Article

Detection of transgenes in gene delivery model mice by adenoviral vector using ddPCR

Takehito Sugasawa[1†], Kai Aoki[2†], Koichi Watanabe[3], Koki Yanazawa[4], Tohru Natsume[5], Tohru Takemasa[3], Kaori Yamaguchi[3], Yoshinori Takeuchi[6], Yasuko Yoshida[7], Katsuyuki Tokinoya[2], Nanami Sekine[4], Kaoru Takeuchi[8], Haruna Ueda[4], Yasushi Kawakami[1], Satoshi Shimizu[3], Kazuhiro Takekoshi[1*]

[1] Laboratory of Laboratory / Sports medicine, Division of Clinical Medicine, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan.; take0716@krf.biglobe.ne.jp (T.S.); y-kawa@md.tsukuba.ac.jp (Y.K.)

[2] Doctoral Program in Sports Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8577, Japan.; fineday0126@gmail.com (K.A.); ilbsk5@yahoo.co.jp (K.Tokinoya.)

[3] Faculty of Health and Sport Sciences, University of Tsukuba, Tsukuba, Japan.; watanabe.koichi.ga@u.tsukuba.ac.jp (K.W.); takemasa.tohru.gm@u.tsukuba.ac.jp (T.T); yamaguchi.kaori.gf@u.tsukuba.ac.jp (K.Yamaguchi.); shimizu.satoshi.fe@u.tsukuba.ac.jp (S.S.)

[4] Master's Program in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-8577, Japan.; s1921312@s.tsukuba.ac.jp (K.Yanazawa.); s1721298@s.tsukuba.ac.jp (N.S.); s1821255@s.tsukuba.ac.jp (H.U.)

[5] Molecular Profiling Research Center for Drug Discovery (molprof), National Institute of Advanced Industrial Science and Technology (AIST).; t-natsume@aist.go.jp

[6] Nutrigenomics Research Group, Faculty of Medicine, University of Tsukuba, Ibaraki 305-8575, Japan.; yoshinori-takeuchi@umin.ac.jp

[7] Department of Medical Technology, Faculty of Health Sciences, Tsukuba International University, 6-20-1 Manabe, Tsuchiura, Ibaraki 300-0051, Japan.; shinbelon@mwa.biglobe.ne.jp

[8] Laboratory of Environmental Microbiology, Division of Basic Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan.; ktakeuch@md.tsukuba.ac.jp

* Correspondence: k-takemd@md.tsukuba.ac.jp (K.Takekoshi); Tel.: +81-029-853-3389

† Tkehito Sugasawa and Kai Aoki contributed equally to this work

Abstract: With the rapid progress of genetic engineering and gene therapy, World Anti-Doping Agency has alerted to gene doping and prohibited its use in sports. However, there is no standard method available yet for detection of transgenes delivered by recombinant adenoviral (rAdV) vectors. Here we aimed to develop a detection method for transgenes delivered by rAdV vectors in a mouse model that mimics gene doping. rAdV vectors containing mCherry gene was delivered in mice through intravenous injection or local muscular injection. After five days, stool and whole blood samples were collected, and total DNA was extracted. As additional experiments, whole blood was also collected from mouse tail tip until 15 days from injection of the rAdv vector. Transgene fragments from different DNA samples were analyzed using semi-quantitative PCR (sqPCR), quantitative PCR (qPCR), and droplet digital PCR (ddPCR). In the results, transgene fragments could directly be detected from blood cell fraction-DNA, plasma-cell free DNA and stool-DNA by qPCR and ddPCR, depending on specimen type and injection methods. We observed that a combination of blood cell fraction-DNA and ddPCR was more sensitive than other combinations used in this model. These results could accelerate the development of detection methods for gene doping.

Keywords: Gene doping, gene therapy, droplet digital PCR, adenoviral vector

1. Introduction

Doping is an act of raising competitive abilities to achieve success by using the substances or methods prohibited in sports [1]. Doping in sports, especially in festivals such as the Olympic Games and in world or local championships for various competitions is considered illegal and against the spirit of the game. The World Anti-Doping Agency (WADA) was established in 1999 and has been involved in scientific research on doping, anti-doping education, development of anti-doping strategies, and monitoring of the World Anti-Doping Code (Code) [2] to ensure soundness and fairness in sports worldwide.

With the rapid progress of genetic engineering technology and gene therapy, WADA has been strongly alerted against gene doping. Since its early days, WADA has added "gene doping" to its prohibited list. Subsequently, in 2004, WADA created a panel of experts on gene doping to investigate the latest advances in the field of gene therapy, and the methods for detecting doping [3]. In January 2018, WADA extended the ban on gene doping to include all forms of gene editing. Therefore, the list of prohibited substances currently includes "gene editing agents designed to alter genome sequences and/or the transcriptional or epigenetic regulation of gene expression" [4]. However, there are no established standard methods for detecting or preventing gene doping till date.

In recent years, genetic engineering technology has rapidly advanced, resulting in progression of gene therapy. In gene therapy, various viral vectors have been frequently devised and applied. Vectors based on recombinant adeno-associated viruses (rAAV) and recombinant adenoviruses (rAdV) have been widely used in clinical trials and animal experiments for investigating gene therapy. For example, rAAV vectors have been applied in the treatment of diseases such as Duchenne muscular dystrophy (DMD) [5], Haemophilia B [6, 7] , and Leber congenital amaurosis (LCA) [8, 9] during clinical trials or animal experiments as a form of gene therapy. Moreover, rAdV vectors also have been applied in gene therapy for the treatment of certain human cancers [10-12]. In china, two rAdV vector-based gene therapy products, namely Gendicine (Shenzhen SiBiono GeneTech Co., Ltd.) [12, 13] and Oncorine（Sunway Biotech Co., Ltd.）, were approved for clinical use in humans to treat head and neck cancer and were released into the commercial market in 2003 and 2006, respectively [14]. Additionally, rAdV vectors have been the most commonly used vectors in approved clinical trials of gene therapy (541 cases, 18% of the total) worldwide till December 2018 [15] (Table 1). It can be assumed that gene doping methods may employ clinical trial methods. Therefore, there is a possibility that rAAV or rAdV vectors, especially rAdV vectors, can be used as gene doping agents to enhance athletic performance by artificially modifying gene expression in specific human organs. In this study, we focused on rAdV vectors since rAdV vectors are most commonly used in clinical trials [15] (Table 1) and are also used as prescription drugs [12-14]. Moreover, we observed that designing rAdV vectors containing transgenes is easier than designing rAAV vectors.

