Doctoral dissertation

Utilization of Taqman qPCR method for detecting gene doping by adenovirus vector

2020

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Table of Contents

This doctoral thesis was revised in two of the following original papers and added the research results up to the present. Study 1 and Study2 is published as the double first authors that contributes equally to this work.

1. Detection of Transgenes in Gene Delivery Model Mice by Adenoviral Vector Using ddPCR
   Takehito Sugasawa, Kai Aoki, Koichi Watanabe, Koki Yanazawa, Tohru Natsume, Tohru Takemasa, Kaori Yamaguchi, Yoshinori Takeuchi, Yuichi Aita, Naoya Yahagi, Yasuko Yoshida, Katsuyuki Tokinoya, Nanami Sekine, Kaoru Takeuchi, Haruna Ueda, Yasushi Kawakami, Satoshi Shimizu and Kazuhiro Takekoshi.
   Genes (Basel). 2019;10(6): 436. <Study 1>

2. The detection of trans gene fragments of hEPO in gene doping model mice by Taqman qPCR assay.
   Kai Aoki, Takehito Sugasawa, Kouki Yanazawa, Koichi Watanabe, Tohru Takemasa, Yoshinori Takeuchi, Yuichi Aita, Naoya Yahagi, Yasuko Yoshida, Tomoaki Kuji, Nanami Sekine, Kaoru Takeuchi, Haruna Ueda, Yasushi Kawakami, Kazuhiro Takekoshi.
   PeerJ. 2020; 8:e8595. <Study 2>

The following original paper is published as the double first authors that contributes equally to this work. This treated as a previous study or whole discussion section.

3. Detection of Multiple Transgene Fragments in a Mouse Model of Gene Doping Based on Plasmid Vector Using Taqman-qPCR Assay.
   Sugasawa T, Aoki K, Kouki Yanazawa and Kazuhiro Takekoshi.
   Genes (Basel). 2020;11(7):E750

I obtained written approvals as reference articles from the other first authors on this doctoral thesis.

List of Tables

List of Figures

Ⅰ. Introduction

Doping is an act of raising competitive abilities to achieve success by using substances or methods prohibited in sports [Japan Anti-Doping Agency (JADA). What Is Anti-Doping?]. 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 (hereafter the Code) [The World Anti-Doping Agency (WADA)] 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. The difference between gene therapy and gene doping for an example is shown in Figure 1. 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 [WADA. Anti-Doping Textbook]. 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" [WADA. World Anti-Doping Code with International Standard, Prohibited List]. However, there are no established standard methods for detecting or preventing gene doping to date. The difference between doping and gene doping, which may be particularly problematic, is shown in Figure 2.

In recent years, genetic engineering technology has rapidly advanced, resulting in the 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) [Duan et al. 2018], hemophilia B [High et al. 2016; Naso et al. 2017], and Leber congenital amaurosis (LCA) [Kumaran et al. 2017; Sharif et al. 2017], 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 [Wold et al. 2014; Lee et al. 2017; Xia et al. 2018]. In China, two rAdV vector-based gene therapy products, namely Gendicine (Shenzhen SiBiono GeneTech Co., Ltd., Shenzhen, China) [Xia et al. 2018; Zhang et al. 2018] and Oncorine (Sunway Biotech Co., Ltd., Taipei, Taiwan), 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 [Liang et al. 2018]. Additionally, rAdV vectors were the most commonly used vectors in approved clinical trials of gene therapy (541 cases, 18% of the total) worldwide until December 2018 [The Journal of Gene Medicine. Charts and Tables, Vectors.] (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, I focused on rAdV vectors, since rAdV vectors are the most commonly used in clinical trials (Table 1), and are also used as prescription drugs. Therefore, the aim of this doctoral dissertation was to establishe the detection methods for gene doping using rAdV vectors.

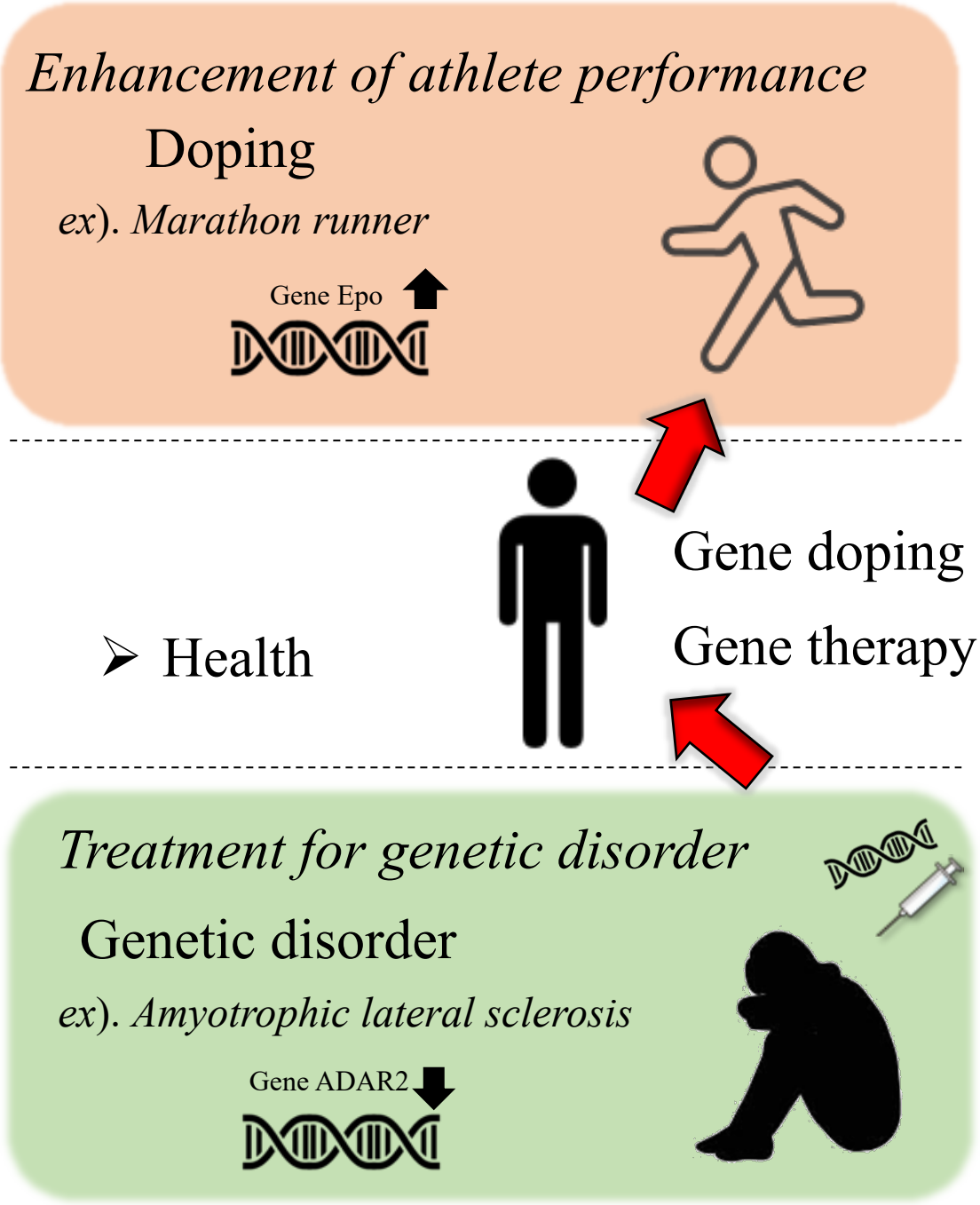

Figure 1. Gene therapy and gene doping

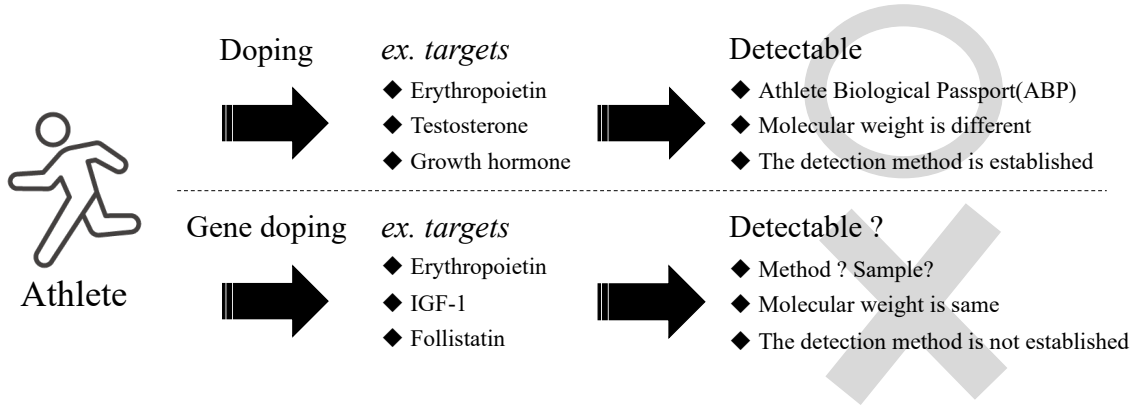

Figure 2. Problems with detection of gene doping

Table 1. Types and relative numbers of the 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

Ⅱ. Previous studies

1. Gene Doping

1) Doping and Anti-Doping

Doping generally refers to the use of prohibited drugs, substances, etc., or prohibited methods to improve athletic performance. Well-known banned drugs include anabolic hormones and growth factors such as testosterone, insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF). Other banned drugs include metabolic regulators, such as AMP-activated protein kinase (AMPK) activators and peroxisome proliferator-activated receptor δ (PPARδ) agonists, and hematopoietic factors, such as hypoxia-inducible factor (HIF) activating agents and erythropoietin (EPO) [WADA 2020]. These drugs have been banned to protect not only the health of athletes but also the fairness of non-doping athletes. Lenskyj has reported that athletes can escape the civil court if they suspect a doping breach; however, if an athlete tests positive for a banned substance, he/she will automatically be presumed guilty [Lenskyj 2018]. Therefore, the judgment on doping by the Court of Arbitration for Sport (CAS) is very strict.

The positive rate for doping investigated by WADA reportedly ranges between 1–2% per year; however, when examined in a detailed and accurate way, it was estimated to be 14–57% [de Hon et al. 2015; Elbe & Pitsch 2018; Ulrich et al. 2018], indicating that potentially a large number of doping cases are not identified.

