the physical process of electroporation [69], but largely due to the introduction of foreign DNA molecules that prime the activation of cytosolic DNA sensors [70, 71, 72]. It is still not clear which plasmid characteristic is responsible for this phenomena, though it does not seem to be sequence, structure, CpG islands content, or even plasmids size dependent [73]. Based on our current and previous results with ORT plasmids, we can confirm that none of the mentioned plasmid characteristics play a big role in cytotoxicity after GET. Although, the absence of antibiotic resistance gene in a plasmid should lower its toxicity according to several studies [24, 74], we were unable to confirm this with our ORT plasmids, at least not *in vitro*. *In vivo*; however, lower immunogenicity is to be expected due to the smaller plasmid part that originates from bacteria (*i.e.*, antibiotic resistance gene) [74].

4. Conclusions

In this study, we present the preparation and testing of antibiotic resistance gene-free plasmids encoding mouse, canine and human IL-12, which comply with the EU regulatory requirements that recommend against the use of antibiotics during the production of clinical grade plasmids. The plasmids are intended for GET studies: the mouse orthologue was prepared to facilitate preclinical evaluation in mouse tumor models, canine for cancer treatment of client-owned dogs, and human for translation into human clinical testing. We showed that tested IL-12 plasmids are stably maintained in bacteria and support sufficient IL-12 expression after *in vitro* GET. These plasmids; therefore, have the potential to proceed to *in vivo* evaluation and, ultimately, translation to clinical studies in Europe, enabled by the absence of the antibiotic resistance gene. However, before translation, plasmid production still needs to be optimized to ensure improved plasmid yield, quality and, above all, standardization of the final antibiotic-free product in good manufacturing practice conditions.

Declarations

Author contribution statement

Maja Cemazar: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Andrej Rencelj; Tanja Jesenko; Tinkara Remic: Performed the experiments and analysed; Interpreted the data.

Gregor Sersa: Contributed reagents, materials, analysis tools or data.

Urska Kamensek: Conceived and designed the experiments; Performed the experiments and analysed; Interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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