Table 1. Types and relative numbers of top seven clinically approved vectors used in gene therapy

Vector	Gene Therapy Clinical Trials	
	Number	%
Adenovirus	541	18.5
Retrovirus	514	17.5
Naked/Plasmid DNA	452	15.4
Lentivirus	278	9.5
Adeno–associated virus	238	8.1
Vaccinia virus	133	4.5
Lipofection	119	4.1
Others	774	26.4
Total	2930	100

This data in Table 1 were obtained from the website of Gene Therapy Clinical Trials Worldwide [15]. The top seven vectors include five viral vectors, with rAdV vectors being the most commonly used vectors. It is believed that these viral vectors can be used for transgene delivery in gene doping.

2. Materials and Methods

2.1. Plasmids and cells

Following plasmids were used in the study. Plasmid pcDNA3.1-Peredox-mCherry was a gift from Gary Yellen [20] (Addgene plasmid # 32383; http://n2t.net/addgene:32383; RRID: Addgene_32383); pENTR4 (Thermo Fisher Scientific); pAd/CMV/V5-DEST (Thermo Fisher Scientific). HEK 293A cells (Thermo Fisher Scientific) were used to clone and amplify recombinant adenoviral (rAdV) vectors.

2.2. Cloning of recombinant adenoviral vectors containing mCherry gene

We selected the mCherry gene, encoding red fluorescent protein, as a transgene. The mCherry gene was amplified by PCR with pcDNA3.1-Peredox-mCherry having 5'-EcoRI and 3'-NotI restriction enzyme sites as a template. The mCherry gene was then cloned into pENTR4 plasmid between EcoRI and NotI sites by digestion by restriction digestion followed by ligation with T4 ligase (Promega). Using Gatewa LR Clonas Enzyme mix (Thermo Fisher Scientific), pENTR4 containing mCherry gene was allowed to react and recombine with pAd/CMV/V5-DEST (destination vector) in an LR reaction to move the mCherry gene into pAd/CMV/V5-DEST plasmid. Subsequently, pAd/CMV/V5-DEST plasmid containing mCherry gene was digested with Pac I restriction enzymes (New England Biolabs), and the resulting liner plasmids were transfected using Lipofectamine LTX Reagent (Thermo Fisher Scientific) into HEK 293A cells cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) containing 10% Fetal Clone III (GE Healthcare) and antibiotics (Nacalai tesque) to synthesize and amplify rAdV vectors containing mCherry gene. Amplified rAdV vectors were purified by CsCl density gradient ultracentrifugation followed by gel filtration, according to the protocol described by Takeuchi et al. [21, 22]. The concentration of rAdV viral particles (VP) was measured on a spectrophotometer. To confirm the expression of functional mCherry protein, HEK 293A cells were seeded at a density of 2.5 × 105 cells per well in 6 well plates and were cultured in DMEM containing 10% Fetal Clone III and antibiotics. After 24 h, the cells were infected with rAdV vectors (2 × 109 VP/ml of medium) to allow the expression of mCherry. After 24 h of induction, red fluorescence of mCherry was analyzed using fluorescence microscope.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 April 2019 doi:10.20944/preprints201904.0285.v1

Peer-reviewed version available at Genes 2019, 10, 436; doi:10.3390/genes10060436

2.3. Animal experiments

Animal experiments in this study were approved by the Animal Care Committee, University of Tsukuba (approval number: 18-118 and 18-474). The overview of the experiments is shown in Fig. 1. Six-week-old IC57BL/6 male mice were purchased from Central Laboratories for Experimental Animals (CLEA). Mice were allowed to grow till they became 10-week-old, with average body weight 26.1 g (SD = ±1.8 g). At this point, they were sacrificed for further experiments.