2) Gene Doping and Gene Doping Targets

Gene therapy is generally aimed at delivering genetic material that encodes proteins necessary for treating diseases. The first gene therapeutic product was recommended for use in Europe [Coghlan 2012], which was followed by various clinical trials [Hu et al. 2017; Ito et al. 2019; Kanemura et al. 2008]. Gene therapy technology has advanced

rapidly in recent years.

Advances in gene therapy technology may pave the way for its use for non-therapeutic purposes. This is named as gene doping and WADA defines it as “non-therapeutic use of genes, genetic elements, and/or cells that have the ability to improve athletic performance.” Gene therapy produces proteins by delivering genetic material encoding them, to cells in the body. The protein expressed in this way may be indistinguishable from the endogenous protein encoded by the chromosomal gene. This is important to achieve the long-term production of proteins for disease treatment. However, the possibility of producing biologically active molecules, such as endogenous proteins, is also attractive to villainous athletes, as it prevents protein detection. Typical target genes include IGF-1, PPARδ, and EPO, which promote muscle assimilation [Harridge & Velloso 2009], regulate muscle fiber type [Wang et al. 2004], and promote hematopoietic signals [Jelkmann 2011], respectively.

3. Erythropoietin (EPO)

1) Characteristics

EPO is a glycoprotein produced by the kidneys that stimulates blood-forming organs, such as the bone marrow, by binding to EPO receptors and activating the Janus kinase (JAK) -2 and signal transducer and activator of transcription (STAT) -5 pathways, which further promote red blood cell production [Debeljak & Sytkowski 2012]. Endogenous EPO production is strongly regulated by hypoxia [Baker & Parise 2016; Jelkmann 2011]. Normally, GATA-2 and NF-κB suppress the transcriptional region of EPO, but when oxygen supply is reduced in the human body, transcription factors called HIFs are activated, and HIFα and HIFβ form a complex that activates EPO transcription [La Ferla et al. 2002].

2) EPO and Sports

Recombinant EPO was initially approved in the United States of America for patients with anemia and chronic kidney disease because of its physiological function. Since then, it has been approved as a therapeutic agent for anemia associated with chemotherapy in cancer patients and the preparation for autotransfusion before surgery. These treatments improve the quality of life by reducing the risk of retransfusion and iron overload [Bonomini et al. 2016]. On the contrary, EPO increases red blood cell production for treating anemia, thus increasing oxygen supply to the muscles and improving athlete performance [Haile et al. 2019]. EPO was first illicitly used in sports in the Tour de France, 1989 [Smeets 2009], and has been repeatedly used since then [Lage et al. 2002]. Following this, EPO use has been prohibited by the IOC since 1990. Currently, EPO is recorded in the WADA prohibited list [Birkeland et al. 2000; McGrath & Cowan 2008; WADA 2020].

Ⅲ. Aim

This study hypothesized that gene doping can be detected with PCR-based methods. Therefore, I examined the methods of gene doping detection using a gene doping mimicked model animal or gene doping model animals. In particular, I examined the optimal method and sample.

<Study 1>

“Detection of Transgenes in Gene Delivery Model Mice by Adenoviral Vector Using ddPCR”

The best method for detecting gene doping is unknown. In particular, how and what samples should be detected has not been established. Therefore, I investigated three types of PCR using a gene doping mimicked model.

<Study 2>

“Detection of Transgene Fragments of hEPO in Gene Doping Model Mice using the Taqman PCR Assay”

Study 1 suggested that ddPCR was the most sensitive detection method, and low blood volume was suitable for sample detection. However, since ddPCR requires a large running cost, I established a detection method using qPCR, which is useful for analyzing multiple samples accurately.

<Study 3>

“Establishment of Gene Doping Model Using Adenovirus Vector Containing Human Erythropoietin, and Its Detection”

Study 2 suggested that Taqman qPCR could detect target gene fragments from blood and

stool samples. Thus, I created a doping model instead of a mimicking model, and used a gene doping detection method to examine it.

This doctoral dissertation discusses the development of a gene doping detection method using above-mentioned studies.

Ⅳ. Study 1

Detection of Transgenes in Gene Delivery Model Mice by Adenoviral Vector Using ddPCR

1. Aim

This study hypothesized that gene doping can detect by PCR based detection methods from low invasive samples. This study was aimed to establish the gene doping mimicking model using cells and animals, and detection method using three types of PCR.

2. Materials and Methods

Cloning of Recombinant Adenoviral Vectors Containing the mCherry Gene

The following plasmids were used in this study: plasmid pcDNA3.1–Peredox–mCherry was a gift from Gary Yellen [Hung et al. 2011] (Addgene plasmid #32383; http : // n2t.net /addgene:32383; RRID: Addgene_32383); pENTR4 (Thermo Fisher Scientific,MA, USA）; and pAd/CMV/V5-DEST (Thermo Fisher Scientific). HEK 293A cells (Thermo Fisher Scientific) were used to clone and amplify recombinant adenoviral (rAdV) vectors. The mCherry gene, having restriction enzyme sites of 5′-EcoRI and 3′-NotI, was amplified by PCR with templated pcDNA3.1–Peredox–mCherry. It was then cloned into a pENTR4 plasmid between EcoRI and NotI sites by restriction enzyme digestion, followed by ligation with T4 ligase (Promega, WI, USA). The sequences of inserted mCherry genes in the pENTR4 plasmids were read using sanger sequencing and confirmed to be correct sequences. Using Gateway LR Clonase Enzyme mix (Thermo Fisher Scientific), and according to the manufacturer protocol, pENTR4 containing the 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 a pAd/CMV/V5-DEST plasmid, which can make rAdV type 5, containing the transgene. Subsequently, a pAd/CMV/V5-DEST plasmid

containing the mCherry gene was digested with Pac I restriction enzymes (New England Biolabs, MA, USA), 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, IL, USA) and antibiotics (Nacalai tesque, Kyoto, Japan) to synthesize and amplify rAdV vectors containing the 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. [Takeuchi et al. 2002; 2007]. The concentration of rAdV viral particles (VP) was measured on a spectrophotometer, according to the method as described previouslyof [Sweeney and Hennessey. 2002]. To confirm the expression of a functional mCherry protein, HEK 293A cells were seeded at a density of 2.5×10^5 cells per well in six-well plates and were cultured in DMEM containing 10% Fetal Clone III and antibiotics. After 24 h, the cells were infected with rAdV vectors (2.8×10^9 VP/mL of medium) to allow the expression of mCherry. After 24 h of induction, the red fluorescence of mCherry was analyzed using fluorescence microscope.

Ethics statement and Animals

Animal experiments in this study were approved by the Animal Experimental Committee, University of Tsukuba (approval numbers: 18–118 and 18–474). Six-week-old C57BL/6 male mice were purchased from the Central Laboratories for Experimental Animals (CLEA, Tokyo, Japan). The mice were allowed to grow until they reached 10 weeks old, with an average body weight of 26.1 g (SD = ±1.8 g). At this point, they were sacrificed for further experiments.

Experimental design

Single detection

The rAdV vectors containing the mCherry gene (2.1×10^{11} VP) were injected into left orbital veins (intravenous; IV group, n = 7) or local muscle (LM group, n = 7) of both the tibialis anterior (TA) muscles of mice under general anesthesia by inhalation agent isoflurane. When rAdV vectors were used intravenously (IV), most of the rAdV transgenes accumulated in the 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 the inferior vena cava using ethylenediaminetetraacetic acid (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℃, wheareas liver and TA muscle samples were stored at -80℃.

Chronological detection

Initially, as pre-samples, 50–100 μL of whole blood was collected from mice tail tip cuttings of 2–3 mm, under general anesthesia by isoflurane. Then, mice were injected with rAdV vectors of the same amounts and by same method as above. After 24 h of injection, whole blood was again collected for the next 15 days, a total of eight times on every other day by the same methods. The collected whole blood was stored at −20 °C until further analysis.

Confirmation of Gene and Protein Expression by Infection of rAdV Vectors In Vivo

For single detection, the total RNA and proteins 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, Shiga, Japan), reverse transcription was carried out to synthesize cDNA. The cDNA synthesized was diluted 10-fold using nuclease-free water. Subsequently, quantitative PCR (qPCR) was performed to confirm *mCherry* expression in different tissues with duplicate measurements, using a KAPA SYBR Fast qPCR kit (NIPPON Genetics, Tokyo, Japan) 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 the total protein, the tissues were homogenized in a lysis buffer （50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA）with an added protease inhibitor cocktail (Nacalai Tesque), and subjected to Western blotting using 10 μg protein sample. After the protein transfer, the membrane was incubated in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) containing 5% Bovine Serum Albumin（BSA） for blocking, and subsequently in anti-mCherry antibody (PM005; MBL) overnight at 4 °C with gentle shaking. The next day, after thorough washes, the membrane was incubated with a 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, TX, USA).

DNA Extraction and Detection of Transgenes Using Three Different PCR Methods

For single detection, total DNA was extracted from collected stool, plasma, and blood cell fractions. A NucleoSpin Plasma XS (Takara Bio) kit was used to isolate plasma cell-free DNA (cfDNA) from 240 μL of plasma. A NucleoSpin Blood (Takara Bio) kit was used to isolate DNA from 200 μL of blood cell fraction. A phenol/chloroform/isoamyl alcohol solution (Nacalai Tesque) was used to isolate DNA from the stool of one piece. The concentration of total extracted DNA was measured, and the final concentration was adjusted to 50 ng/μL for stool- and blood cell fraction DNA. Since plasma cfDNA had a very low concentration, it was used as an undiluted solution. For chronological detection, the 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 single detection, semi-quantitative PCR (sqPCR), real-time quantitative PCR (qPCR), and droplet-digital PCR (ddPCR) were performed to detect transgene fragments. For DNA samples of chronological detection, only ddPCR was performed. All primer sequences used in these PCR methods are given in Table 2.

Semi-Quantitative PCR (sqPCR)

Kod Plus (TOYOBO, Osaka, Japan) reagent was used to perform sqPCR. The 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 for 2 min; 98 °C for 10 s, 60 °C for 30 s and 68 °C for 30 s, for 35 cycles; and then 4 °C for infinite hold. The amplicons were subjected to electrophoresis and visualized using ethidium bromide in a LAS-4000 transilluminator (GE Healthcare).