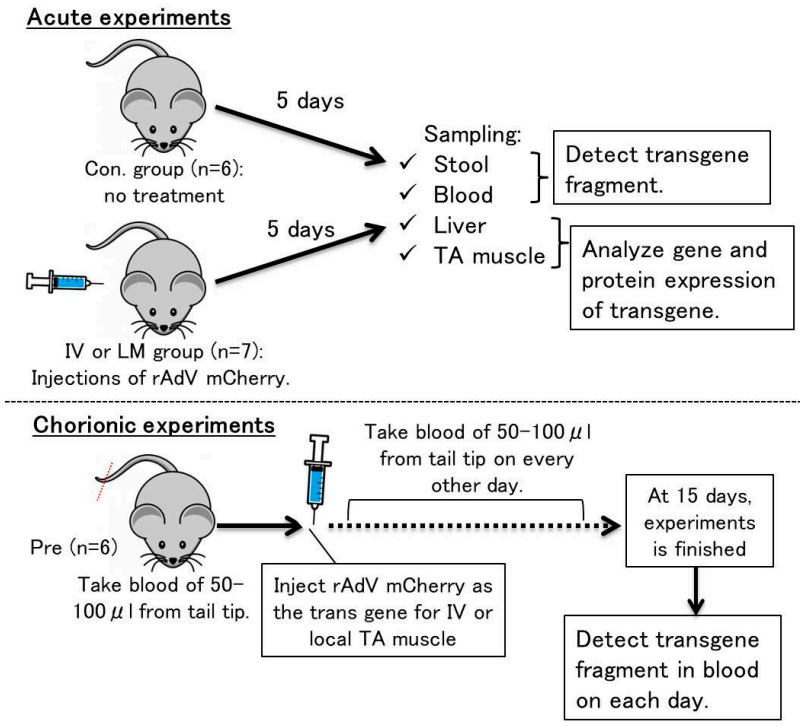

Figure 1. Overviews of animal experiments carried out in this study

2.3-1. Acute experiments

The rAdV vectors containing mCherry gene (1.5×10^{11} VP) were injected into left orbital veins (intravenous; IV group, n = 7) or local muscle (LM group, n = 7) of both tibialis anterior (TA) muscle (injected half amount to one of TA muscle) of mice under general anesthesia by inhalation agent isoflurane. When rAdV vectors were used intravenously (IV), most of the rAdV transgenes accumulated in liver. Control mice were left untreated (Con. Group, n = 6). After five days of injection, mice were placed in an empty cage and were allowed to defecate. Stool samples were quickly collected into microtubes and placed on ice. After collecting stool samples from experimental mice, whole blood was extracted from inferior vena cava using EDTA as an anticoagulant. During this procedure, mice were given general anesthesia by inhalation agent isoflurane. After blood collection, the mice were euthanized. Whole blood was then centrifuged and separated into plasma and blood cell fraction. Liver and TA muscle were also harvested to check gene and protein expression after infection with rAdV vectors. Collected stool samples, plasma samples, and blood cell fractions were stored at -20°C, whereas liver and TA muscle samples were stored at -80°C till further analysis.

2.3-2. Chronic experiments

Initially, as pre-samples, 50-100 µl of whole blood was collected from mice tail tip cutting 2-3 mm under general anesthesia by isoflurane. And then, mice were injected the rAdV vectors of same amounts by same method. After 24 h of injection, whole blood was again collected for next 15 days, total was eight times, on every other day by same methods. Collected whole blood was stored at -20°C till further analysis.

2.4. Confirmation of gene and protein expression by infection of rAdV vector in vivo

For acute experiments, total RNA and protein were extracted from isolated liver and TA muscle tissues as follows. Sepasol-RNA I Super G (Nacalai Tesque) was used for total RNA extraction, according to the manufacturer's instructions. Using 500 ng of total RNA and PrimeScrip RT Master Mix (Takara Bio), reverse transcription was carried out to synthesize cDNA. The cDNA synthesized was diluted 10-fold using nuclease-free water. Subsequently, qPCR was performed to confirm mCherry expression in different tissues with duplicate measurements using KAPA SYBR Fast qPCR kit (NIPPON Genetics) for 18S ribosomal RNA and PrimeTime Gene Expression Master Mix (Integrated DNA Technologies) for mCherry, normalized to 18S ribosomal RNA expression with delta CT calculations. Primer sequences are given in table 2. To extract total protein, the tissues were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA] with added protease inhibitor cocktail (Nacalai Tesque) and subjected to Western blotting using 10 µg protein sample. After protein transfer, the membrane was incubated in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) containing 5% BSA for blocking and subsequently in anti-mCherry antibody (PM005; MBL) overnight at 4°C with gentle shaking. Next day, after thorough washes, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase for 1 h with gentle shaking. Finally, the protein bands of mCherry were visualized with ECL Select Western Blotting Detection Reagent (GE Healthcare) using LAS-4000 software (GE Healthcare). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control (C65; Santa Cruz Biotechnology).

2.5. DNA extraction and detection of transgene using three different PCR methods

For acute experiments, total DNA was extracted from collected stool, plasma, and blood cell fractions. NucleoSpin Plasma XS (Takara Bio) kit was used to isolate plasma-cell free DNA (cfDNA) from 240 µl of plasma. NucleoSpin Blood (Takara Bio) kit was used to isolate DNA from 200 µl of blood cell fraction. phenol/chloroform/isoamyl alcohol solution (Nacalai Tesque) was used to isolate DNA from stool of one piece. Concentration of total extracted DNA was measured and final concentration was adjusted to 50 ng/µl for stool- and blood cell fraction-DNA. Since plasma cfDNA had very low concentration, it was used as an undiluted solution. For chronic experiments, total DNA was extracted from 50-100 µl of whole blood using NucleoSpin Blood and its final concentration was adjusted to 50 ng/µl.

Using DNA samples of acute experiments, Semi-quantitative PCR (sqPCR), Real-time quantitative PCR (qPCR) and Droplet-digital PCR (ddPCR) were performed to detect transgene fragments. For DNA samples of chronic experiments, only ddPCR was performed. All primer sequences used in these PCR methods are given in Table 2.

2.6. Semi-quantitative PCR (sqPCR)

Kod Plus (TOYOBO) reagent was used to perform sqPCR. Template volume and primer concentrations were 1 µl and 300 nM, respectively, for a total reaction volume of 10 µl per sample. The conditions maintained in the thermal cycler were 94°C -2 min, 98°C -10 s/60°C -30 s/68°C -30 s for 35 cycles, 4°C -infinite hold. The amplicons were subjected to electrophoresis and visualized using ethidium bromide in LAS-4000 transilluminator (GE Healthcare).

2.7. Real-time quantitative PCR (qPCR)

PrimeTime Gene Expression Master Mix (Integrated DNA Technologies) reagent was used to perform qPCR. Template volume and primer and probe concentrations were 2 µl, 500 nM and 250 nM, respectively for a total reaction volume of 10 µl per sample. pcDNA3.1-Peredox-mCherry plasmids (10 pg/µl) was used as a standard DNA to perform absolute quantification. The conditions maintained in the thermal cycler were 95°C -3 min, 95°C -3 s/60°C -30 s for 35 cycles. Melting curve was analyzed on QuantStudio 5 Real-Time PCR Systems (Thermo Fisher Scientific). All samples were measured in duplicate and R2 of standard curve was equal to 0.98.

254
255 2.8. Droplet-digital PCR (ddPCR)
256 ddPCR Supermix for Probes and Droplet Generator oil (Bio Rad) were used to form droplets.
257 Template volume and primer and probe concentration were 1 µl, 500 nM and 250 nM, respectively
258 for a total reaction volume of 20 µl per sample. Droplets were formed by automated droplet generator
259 (Bio-Rad). The conditions maintained in the thermal cycler were 95°C -10 min, 94°C -30 s/60°C -1 min
260 for 40 cycles, 4°C -5 min, 90°C -5 min, 4°C -infinite hold. The droplets were analyzed PCR-positive or
261 PCR-negative by QX200 Droplet Digital PCR System (Bio Rad). DNA samples from chronic
262 experiments were also subjected to ddPCR using a similar method. All samples were measured in
263 duplicate.
264
265 **Table 2.** Primer sequences used in this study

Methods	Targets		Sequences	Predicted size (bp)
sqPCR	mCherry gene body	Forward	CACGAGTTCGAGATCGAGGG	234
		Reverse	GCCGTCCTCGAAGTTCATCA	
sqPCR	CMV promoter	Forward	CACGCCCATTGATGTACTGC	247
		Reverse	ACGCCAATAGGGACTTTCCA	
qPCR, ddPCR: Taq man probe assay	mCherry gene body	Forward	GGCACCAACTTCCCCTCC	115
		Probe	56FAM/CATGGTCTT/ZEN/CTTC TGCAT/3IABkFQ	
		Reverse	TCTGCTTGATCTCGCCCTTC	
qPCR: SYBR green assay	18s rRNA	Forward	AGTCCCTGCCCTTTGTACACA	70
		Reverse	CGATCCGAGGGCCTCACTA	

266
267
268
269 2.9. Statistics
270 Bar graph shows average ± SD and plots of individual values. The tables show individual
271 absolute values and median. Bar graph and table data were subjected to Kruskal-Wallis H test (one-
272 way ANOVA of ranks) followed by two-stage Benjamini, Krieger, & Yekutieli FDR procedure as a
273 post-hoc test using GraphPad Prism ver. 7.04. P value less than 0.05 was considered to be statistically
274 significant

275 **3. Results**

276 3.1. mCherry gene and protein were sufficiently expressed both in vitro and in vivo

277 The red fluorescent signal of mCherry protein after infection with rAdV vectors was confirmed
278 in HEK 293A cells (Figure 2A). In acute experiments, RNA and protein expression of mCherry were
279 also confirmed in liver and TA muscle in mice infected with rAdV (Figure 2B, C).

280

281 3.2. The three PCR methods showed each characteristic and could detect transgene fragments in
282 acute experiments

283 In sqPCR, transgene fragments were detected with strong signals for blood cell fraction-DNA
284 and very weak signals for other DNA samples which could not be clearly distinguished into
285 positive or negative signals. (Figure 3)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 April 2019 doi:10.20944/preprints201904.0285.v1

Peer-reviewed version available at Genes 2019, 10, 436; doi:10.3390/genes10060436

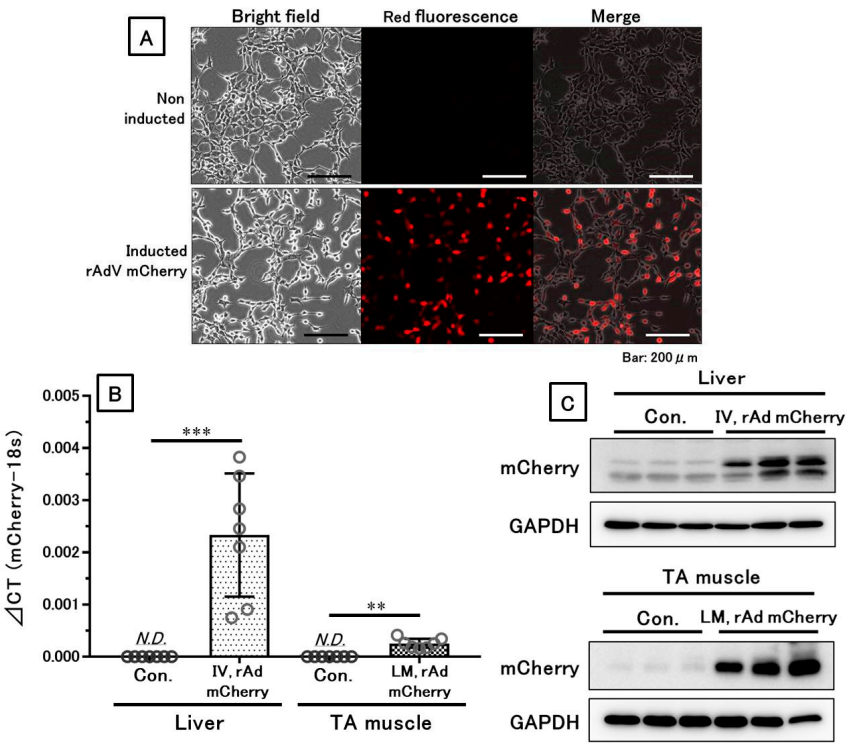

Figure 2. Confirmed gene and protein expression by the rAdV vector. RNA and functional protein were sufficiently expressed both in vitro and in vivo. (A) Functional protein expression of mCherry in HEK 293A cells. (B) Gene expression of mCherry in liver or tibialis anterior (TA) muscle detected by qPCR. (C) Protein expression of mCherry in liver or TA muscle shown by western blotting of representative samples. IV means intravenous injection and LM means local muscular injection of the rAdV vectors in the TA muscle. **: p<0.01, ***: p<0.001

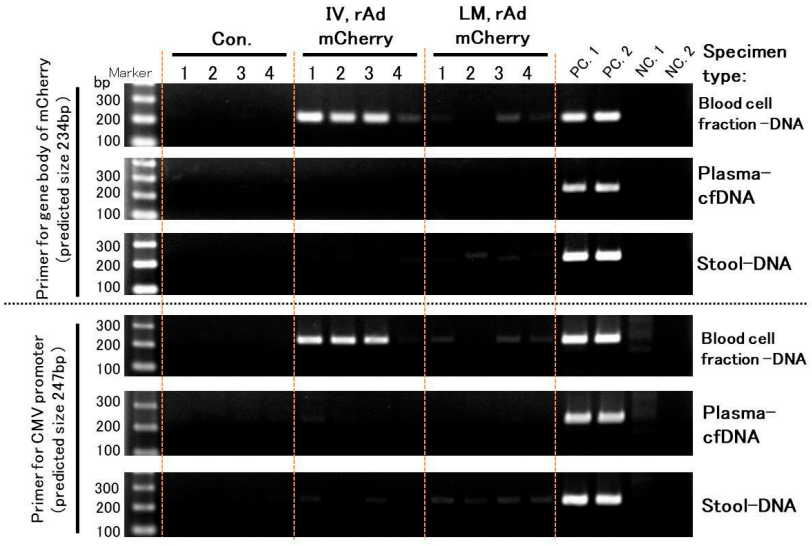

Figure 3. Detection of transgene fragments in reprehensive samples on sqPCR. Blood cell fraction-DNA samples show strong signal, but others show very weak signals. IV means intravenous injection, and LM means local muscular injection of the rAdV vectors in the tibialis anterior (TA) muscle. PC. 1: Positive control of rAdV DNA containing mCherry gene (10 pg/μl). PC. 2: Positive control of liver DNA containing mCherry gene with rAdV induction (10 ng/μl). NC. 1: negative control of mouse till DNA (50 ng/μl). NC. 2: Distilled water (DW) as negative control.