Real-Time Quantitative PCR (qPCR)

PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, IA, USA) reagent was used to perform qPCR. The template volume, primer, and probe concentrations were 2 µL, 500 nM, and 250 nM, respectively, for a total reaction volume of 10 µL per sample. The pcDNA3.1–Peredox–mCherry plasmids (10 pg/µL) were used as standard DNA to perform absolute quantification. The conditions maintained in the thermal cycler were 95 °C for 3 min; and 95 °C for 3 s and 60 °C for 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 the coefficient of determination（R2） of the standard curve was equal to 0.98.

Droplet Digital PCR (ddPCR)

To form droplets, ddPCR Supermix for Probes and Droplet Generator oil (Bio Rad, CA, USA) were used. The template volume, primer, and probe concentration were 1 µL, 500 nM, and 250 nM, respectively, for a total reaction volume of 20 µL per sample. Droplets were formed by an automated droplet generator (Bio Rad). The conditions maintained in the thermal cycler were 95 °C for 10 min; 94 °C for 30 s and 60 °C for 1 min, for 40 cycles; 4 °C for 5 min; 90 °C for 5 min; and 4 °C -infinite hold. The droplets were analyzed as PCR-positive or PCR-negative by QX200 Droplet Digital PCR System (Bio Rad). DNA samples from chronic experiments were also subjected to ddPCR using a similar method. All samples were measured in duplicate.

Statistical analysis

The bar graph shows average ± SD and plots of individual values. The tables show individual absolute values and the median. The bar graph and table data were subjected to

a Kruskal–Wallis H test (one-way ANOVA of ranks), followed by a two-stage Benjamini, Krieger, and Yekutieli False Discovery Rate（FDR） procedure as a post-hoc test, using GraphPad Prism version 7.04. A p value less than 0.05 was considered to be statistically significant.

Table 2. Primer sequences used in Study1

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

3. Results

The mCherry Gene and Protein Were Sufficiently Expressed Both in Vitro and in Vivo

The red fluorescent signal of mCherry protein after infection with rAdV vectors was confirmed in HEK 293A cells (Figure 3A). In acute experiments, RNA and protein expression of mCherry were also confirmed in the liver and TA muscle in mice infected with rAdV (Figure 3B, C).

The Three PCR Methods Showed Each Characteristic and Could Detect Transgene Fragments in single detection

In sqPCR, transgene fragments were detected with strong signals for blood cell fraction DNA, as well as very weak signals for other DNA samples that could not be clearly distinguished into positive or negative signals (Figure 4).

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, while no fragments were detected in the plasma and stool DNA of the LM group. Additionally, variations between individual values were very large (Table 3).

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 5, Table 4).

ddPCR on Chronic Experiments Showed a Possibility of Detecting Transgenes Repeatedly

From the results of the single detection, a combination method of ddPCR and blood cell fraction DNA had the highest sensitivity for detecting transgene fragments, and it was hypothesized that performing ddPCR with whole blood DNA is useful for detecting transgene fragments over several days. Therefore, we performed the combination method in the chronological detection. In the results, 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 for approximately seven days in the IV group, and for five days in the LM group (Figure 6, Table 5). Additionally, variations between individual values were very large.

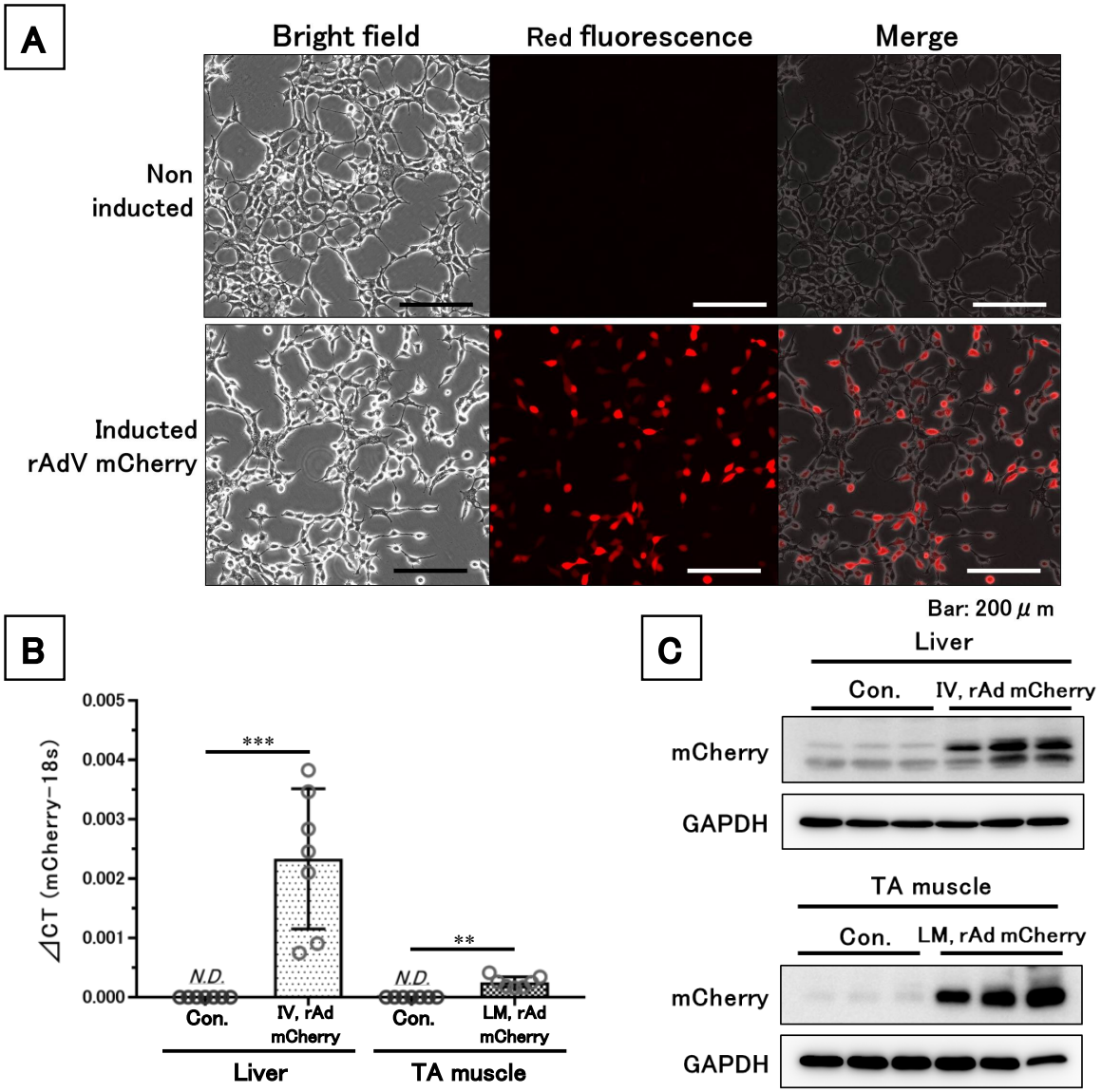

Figure 3. Confirmed gene and protein expression by the recombinant adenoviral (rAdV) vector. RNA and functional proteins were sufficiently expressed both in vitro and in vivo. (**A**) Functional protein expression of mCherry in HEK 293A cells. (**B**) Gene expression of *mCherry* in the liver or tibialis anterior (TA) muscle, detected by qPCR. (**C**) Protein expression of mCherry in the 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. **: $p < 0.01$, ***: $p < 0.001$.

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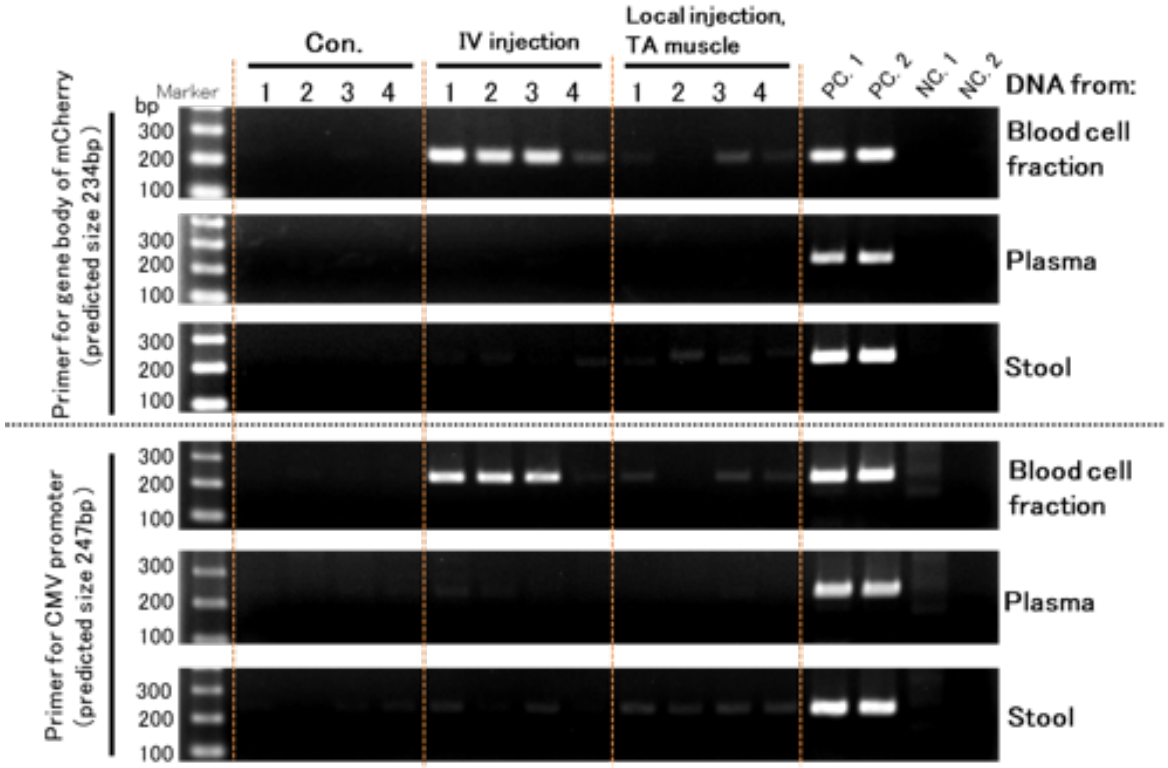

Figure 4. Detection of transgene fragments in representative samples using semi-quantitative PCR (sqPCR). Blood cell fraction DNA samples show strong signals, but other samples show very weak signals. IV means intravenous injection, and LM means local muscular injection of the rAdV vectors in the tibialis anterior (TA) muscle. PC. 1: positive control of rAdV DNA containing the mCherry gene (10 pg/μL). PC. 2: positive control of liver DNA containing the mCherry gene with a rAdV induction (10 ng/μL). NC. 1: negative control of mouse DNA (50 ng/μL). NC. 2: Distilled water (DW) as a negative control.