In the qPCR using the TaqMan probe, transgene fragments were detected in all specimens in the IV group with strong signals from the blood cell fraction-DNA. However, small amounts of transgene fragments were observed in the blood cell fraction DNA but no fragments were detected in plasma and stool-DNA of the LM group. Additionally, variations between individual values were very large (Table 3).

Table 3. Detection of transgene fragments on qPCR

qPCR Group	Mouse No.	Copy/μl of transgene: Blood cell fraction–DNA	Plasma–cfDNA	Stool–DNA
Con.	1	0.0	0.0	0.0
	2	0.0	0.0	0.0
	3	0.0	0.0	0.0
	4	0.0	0.0	0.0
	5	0.0	0.0	0.0
	6	0.0	0.0	0.0
	Median	*0.0*	*0.0*	*0.0*
IV, rAdV mCherry	1	2528.2	33.4	3.7
	2	390.3	3.3	0.0
	3	1808.4	8.5	1.6
	4	217.9	5.1	0.0
	5	230.4	4.9	0.9
	6	26.2	5.5	0.8
	7	63.1	4.0	2.6
	Median	*230.4*[a, b]	*5.1*[a, b]	*0.9*[a, b]
LM, rAdV mCherry	1	56.2	0.0	1.8
	2	11.8	0.0	0.0
	3	16.5	0.0	0.0
	4	30.7	0.0	0.0
	5	15.9	0.0	0.0
	6	31.7	0.0	0.0
	7	28.3	0.0	0.0
	Median	*28.3*[c]	*0.0*	*0.0*

A high amount of transgene fragments was detected in the blood cell fraction-DNA in the IV group. IV means intravenous injection and LM means local muscular injection of the rAdV in the tibialis anterior (TA) muscle. a: $p<0.01$ vs Con. within same specimens. b: $p<0.05$ vs Local injection within same specimens. c: $p<0.05$ vs Con. within same specimens.

For ddPCR, transgene fragments were detected in all specimens in the IV group, with strong signals for DNA from the blood cell fraction. However, in the LM group, small amounts of transgene fragments were observed in the blood cell fraction-DNA and significantly lower amounts were found in stool-DNA; fragments were not detected in plasma cfDNA and stool-DNA. Additionally, variations between individual values were also very large (Figure 4, Table 4).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 25 April 2019 doi:10.20944/preprints201904.0285.v1

Peer-reviewed version available at Genes 2019, 10, 436; doi:10.3390/genes10060436

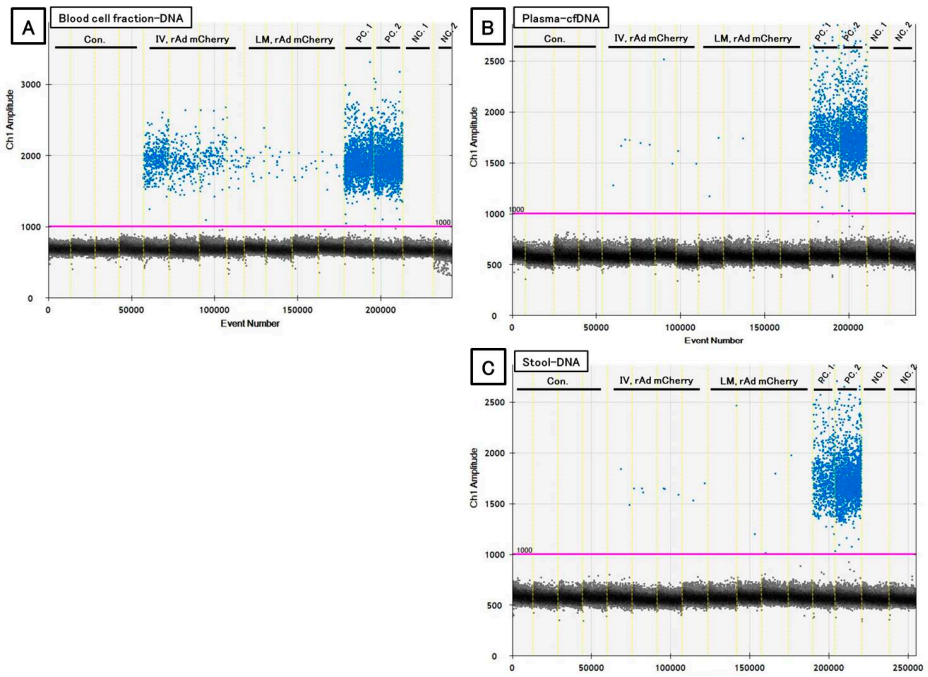

Figure 4. Representative 1-D plot data showing detected transgene in each specimen by ddPCR reactions. This data shows representative samples. (A) Blood cell fraction-DNA. (B) Plasma-cfDNA. (C) Stool-DNA. The blue plots denote positive and black denote negative existence of transgene fragments. IV means intravenous injection and LM means local muscular injection of the rAdV in the tibialis anterior (TA) muscle. PC. 1: Positive control of rAdV DNA containing mCherry gene (10 pg/μl). PC. 2: Positive control of liver DNA with induced rAdV containing mCherry gene (10 ng/μl). NC. 1: negative control of mouse till DNA (50 ng/μl). NC. 2: Distilled water (DW) as negative control.

Table 4. Detection of transgene fragments on ddPCR.

ddPCR Group	Mouse No.	Copy/μl of transgene: Blood cell fraction-DNA	Plasma-cfDNA	Stool-DNA
Con.	1	0.0	0.0	0.0
	2	0.0	0.0	0.0
	3	0.0	0.8	0.7
	4	0.0	0.0	0.0
	5	0.6	0.0	0.0
	6	0.0	0.8	0.0
	Median	*0.0*	*0.0*	*0.0*
IV, rAdV mCherry	1	4460.0	19.2	2.3
	2	620.7	0.7	2.2
	3	2873.3	6.0	5.5
	4	276.7	4.3	0.0
	5	190.0	2.3	0.0
	6	13.7	1.9	0.8
	7	42.7	2.5	1.5
	Median	*276.7[a, b]*	*2.5[a, b]*	*1.5[a]*
LM, rAdV mCherry	1	34.0	0.0	0.7
	2	5.5	0.0	0.0
	3	4.5	3.0	0.7
	4	12.9	1.4	0.0
	5	3.8	0.0	0.7
	6	16.5	0.0	1.4
	7	11.5	0.0	1.5
	Median	*11.5[c]*	*0.0*	*0.7[d]*

Transgene fragments were detected in blood cell fraction-DNA on IV group. IV means intravenous injection and LM means local muscular injection of the rAdV in tibialis anterior (TA) muscle. a: $p<0.01$

vs Con. within same specimens.b: p<0.05 vs Local injection within same specimens. c: p<0.05 vs Con. within same specimens. d: p=0.089 vs Con. within same specimens.

3.3. ddPCR on chronic experiments showed possibility to detect transgenes repeatedly

Using ddPCR for whole blood DAN in chronic experiments, transgene fragments could be detected. The transgene fragments mainly existed between one and three days, especially in the IV group, but decreased sharply after three days. The fragments could be detected approximately for seven days in the IV group and for five days in the LM group (Figure 5, Table 5). Additionally, variations between individual values were very large.

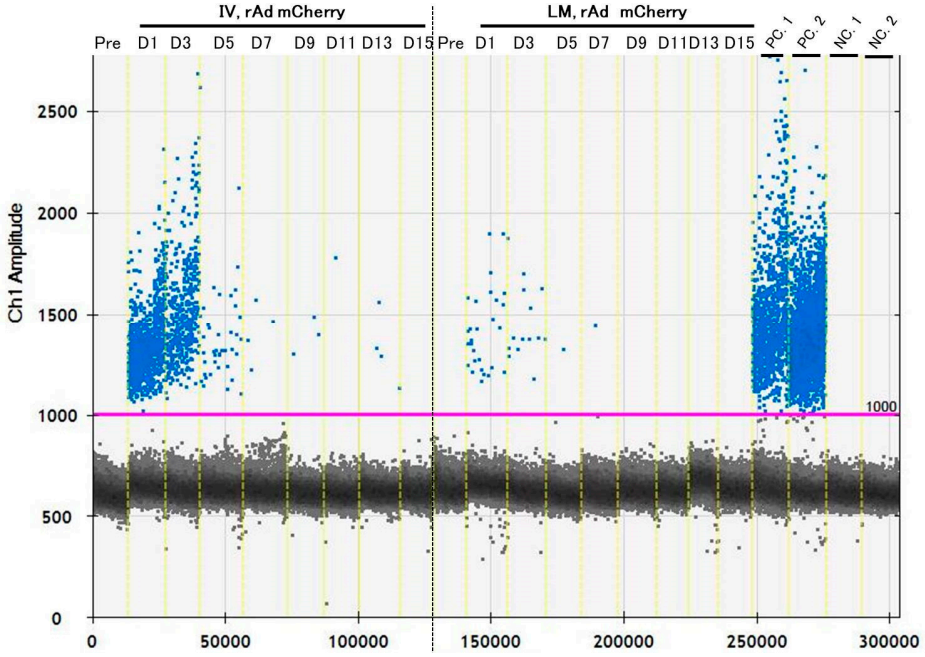

Figure 5. 1-D plot data detected transgene fragments on ddPCR reactions until 15 day from injection of the rAdV vectors. These plots represent blood cell fraction-DNA pooled each day. Transgene fragments could be detected repeatedly. Especially, detection of fragments was higher on day 1 (D1) and 2 (D2). The blue plots denote positive and black denote negative presence of transgene fragments. IV means intravenous injection and LM means local muscular injection of the rAdV in tibialis anterior (TA) muscle. PC. 1: Positive control of rAdV DNA containing mCherry gene (10 pg/μl). PC. 2: Positive control of liver DNA containing mCherry gene with induced rAdV (10 ng/μl). NC. 1: negative control of mouse till DNA (50 ng/μl). NC. 2: Distilled water (DW) as negative control.

355 **Table 5.** Repeated detection of transgene fragments on ddPCR

		Copy/μl of transgene								
	Mouse no.	Pre	1 day	3 days	5 days	7 days	9 days	11 days	13 days	15 days
IV, rAdV mCherry	1	0.0	817.0	1398.0	48.0	4.3	1.4	0.0	0.8	0.0
	2	0.0	890.0	209.0	25.0	2.4	0.0	0.7	3.4	0.7
	3	0.8	1108.0	796.0	10.3	0.9	0.0	0.0	10.4	0.0
	4	2.0	1422.0	298.0	13.7	1.4	2.7	1.7	0.0	0.0
	5	0.7	1900.0	1132.0	62.0	10.5	1.4	1.6	1.5	0.8
	6	0.0	4800.0	1261.0	39.0	7.1	2.0	0.7	0.9	1.5
	Median	*0.4*	*1265.0*	*964.0*	*32.0*	*3.4*	*1.4*	*0.7*	*1.2*	*0.4*
	p− values vs Pre		*0.0002*	*0.0004*	*0.0063*	*0.0911*	*0.4181*	*0.5886*	*0.2992*	*0.6177*
LM, rAdV mCherry	1	0.0	70.0	66.0	7.3	0.7	0.8	0.0	0.0	0.0
	2	0.9	149.0	24.0	5.0	1.6	0.7	0.0	0.0	0.0
	3	0.0	19.0	7.2	4.2	0.7	0.0	0.0	0.0	0.0
	4	0.0	10.4	4.6	0.8	0.8	0.7	0.0	0.0	0.0
	5	0.0	8.6	9.0	0.0	0.8	0.8	0.0	1.5	0.0
	6	0.0	5.2	27.0	4.1	0.0	0.7	0.0	0.0	0.0
	Median	*0.0*	*14.7*	*16.5*	*4.2*	*0.8*	*0.7*	*0.0*	*0.0*	*0.0*
	p− values vs Pre		*0.0012*	*0.0012*	*0.0666*	*0.2576*	*0.2902*	*0.5904*	*0.7753*	*0.5907*

356

357 Transgene fragments could be detected consistently after one and three days post-transfection,
358 especially in the IV group, but decreased sharply after three days with elapsed time. IV means
359 intravenous injection and LM means local muscular injection of the rAdV in tibialis anterior (TA)
360 muscle.