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Table 3. Detection of transgene fragments from qPCR.

		Copy/μl of transgene		
Group	**Mouse No.**	**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 number 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. control (con.) within same specimens. b: $p < 0.05$ vs. local injection within same specimens. c: $p < 0.05$ vs. con. within same specimens.

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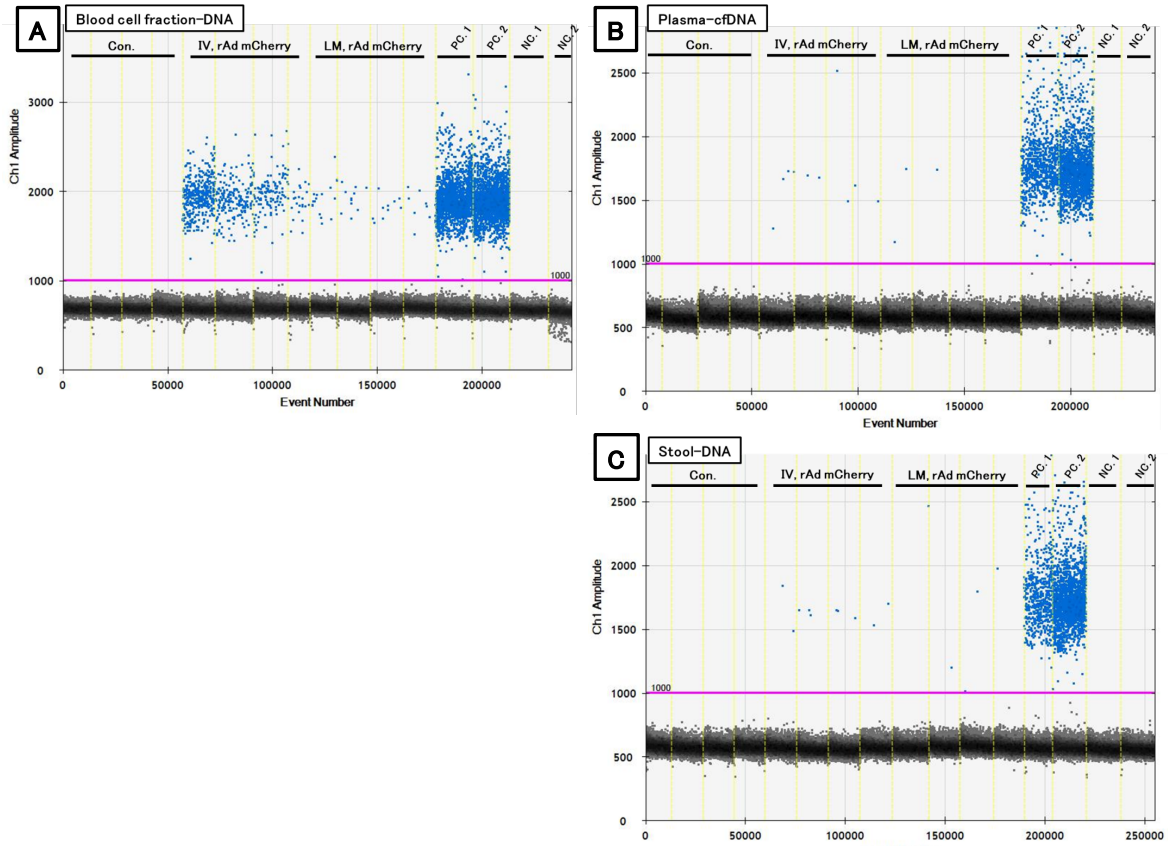

Figure 5. One-dimensional (1-D) plot data showing the detected transgene in each specimen by droplet digital PCR (ddPCR) reactions. (A) Blood cell fraction DNA. (B) Plasma plasma cell-free DNA (cfDNA). (C) Stool DNA. The blue plots denote positive and black ones denote the negative existence of transgene 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 the mCherry gene (10 pg/μL). PC. 2: positive control of liver DNA with induced rAdV containing the mCherry gene (10 ng/μL). NC. 1: negative control of mouse DNA (50 ng/μL). NC. 2: distilled water (DW) as a negative control.

Accepted by *Genes(Basel)*(Sugasawa and Aoki et al. 2019)

Table 4. Detection of transgene fragments from droplet digital PCR (ddPCR).

		Copy/μl of transgene		
Group	**Mouse No.**	**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]

A high number 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. control (con.) within same specimens. b: $p < 0.05$ vs. local injection within same specimens. c: $p < 0.05$ vs. con. within same specimens.

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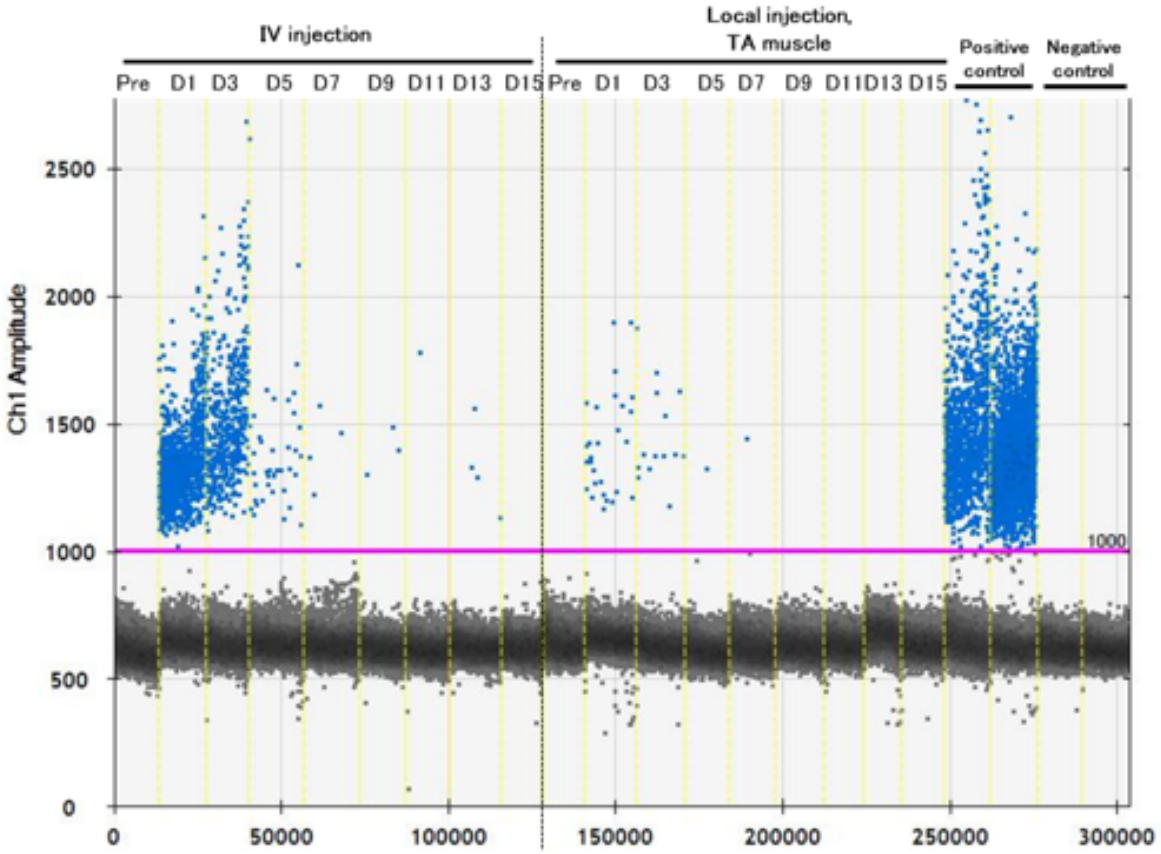

Figure 6. 1-D plot of data showing detected transgene fragments in ddPCR reactions until 15 day after injection of the rAdV vectors. These plots show a mean positive for the transgenes in whole blood DNA pooled from six mice each day. Transgene fragments could be detected repeatedly. In particular, the detection of fragments was higher on days 1 (D1) and 2 (D2). The blue plots denote a positive presence and black plots denote a negative presence 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 the mCherry gene (10 pg/μL). PC. 2: positive control of liver DNA containing the mCherry gene with induced rAdV (10 ng/μL). NC. 1: negative control of mouse DNA (50 ng/μL). NC. 2: distilled water (DW) as a negative control. Accepted by *Genes(Basel)*(Sugasawa and Aoki et al. 2019)

Table 5. Repeated detection of transgene fragments using 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***

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

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4. Discussion

Development of a gene delivery model using adenovirus vectors in cells and mice

First, I aimed to develop a gene delivery model using an adenovirus vector. To check whether the target gene, mCherry, was inserted into the adenoviruses, I transfected them into HEK293 cells and checked their fluorescence. The results confirmed red fluorescence in HEK293 cells 24 h after transfection (Figure 3A). To confirm whether gene and protein expression was normally induced in vivo, I confirmed gene and protein expression in the liver of group IV and tibialis anterior muscle of group LM in acute experiments. Gene and protein expression of mCherry was similarly induced in vivo (Figure 3B, C). Therefore, I believe that the gene delivery model using adenoviruses was developed successfully, and the model mimicked gene doping. In Study 1, I used this model as the gene doping model and performed the following experiments.

Detection of transgene fragments using three PCR methods

Useful samples for detecting gene doping may include urine and blood, which are currently also used for detecting drug doping. However, it would be difficult to distinguish between the proteins produced in the body during gene doping based on the differences in molecular weight or structure, unlike that during drug doping. In addition, a biopsy of the muscle where a recombinant gene is injected, or biopsy of the liver as the target secretory organ after intravenous adenovirus injection, is also thought to be virtually impossible in athletes due to the invasiveness of this procedure. Therefore, I hypothesized that liquid biopsy used for the diagnosis or monitoring of cancer—analyzing specific DNA fragments in cancer cells using plasma cfDNA [Adriana et al. 2020]—may be useful for detecting the transgene fragments. Accordingly, in this study, I attempted to detect the transgene fragments using plasma cfDNA in single detection, using liquid biopsy. However, the

number of fragments detected in the plasma cfDNA was significantly lower than that in the blood cell fraction DNA isolated from group IV. In addition, I did not detect any fragments from the blood cell fraction DNA isolated from group LM (Figure 4). I used small mice in this study and 240 μL sample to extract cfDNA, which resulted in a low DNA yield. Therefore, it is likely that the transgene fragments would be detected with high sensitivity if the amount of plasma used to extract cfDNA is increased. However, sqPCR has a low sensitivity for detecting transgene fragments; qPCR is the most common PCR method. Despite the failure of sqPCR to detect recombinant genes in cfDNA and stool, qPCR detected them in group IV. However, similar to sqPCR, the LM group failed to detect the transgene from cfDNA and stool (Table 3). On the contrary, ddPCR could detect transgenes in all the samples in group IV, while the LM group was also able to detect significantly in all samples except cfDNA (Table 4, Figure 5). Some of the samples were also detectable in cfDNA, although the difference was not significant. Therefore, I used ddPCR for chronological detection, as it was the most sensitive among the three types of PCR.

In chronological detection, group IV was able to detect significantly until 5 days, and some individuals were able to detect until 7 days, although the difference was not significant. On the contrary, in the LM group, the detection was significant until 3 days, and some individuals were detected until 5 days (Table 5, Figure 6). A recent study reported that ddPCR can detect the hEPO plasmid gene until 3 weeks using microminipig [Tozaki et al. 2018]. This difference in the detection period may be due to differences in the animal species used, transgene dosage regime, and dosage. Surprisingly, in ddPCR experiments, positive droplets were detected in the DNA of rAdV-negative samples, although mice in the injected and control groups were completely separated. Additionally, ddPCR is believed to be highly precise and sensitive for the quantification of absolute values, according to Bio-Rad Laboratories, Inc. Therefore, there is a possibility of

cross-contamination by transgenes present in positive samples. Such cross-contamination must be avoided during the examination of gene doping.

My findings were that ddPCR had the highest detection sensitivity, followed by qPCR and sqPCR. In particular, with regard to its application to doping detection, it was impossible to use sqPCR owing to its low sensitivity. On the contrary, the sensitivity of gene doping detection from whole blood was significantly high. Therefore, I believe that using ddPCR on whole blood is the most sensitive method to detect gene doping. However, ddPCR is costly and time-consuming despite having the highest sensitivity. Currently, ddPCR does not apply to doping tests that require multiple samples; therefore, in Studies 2 and 3, I proceeded with the qPCR-based analysis.

5. Summary

In Study 1, I detected gene doping in a minimally invasive blood sample. In particular, detection using ddPCR was highly sensitive, but qPCR also detected it.

Ⅴ. Study 2

The detection of trans gene fragments of hEPO in gene doping model mice by Taqman PCR assay.

1. Aim

This study hypothesized that PCR-based detection methods, in particular Taqman qPCR, could detect gene fragments of hEPO. This study was aimed to examin the accurancy of Taqman qPCR using plasmid gene doping model mice.

2. Materials and Methods

Animal

Animal experiments in this study were approved by the Animal Experiment Committee, University of Tsukuba (approval numbers: 19-163 and 19-425). Four-week-old C57BL/6 male mice, weighing 10.3–13.9 g, were purchased from the Central Laboratories for Experimental Animals (Tokyo, Japan). The mice were bred and maintained in an air-conditioned animal house under specific pathogen-free conditions and subjected to 12/12-h light and dark cycles. The mice were fed standard mice pellet and water ad libitum. When the mice were 5 weeks of age, 50–100 μL whole blood was collected from the tail tip from an approximately 1 mm cut into a microtube containing EDTA 2Na while the mice were under inhalation-general anesthesia by isoflurane. One piece of stool was also collected from the mice. The mice were returned to normal breeding after blood collection. Blood and stool samples were stored at −20℃ until further analysis. After 3 weeks, when the mice were aged 8 weeks, plasmids containing hEPO gene were injected as described below.

Naked plasmids injection and collection of samples (blood and stool)

Plasmid of pcDNA3.1(-) containing hEPO gene without introns derived from cytomegalovirus-promoter (phEPO) was purchased from GenScript Biotech Corp. (Nanjing, China). This plasmid was injected into the mice by intravenous (IV), intraperitoneal (IP), or local muscular (LM) injection. A total of 50 μg plasmid was injected into each mouse (volume; 50 μL, concentration; 1,000 ng/μL). Approximately 50-μL blood samples were collected at 1, 2, 3, 6, 12, 24, and 48 h after injection and 1 piece of stool sample was collected at 6, 12, 24, and 48 h after injection.

DNA extraction and sample preparation for qPCR assays

Total DNA was extracted from collected whole blood and the piece of stool. A NucleoSpin Blood kit (Takara Bio, Shiga, Japan) was used to isolate DNA from 50–100 μL of whole-blood. A phenol/chloroform/isoamyl alcohol solution (Nacalai Tesque, Kyoto, Japan) was used to isolate DNA from the stool sample. The concentration of total extracted DNA was measured with a NanoDrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), and the final concentration was adjusted to 10 ng/μL using elution buffer or distilled water (DW) for whole blood and stool-DNA. As a negative control, only elution buffer or DW was prepared.

Primer design

To detect transgene fragment by phEPO, forward and reverse primers for the hEPO gene for both Taqman qPCR and SYBR Green qPCR assays were designed including exon-exon junctions within exon 2–4 to prevent amplification of genomic DNA from both mouse and human and ensure specificity for phEPO for in silico PCR analysis. For Taqman qPCR, primers were designed to include double quenching systems. The primers and probe were

synthesized by Integrated DNA Technologies (Coralville, IA, USA) and the sequences are shown at Table 6.

Real-time quantitative PCR (qPCR) for trans-gene fragments

PrimeTime Gene Expression Master Mix (Integrated DNA Technologies) reagent was used to perform Taqman qPCR. The template volume, primer pairs, and probe concentrations were 2 μL, 100 nM, and 50 nM, respectively, for a total reaction volume of 10 μL per sample. KAPA SYBR® FAST qPCR Master Mix (KK 4602, Kapa Biosystems, Wilmington, MA, USA) reagent was used to perform SYBR Green qPCR. The template volume and primer pair concentrations were 2 μL and 100 nM, respectively, for a total reaction volume of 10 μL per sample. The phEPO concentration was 10 pg/μL (1.54×10^9 copy/μL) for use as a standard material to perform absolute quantification of the copy number of trans-gene fragments. As negative controls, 2 μL of DW was used. The conditions maintained in the thermal cycler were 95°C for 20 s; and 95°C for 1 s and 60°C for 20 s, for 35 cycles in the Taqman probe assay and 95°C for 20 s; 95°C for 30 s, and 60°C for 30 s for 35 cycles followed by melting curve analysis in the SYBR Green assay in a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). All samples were measured in duplicate, and the coefficient of determination (R2) of the standard curve was equal to 0.98.

Specificity and accuracy of Taqman qPCR assay

To confirm the accuracy and specificity of the Taqman qPCR assay for trans-genes introduced by phEPO, blood-DNA and stool-DNA samples at each time point were pooled; negative control DNA samples were prepared using genomic DNA isolated by phenol chloroform extraction from 5 human cell lines: HK-2 cells (CRL-219, ATCC, Manassas,

VA, USA), human embryonic fibroblasts (HEF cells: JCRB 1006.7, JCRB Cell Bank; original developers: Kouchi and Namba), human embryonic kidney cells (HEK293 cells: Thermo Fisher Scientific), HuH-7 cells (JCRB0403, JCRB Cell Bank; original developers: Nakabayshi, H. & Sato, J.), and human mesenchymal stem cells (HMS cells: UE6E7T-2; JCRB 1133, original developer: Umezawa, A.). The concentration of the negative control DNA samples was adjusted to 10 ng/μL. These DNA samples were subjected to Taqman qPCR assay again as described above. After Taqman qPCR, the amplicons were subjected to electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide to confirm the amplicon size. The remaining amplicons of blood-DNA were cleaned up using NucleoSpin Gel and a PCR Clean-up kit (Takara Bio). Because amplicons of non-specific sizes were detected in the stool-DNA after Taqman qPCR, the remaining amplicons were subjected to electrophoresis in a 2% agarose gel and visualized; amplicons of the target size were cut from the gel and their DNA was extracted using NucleoSpin Gel and PCR Clean-up kit. The cleaned amplicon-DNA were subjected to Sanger sequencing performed by FASMAC, Inc. (Kanagawa, Japan). The sequencing data were analyzed using CLC Sequence Viewer ver. 8.0 and BioEdit Ver. 7.0.5.3 to determine whether the amplicon sequences matched the transgene of phEPO.

Statistics

Data are shown as the mean ± SEM. All data were subjected to Kruskal–Wallis H test (one-way analysis of variance of ranks), followed by a two-stage Benjamini, Krieger, and Yekutieli false discovery rate procedure as a post-hoc test using GraphPad Prism version 7.04 (GraphPad, Inc., La Jolla, CA, USA). A p value less than 0.05 was considered as statistically significant.

Table 6. Primer sequences used in Study2

Methods	Targets		Sequences	Predicted size (bp)
qPCR Taqman probe assay	Human Erythropoietin	Forward	CGA GAA TAT CAC GAC GGG CT	126
		Probe	56FAM/TATCACTGT/ZEN/CCCAGA CACCAAAGTT/3IABkFQ/	
		Reverse	CAG ACT TCT ACG GCC TGC TG	
qPCR SYBR green assay	Human Erythropoietin	Forward	CGA GAA TAT CAC GAC GGG CT	126
		Reverse	CAG ACT TCT ACG GCC TGC TG	

3. Results

hEPO transgene was detected in two different samples by qPCR

In the blood specimens, using the Taqman probe, transgene fragments were detected at 1, 2, 3, 6, and 12 h in the IV and LM groups and at 1, 2, 3, 6, 12, and 48 h in the IP group. In contrast, in the SYBR green assay, they were detected at 1, 2, 3, and 6 h in the IV and LM groups, but were not detected in the IP group (Table 7). In the stool specimens, using the Taqman probe, they were detected at all time points in the IV and LM groups and at 6 and 12 h in the IP group. In contrast, using SYBR green, the trans-gene fragments were not detected (Table 8).