361

362 **4. Discussion**

363 We tried to develop a detection method for transgenes delivered by rAdV vectors in gene
364 delivery mouse model. We successfully detected transgenes containing mCherry gene in blood
365 fraction-DNA, plasma cfDNA, stool-DNA, and whole blood-DNA using sqPCR, qPCR and ddPCR.
366 Both IV and LM groups showed gene expression and functional protein expression in liver tissue and
367 TA muscle. Therefore, our model mimics gene delivery model for liver or local muscle, which can
368 potentially be used in gene doping. However, the model is not a true representative of gene doping,
369 since the aim was not to enhance muscle endurance or power of mice. Nevertheless, this model
370 resembles gene doping in terms of rAdV vectors since functional protein was successfully expressed
371 in liver or local muscle tissue. Hence, it is believed that this model can be useful for developing novel
372 detection methods for transgene fragments in gene doping.

373 After comparing combinations of three different PCR methods and three different specimens, it
374 was observed that a combination of ddPCR and blood cell fraction-DNA was highly sensitive for
375 detection of transgene. However, this method has a limitation of being invasive. Moreover, we
376 detected transgene fragments in stool-DNA samples as well, although in low amounts. Therefore,
377 there is possibility that non-invasive sampling for gene doping could be achieved by using stool-
378 DNA.

379 We observed that transgene fragments predominantly exist in blood fraction. Therefore, it is
380 recommended to use blood cell fraction for examination of gene doping by AdV vectors.
381 Additionally, it is also reported that rAdV vectors are recognized by neutrophils, monocytes or
382 macrophages or are attached to red blood cells. To solve these questions, further experiments, such
383 as sorting of different types of cells using flow cytometry are needed.

384 We hypothesized that the concept of liquid biopsy for diagnosis or monitoring of cancer, that is
385 to analyze specific DNA fragments in cancer cells using plasma-cfDNA, would be useful for the

detection of transgene fragments. Therefore, initially in this study, we tried to detect the transgene fragment using plasma-cfDNA in acute experiments using the concept of liquid biopsy. However, the amount of fragments detected in plasma-cfDNA was much lower than that in blood cell fraction-DNA isolated from IV group. Additionally, no fragment was detected in LM group. We used relatively smaller mice in our experiments and only 240 μl of sample was used to extract cfDNA, resulting in low yield of DNA. Therefore, it is likely that if the amount of plasma used to extract cfDNA is increased the detection of transgene fragments could be more sensitive.

It has been reported that human candidate genes at a high risk of gene doping include EPO, insulin-like growth factor-1 (IGF-1), hypoxia inducible factor-1 (HIF-1), vascular endothelial growth factor-a (VEGF-A) and follistatin (FST) etc. [23, 24]. Gene doping may enhance their expression. Moreover, silencing the gene expression of myostatin by RNA interference (RNAi) technology may lead to a risk of artificially increased muscle mass [23, 24], since animal experiments have shown increase in muscle mass using naked plasmid-DNA expressed as a small hairpin RNA (shRNA) [16] or cholesterol-conjugated small interfering RNA (siRNA) [25]. Therefore, in near future, it is necessary to develop methods that can detect multiple different genes and different vectors simultaneously. In order to achieve this, a comprehensive analysis by Next Generation Sequencing (NGS) technology may be necessary.

Surprisingly, in ddPCR experiments, positive droplets were detected in DNA samples of rAdV-negative samples, although mice of the injected group and control group were completely separated. Additionally, ddPCR is believed to be highly precise and sensitive for quantification of absolute values according to Bio-Rad Laboratories, Inc. Therefore, there is a possibility of cross-contamination by transgenes of positive samples. Such cross-contamination must be absolutely avoided during examination of gene doping. Therefore, we have challenge to apply robot technology that resembles human moves for examination of gene doping. The robot named “Maholo,” developed by LabDroid (Robotic Biology Institute), demonstrates the concept that humanoid robots in laboratory can solve problems such as numerous labor-intensive tasks required in high-throughput research as well as the dangers (and costs) associated with experiments involving pathogens and harmful reagents [25]. Maholo can perform various tasks ranging from automating sample preparations including move of pipetting, moving samples, dispensing, opening and closing of μ tube, using centrifugation machine and aspirating medium etc. to measurement of number of transgene fragments in a clean room with low risk of artificial contaminations. Additionally, the robot can handle a large number of samples automatically. Currently, we have tested and established practicality and developed a computer system to apply testing for the detection of transgene fragments in our model mice by Maholo (Figure 6). This technology might be used in human application in future.

Figure 6. LabDroid; Maholo. We tried to apply robot technology to examine gene doping in our mouse model, aiming for human application.

5. Conclusions

In the present study, transgene fragments could directly be detected from blood cell fraction-DNA, plasma-cfDNA, and stool-DNA by qPCR and ddPCR methods in gene delivery mice model, depending on specimen type and injection methods. Additionally, it was observed that a combination of blood cell fraction-DNA and ddPCR is more sensitive than other combinations used in this model. These results could accelerate the establishment of examination methods for gene doping.

Author Contributions: T.S. conceived and designed the experiments; T.S., K.A. and K.Yanazawa. performed the experiments: T.S. and K.A. drafted the manuscript; T.S., K.A., K.Yanazawa., K.Tokinoya., N.S., H.U. and Y.Y. analyzed the data; K.W., T.N., T.T., K.Yamaguchi., Y.T., K.Takeuchi., Y.K. and S.S. provided critical comments and contributed to the discussion of the results; T.S., Y.Y. and K.Takekoshi. edited and revised the manuscript. All authors approved the final version of the manuscript.