Taqman qPCR specifically and accurately detected the transgene

After confirming the specificity and accuracy of the Taqman qPCR assay for detecting trans-gene fragments, a specific amplification curve was detected in pooled blood-DNA and stool-DNA samples, while no amplification was observed in any human cell lines or DW as the negative controls (Figure. 7). In agarose gel electrophoresis, a single band for the amplicon was detected in all whole blood-DNA samples by Taqman qPCR (Figure. 8). In contrast, stool-DNA samples showed two bands for the amplicon, indicating that one was the hEPO gene and the other was a non-specific amplicon. No amplicon band was detected in any of the human cell lines or DW as negative controls (Figure. 8). Further analysis to confirm the specificity and accuracy of Taqman qPCR was conducted by Sanger sequencing. In whole blood samples, separated and single peak sequences were detected in the IV, LM, and IP specimens. In the stool samples, no single sequence was detected in any group. Concordances between hEPO-reference and amplicon sequences in all samples were high (Figure. 9, 10).

Table 7. Detection of transgene fragments in Blood samples by Taqman qPCR and SYBR Green qPCR.

Method	Group	Copy/μL of Transgene							
		Pre	1h	2h	3h	6h	12h	24h	48h
Taqman qPCR	IV	0	739964.7** ± 423342.3	27929.9** ± 6674.9	53345** ± 23608.6	9274.3** ± 2449.9	7948** ± 7408.7	26.5 ± 8.4	41.4 ± 12.8
	LM	0	84334** ± 12302.6	21906.5** ± 7682.8	58617.3** ± 15427.5	14651.9** ± 4555.5	209.8** ± 48.1	5.5 ± 1.5	48.7 ± 18.4
	IP	0	135428.1** ± 71292.2	2655.3** ± 1697.2	2553.3** ± 1266.9	532.2** ± 318.7	611.6** ± 542.5	5.3 ± 0.9	337.7** ± 247.4
SYBR qPCR	IV	34.9 ± 4.6	423512.2** ± 249442.4	3424.8** ± 963.9	15471.8** ± 8637.2	2249.8** ± 634.1	1455.3 ± 1370.1	45.4 ± 6.9	23.8 ± 2.2
	LM	35.8 ± 2.4	31633.2** ± 5109.2	3114* ± 1340.4	16818 ± 4902.1	4397.9* ± 1546.8	44.9 ± 8.4	25.7 ± 1.2	28.7 ± 3.1
	IP	53.4 ± 3.9	71880.8 ± 44500.8	235.3 ± 152.1	441.6 ± 217.9	107.9 ± 57.9	74.6 ± 53.3	27.6 ± 1.7	54.1 ± 25.8

Transgene fragments were detected from all samples in Taqman qPCR. IV means intravenous injection, LM means local muscular injection and IP means intraperitoneal injection of the hEPO coding naked plasmid. **: $p<0.01$ vs Pre in each groups. *: $p<0.05$ vs Pre in each groups.
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Table 8. Detection of transgene fragments in stool samples by Taqman qPCR and SYBR Green qPCR.

Method	Group	Copy/μL of Transgene				
		pre	6h	12h	24h	48h
Taqman qPCR	IV	0	30.0 ± 11.4**	85.4 ± 39**	967.5 ± 388.3**	10.0 ± 1.4**
	LM	0	15.1 ± 4.9**	85.8 ± 30.8**	913.9 ± 263**	43.2 ± 12.2**
	IP	0	1809.6 ± 1587.9**	180.8 ± 148.1**	0	0
SYBR qPCR	IV	426.9 ± 159.8	65.4 ± 16.6	111.2 ± 18.3	239.9 ± 62.5	34 ± 5.4
	LM	762.4 ± 239.3	44 ± 5.7	117.7 ± 23.6	263.8 ± 56.1	46.1 ± 7.1
	IP	885.1 ± 403.6	1111.6 ± 965.2	103.8 ± 35	110.1 ± 10.2	51.6 ± 8.4

Transgene fragments were detected from all samples in Taqman qPCR. IV means intravenous injection, LM means local muscular and IP means intraperitoneal injection of the hEPO coding naked plasmid. **: $p<0.01$ vs Pre in each groups. *: $p<0.05$ vs Pre in each groups.
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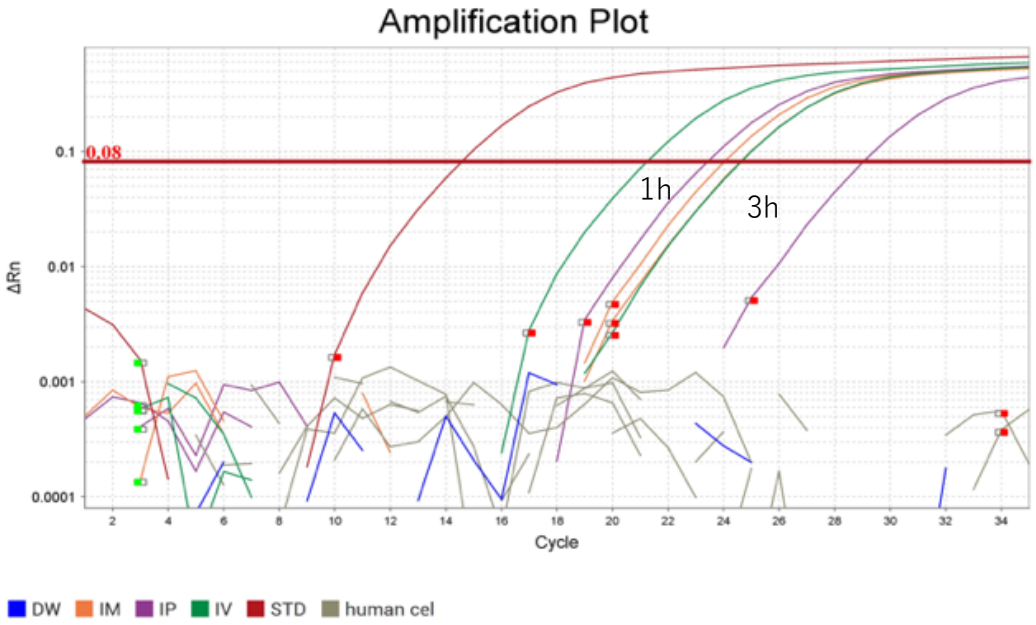

Figure 7. Amplification Plot in Blood sample and human cells. Specific amplification was detected in blood samples. There were no amplifications in human cells and Distilled water (DW). Accepted by *PeerJ* (Aoki and Sugasawa et al. 2020 modified)