Acknowledgments: This work was supported by the grant from promotional business of doping prevention activities, Japan Sports Agency (JSA). We express our gratitude to JSA.

Conflicts of Interest: This work was supported by the grant from JSA that is a non-profit organization in Japan. There were no conflicts of interest in this work.

References

1. Japan Anti-Doping Agency (JADA). *What is anti-doping?* March 12, 2019. Available from: https://www.playtruejapan.org/about/.
2. The World Anti-Doping Agency (WADA). *Who We Are?* March 12, 2019. Available from: https://www.wada-ama.org/en/who-we-are.
3. The World Anti-Doping Agency (WADA). Anti-doping textbook. Montreal, Quebec, Canada: WADA; 2016.
4. The World Anti-Doping Agency (WADA). World anti-doping code with international standard, prohibited list. Montreal, Quebec, Canada: WADA; 2018.
5. Duan D. Systemic AAV Micro-dystrophin Gene Therapy for Duchenne Muscular Dystrophy. *Mol Ther*. 2018;26(10):2337-2356. doi: 10.1016/j.ymthe.2018.07.011
6. High KA, Anguela XM. Adeno-associated viral vectors for the treatment of hemophilia. *Hum Mol Genet*. 2016;25(R1):R36-R41. doi: 10.1093/hmg/ddv475
7. Naso MF, Tomkowicz B, Perry WL, Strohl WR. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs*. 2017;31(4):317-334. doi: 10.1007/s40259-017-0234-5
8. Kumaran N, Moore AT, Weleber RG, Michaelides M. Leber congenital amaurosis/early-onset severe retinal dystrophy: Clinical features, molecular genetics and therapeutic interventions. *Br J Ophthalmol*. 2017;101(9):1147-1154. doi:10.1136/bjophthalmol-2016-309975
9. Sharif W, Sharif Z. Leber's congenital amaurosis and the role of gene therapy in congenital retinal disorders. *Int J Ophthalmol*. 2017. doi: 10.18240/ijo.2017.03.24
10. Wold W, Toth K. Adenovirus Vectors for Gene Therapy, Vaccination and Cancer Gene Therapy. *Curr Gene Ther*. 2014;13(6):421-433. doi: 10.2174/1566523213666131125095046
11. Lee CS, Bishop ES, Zhang R, et al. Adenovirus-mediated gene delivery: Potential applications for gene and cell-based therapies in the new era of personalized medicine. *Genes Dis*. 2017;4(2):43-63. doi: 10.1016/j.gendis.2017.04.001
12. Xia Y, Du Z, Wang X, et al. Treatment of Uterine Sarcoma with rAd-p53 (Gendicine) Followed by Chemotherapy: Clinical Study of TP53 Gene Therapy. *Hum Gene Ther*. 2018;29(2):242-250. doi: 10.1089/hum.2017.206
13. Xu X, Lin H, Li X, et al. The First Approved Gene Therapy Product for Cancer Ad- p53 (Gendicine): 12 Years in the Clinic. *Hum Gene Ther*. 2018;29(2):160-179. doi: 10.1089/hum.2017.218
14. Liang M. Oncorine, The World First Oncolytic Virus Medicine and its Update in China. *Curr Cancer Drug Targets*. 2018;18(2):29189159. doi: 10.2174/1568009618666171129221503
15. The Journal of Gene Medicine. Charts and tables, Vectors. http://www.abedia.com/wiley/vectors.php. Accessed March 11, 2019.

16. Takemasa T, Yakushiji N, Kikuchi DM, et al. Fundamental study of detection of muscle hypertrophy-oriented gene doping by myostatin knock down using RNA interference. *J Sport Sci Med*. 2012;11(2):294-303.
17. Tozaki T, Kikuchi M, Hirota K, et al. Digital PCR detection of plasmid DNA administered to the skeletal muscle of a microminipig: a model case study for gene doping detection. *BMC Res Notes*. 2018;11(1). doi: 10.1186/s13104-018-3815-6
18. Moser DA, Braga L, Raso A, Zacchigna S, Giacca M, Simon P. Transgene detection by digital droplet PCR. *PLoS One*. 2014;9(11). doi: 10.1371/journal.pone.0111781
19. Meng Q-L, Shen Y-W, Liu X-M, et al. Detection of exogenous gene doping of IGF-I by a real-time quantitative PCR assay. *Biotechnol Appl Biochem*. 2016;64(4):549-554. doi: 10.1002/bab.1518
20. Hung YP, Albeck JG, Tantama M, Yellen G. Imaging cytosolic NADH-NAD + redox state with a genetically encoded fluorescent biosensor. *Cell Metab*. 2011;14(4):545-554. doi: 10.1016/j.cmet.2011.08.012
21. Takeuchi Y, Yahagi N, Nakagawa Y, et al. In vivo promoter analysis on refeeding response of hepatic sterol regulatory element-binding protein-1c expression. *Biochem Biophys Res Commun*. 2007;363(2):329-335. doi: 10.1016/j.bbrc.2007.08.165
22. Takeuchi Y, Yahagi N, Aita Y, et al. KLF15 Enables Rapid Switching between Lipogenesis and Gluconeogenesis during Fasting. *Cell Rep*. 2016;16(9):2373-2386. doi: 10.1016/j.celrep.2016.07.069
23. Brzeziańska E, Domańska D, Jegier A. Gene doping in sport - Perspectives and risks. *Biol Sport*. 2014;31(4):251-259. doi: 10.5604/20831862.1120931
24. Neuberger EWI, Simon P. Gene and Cell Doping: The New Frontier - Beyond Myth or Reality. *Med Sport Sci*. 2017;62:91-106. doi:10.1159/000465456
25. Yachie N, Robotic Biology Consortium, Natsume T. Robotic crowd biology with Maholo LabDroids. Nat Biotechnol. 2017 Apr 11;35(4):310-312. doi: 10.1038/nbt.3758