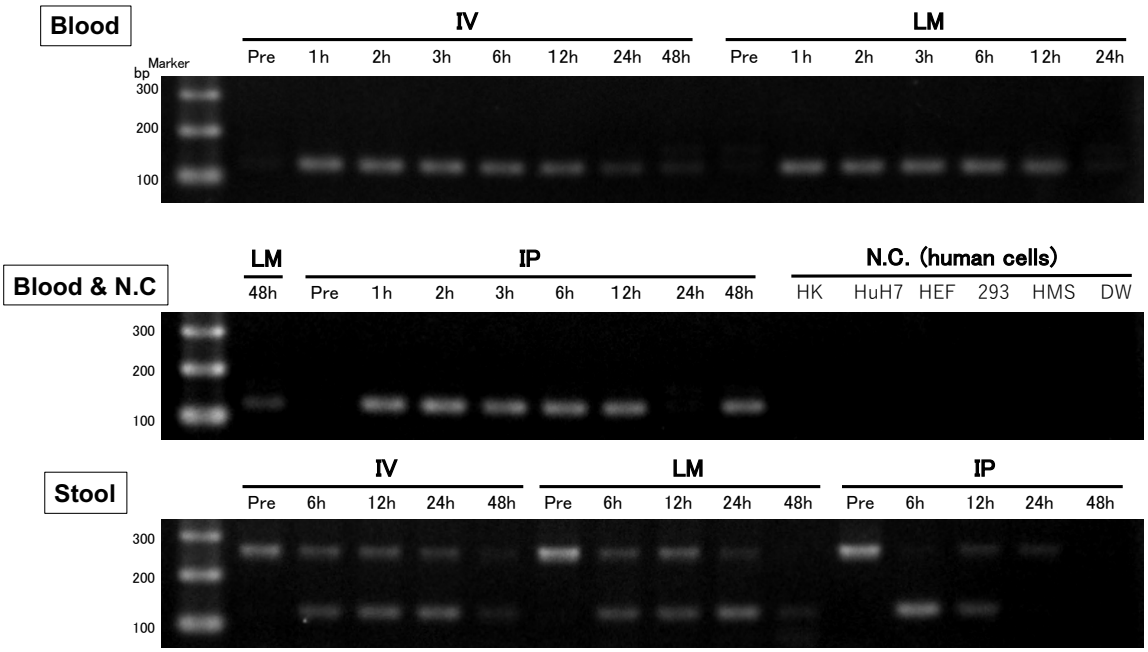

Figure 8. Confirmation of the accuracy and specificity in Taqman qPCR using agarose gel electrophoresis. Single band was detected from blood samples, but two bands were detected from stool samples. There was no band in N.C.(human cells). IV means intravenous injection, LM means local muscular and IP means intraperitoneal injection of the hEPO coding naked plasmid. HK: HK-2 cells(10ng/μl), HuH7: HuH-7 cells(10ng/μl), HEF: Human embryonic fibroblasts cells(10ng/μl), 293: Human embryonic kidney 293 cells(10ng/μl), HMS: Human mesenchymal stem cells(10ng/μl), DW: Distilled water.
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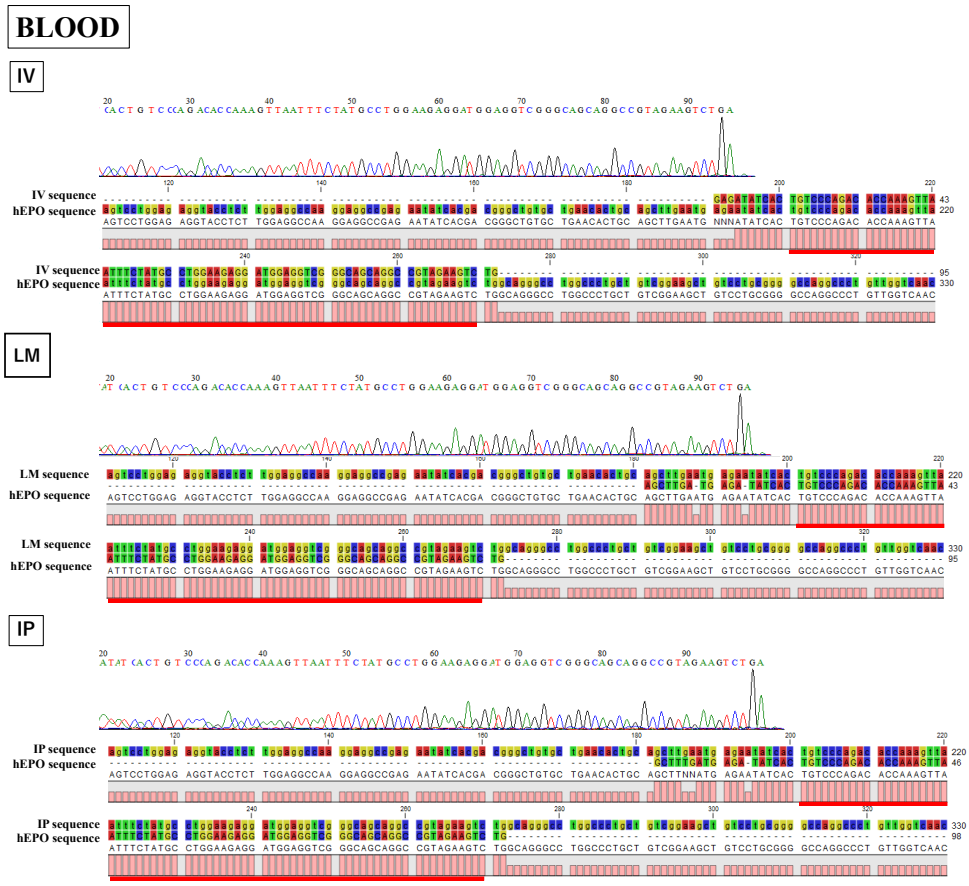

Figure 9. Confirmation of the accuracy and specificity in Taqman qPCR from blood samples using Sanger sequence methods. Single peak was detected from all samples and concordances between hEPO-reference and amplicon sequences in all samples were high level. IV means intravenous injection, LM means local muscular and IP means intraperitoneal injection of the hEPO coding naked plasmid. Red lines indicate the matching sequences between hEPO-reference and amplicon sequences.
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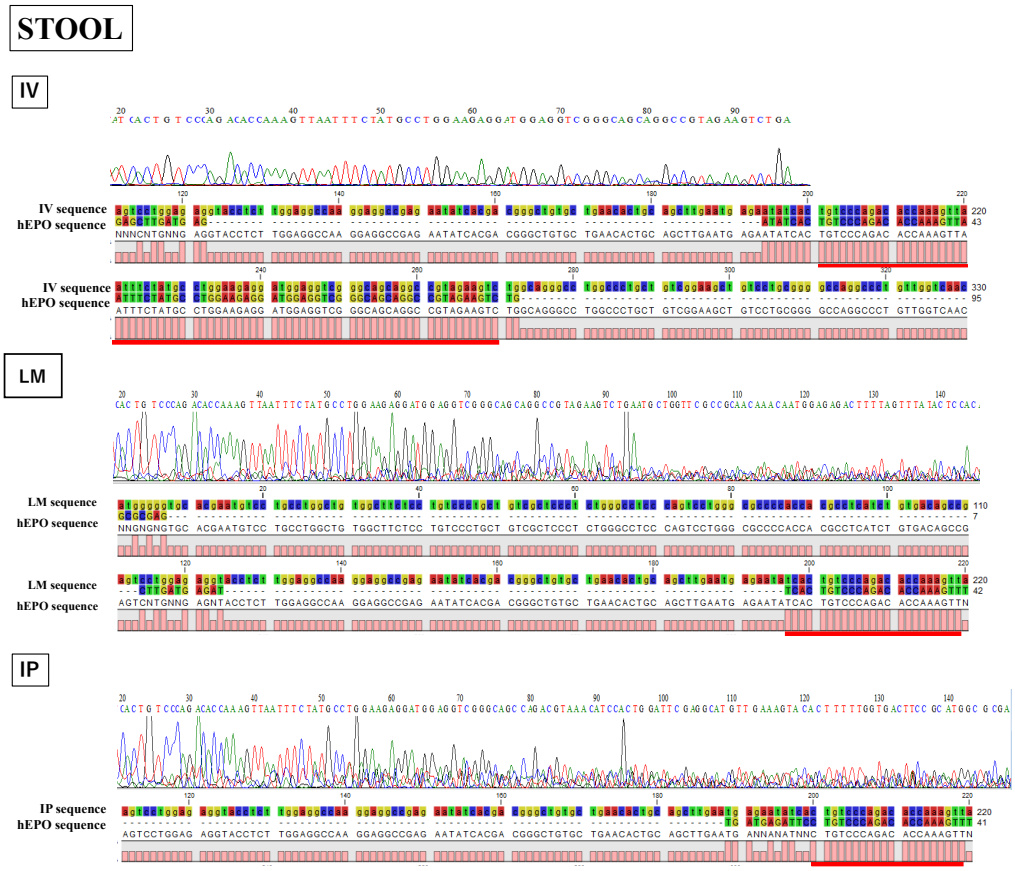

Figure 10. Confirmation of the accuracy and specificity in Taqman qPCR from stool samples using Sanger sequence methods. Single peak was detected from only IV sample, but concordances between hEPO-reference and amplicon sequences in all samples were confirmed. IV means intravenous injection, LM means local muscular and IP means intraperitoneal injection of the hEPO coding naked plasmid. Red lines indicate the matching sequences between hEPO-reference and amplicon sequences.
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4. Discussion

Study 1 suggested the usefulness of ddPCR, but due to the problem (high cost, detection time, process capabilities) of its application to the testing field, Study 2 aimed to establish a method for detecting gene doping by qPCR targeting the hEPO transgene. In particular, I focused on the specificity and accuracy of Taqman qPCR, and also examined the differences in detection period and accuracy by route of administration and sample.

Detection of gene doping using hEPO plasmids and detection periods

I developed a method for detecting the transgene delivered by a naked plasmid coding for hEPO and examined the differences in detection sensitivity depending on the route of administration, as various organs may be targeted by the gene. Transgene fragment was detected following IV, IP, or LM injection by two qPCR methods. Tozaki et al. reported that plasmid injection into the muscle could be detected by ddPCR until 2–3 weeks after injection in micro mini pigs [Tozaki et al. 2018]. In contrast, hEPO transgene fragments were detected in IP blood samples until 48 h after injection in this study. There are three reasons for this difference: (i) the injection amount was lower than that used in the previous study (previous: 250 μg, this study: 50 μg); (ii) Original blood sample volume (previous: 4 mL, this study: 50 μL); (iii) species differences (metabolic speed can differ because of enzyme activity differences); and (iv) the PCR method and sensitivity show some differences (previous: ddPCR/high sensitivity, this study: Taqman qPCR/normal). However, ddPCR is more costly than Taqman qPCR as mentioned above, and thus ddPCR cannot be used to analyze large numbers of samples. In this study, we compared the Taqman qPCR and SYBR Green PCR assay to detect gene doping. As a result, Taqman qPCR detected higher levels of the gene than the SYBR Green assay and for a longer period of time. Therefore, this model can be used for gene doping detection using hEPO

transgene.

Based on the results of qPCR, stool samples are useful for gene doping inspection because they can be collected non-invasively. The collection of blood samples is invasive; however, transgene fragments were detected from a smaller sample volume. Hence, blood and stool samples are useful for tansgene detection.

Confirmation of specificity and accuracy of Taqmanq PCR

Accuracy of the Taqman assay by three methods using blood and stool samples were confirmed by (i) specific amplifications in the Taqman assay (Figure 7), (ii) transgene molecular size by electrophoresis (Figure 8), and (iii) transgene DNA sequence by Sanger sequencing (Figure 9, 10). The hEPO amplification products and DNA sequence agreed with the reference in all blood samples. In particular, the lack of amplification of hEPO from the DNA of human-derived cell lines suggests that there is no amplification of EPO from the genome DNA. In other words, this indicates that it was able to distinguish between transgenes and genome DNA fragments. In the present study, primers and probes were created between exon 2-4 based on the study of Baoutina et al [Baoutina et al. 2013]. Consistent with previous study, no genomic DNA was amplified. In contrast, in the stool sample, several non-specific bands were observed in agarose gel electrophoresis and waveform disturbances were detected in the Sanger method (Figure 8, 10). The stool sample may have contained a high level of enterobacteria DNA. Although Sanger sequencing did not provide accurate results, Taqman qPCR was specific and thus may be adapted for transgene detection.

Primer dimers formed in SYBR Green reactions cause decrease the sensitivity of real-time PCR. This problem can be avoided by using a specific detection system with Taqman MGB probes, where non-specific amplification is not detected because of the

binding of the specific MGB probe only to a 100% complementary nucleotide sequence [Orlando, Pinzani & Pazzagli. 1998].

5. Summary

Study 2 showed that the Taqman probe method was more specific and accurate than the SYBR Green method for gene doping in qPCR. In addition, my finding confirmed that the hEPO gene, which is the target of gene doping, could be detected separately from the genomic DNA.

VI. Study 3

Estabalishment of gene doping model using adenovirus vector containing human erythropoietin and its detection.

1. Aim

This study hypothesized that hEPO over expression using adenovirus vector containing hEPO can be a gene doping model. This study was aimed to establishe the gene doping model and to detect the transgene fragments from this model.

2. Materials and Methods

Cloning of Recombinant Adenoviral Vectors Containing the Epo Gene

Plasmid of pcDNA3.1(-) containing hEPO gene without introns derived from cytomegalovirus-promoter was purchased from GenScript Biotech Corp. (Nanjing, China). Cloning methods were refered to Study1.

Ethics statement and Animals

Animal experiments in this study were approved by the Animal Experimental Committee, University of Tsukuba (approval numbers: 20-361). 9-week-old ICR male mice were purchased from the Central Laboratories for Experimental Animals (CLEA, Tokyo, Japan). The mice were bred and maintained in an air-conditioned animal house under specific-pathogen-free conditions and subjected to a 12/12 h light and dark cycle. The mice were fed standard mouse pellets and water ad libitum. The mice were allowed to grow until they reached 10 weeks old, with an average body weight of 37.2g (SD = ±1.2 g). At this point, they were used for experiments.

Establishment of gene doping model using adenovirus vector

Confirmation of gene doping phenotype

EPO increases the red blood cell count. Therefore, I investigated the changes in blood cell parameters after injecting an adenovirus vector containing hEPO. I intravenously injected 2.2×10^{11} VP adenovirus containing mCherry (Control) or hEPO and measured the blood cell parameters at 3, 5, 7, and 14 days. I collected 60 μL blood, from the tail tip, into a 1.5 mL tube with 10 μL EDTA-2Na as the pre-sample. Subsequently, I diluted the blood sample with an equal volume of PBS, and measured the blood cell parameters using an automatic cell counter (Celltac α MEK6458; NIHON KOHDEN CORPORATION; Tokyo, Japan).

in vivo confirmation of hEPO expression

To confirm the gene doping model animal, adenovirus containing mCherry or hEPO was injected by intravenous and virus volumes were 2.2×10^{11}VP. Mice were sacrificed at 5 days later after injection and liver was harvested to analyze the hEPO gene expression and protein expression.

Sepasol-RNA I Super G (Nacalai Tesque) was used for total RNA extraction, according to the manufacturer's instructions. Using 400 ng of total RNA and PrimeScrip RT Master Mix (Takara Bio, Shiga, Japan), reverse transcription was carried out to synthesize cDNA. Subsequently, qPCR was performed to confirm hEPO expression, using a KAPA SYBR Fast qPCR kit (NIPPON Genetics), normalized to 36B4 expression with delta CT calculations. Primer sequences using Study3 are given in Table 9.

Total proteins were extracted from liver with radio immuno precipitation assay (RIPA) buffer (1% NP-40, 0.1% SDS, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl including proteinase inhibitor tablets (cOmpleteTM mini, Roche, Basel, Switzerland)).

Lysates were centrifuged at 15,000 g for 10 min at 4 ◦C. Total protein concentrations for each sample were measured by BCA protein assay kit (Takara, Japan), and 10 μg/lane of total protein were used for 10% SDS-polyacrylamide gel electrophoresis. In Western blot analysis, the primary antibodies used were mouse monoclonal Erythropoietin antibody (Santacruz Biotechnology, sc-5290, 1:100) and β -actin antibody (Santacruz Biotechnology, sc-81178, 1:1000). The horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology, #7076, US) were used as secondary antibodies. Signals were detected using the chemiluminescence reagent (EzWestLumi One, ATTO, Tokyo, Japan). Blots were scanned using a LAS-4000 (GE Healthcare, UK).

Chronological detection of transgene fragments

Chronological detection of transgene fragments was performed as described by Sugasawa et al [Sugasawa et al. 2020]. Namely, using 15 untreated ICR mice, about 2 mm of the tail tip was cut, after which about 50 μL of whole blood, just a single drop as a pre-experiment specimen, was collected into a 1.5-mL microtube including 150 μL of PBS-EDTA 2Na mixture. Then, the collected blood was quickly put on ice and stored at − 20 ◦C until further analysis. Simultaneously, the stools were also collected and stored the same way as the blood. One week later, the hEPO was injected by intravenous. Subsequently, one drop of whole blood and stools were collected at 1, 3, 6 and 12h, and 1, 2, 3, 4, 5, 6 and 7days after the injection. A phenol/chloroform/isoamyl alcohol solution (Nacalai Tesque, Kyoto, Japan) was used to extract total DNA from the blood and stool in accordance with the manufacturer's instructions and then blood sample were adjusted to 10 ng/μL or 100ng/μL and stool samples were adjusted to 10 ng/μL. Using the total DNA, qPCR was performed to detect hEPO transgene fragments. The phEPO concentration was 10 pg/μL (1.54×10^9 copy/μL) for use as a standard material to perform absolute

quantification of the copy number of trans-gene fragments. PCR condition was referred to Study 2.

Statistics

Data are shown as the median or mean ± SEM. For comparsion of blood cell parameters, two-way analysis of variance followed by Dunnett's test as a pst-hoc test was used. For comparsion of Epo gene and protein expression, Mann-Whitney U test was used. For comparsion of chronological detection, data were subjected to Kruskal–Wallis H test (one-way analysis of variance of ranks), followed by a two-stage Benjamini, Krieger, and Yekutieli false discovery rate procedure as a post-hoc test using GraphPad Prism version 8.3.1 (GraphPad, Inc., La Jolla, CA, USA). A p value less than 0.05 was considered as statistically significant.

Table 9. Primer sequences used in Study3

Methods	Targets		Sequences
qPCR Taqman probe assay	Human Erythropoietin	Forward	CGA GAA TAT CAC GAC GGG CT
		Probe	56FAM/TGAGAATAT/ZEN/CACTGT CCCAGACACC/3IABkFQ/
		Reverse	CAG ACT TCT ACG GCC TGC TG
qPCR SYBR green assay	Human Erythropoietin	Forward	CGA GAA TAT CAC GAC GGG CT
		Reverse	CAG ACT TCT ACG GCC TGC TG
	36B4	Forward	CACTGGTCTAGGACCCGAGAA
		Reverse	AGGGGGAGATGTTCAGCATGT

3. Results

Adenovirus vector containing hEPO upregulate the blood cell parameters

To confirm the effects of hEPO, we measured the blood cell parameters. In white blood cell (WBC), Epo group was higher than Con group at 5, 7 and 14 day (interaction $p<0.01$). In red blood cell (RBC), Epo group was higher than Con group at 7 and 14 day (interaction $p<0.01$). In hemoglobine (HGB), Epo group was higher than Con group at 5, 7 and 14 day (interaction $p<0.01$).

Adenovirus vector containing hEPO upregulate the expression level of Epo gene and protein in liver

Since adenovirus has the property of collecting in the liver after injection, Epo gene expression and protein expression in the liver were measured. Both gene and protein expression were significantly higer in Epo group.

Transgene fragments were detected until 7 days later after injection using low invasity sample

Transgene fragments were detected until 4 day after injection in blood sample. In stool sample, transgene fragments were detected until 1day after injection. We also considered increasing the amount of DNA used for PCR. The detection limit has been extended to 7 day after injection in blood sample.

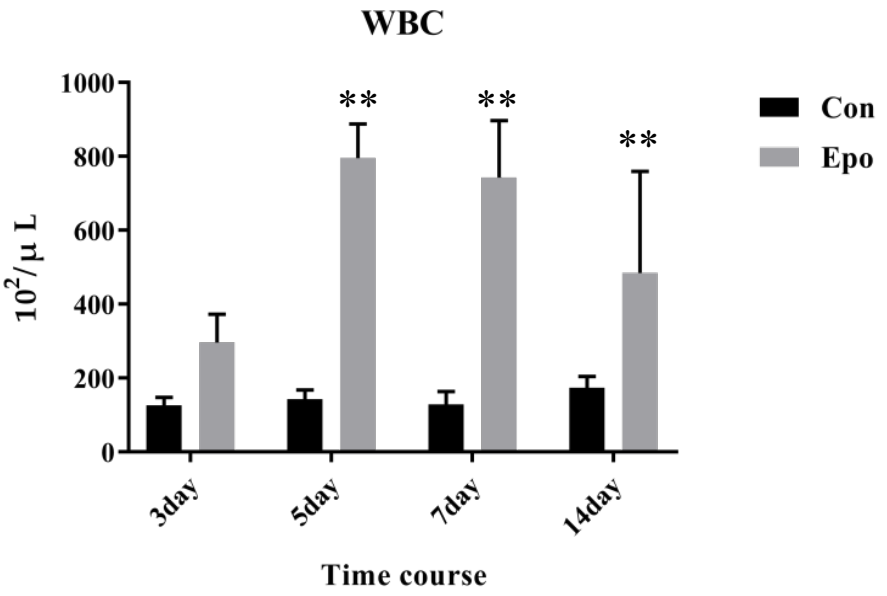

Figure 11. Changes in white blood cell after adenovirus vector injection. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. **: $p<0.01$ vs 3day in each groups. Con: Control (mCherry), n=10; Epo: Erythropoietin, n=10

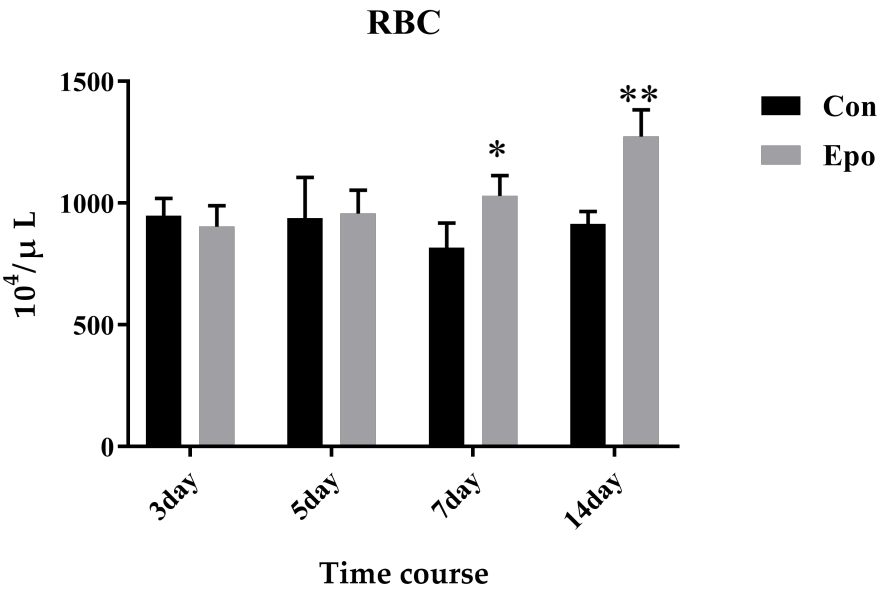

Figure 12. Changes in red blood cell after adenovirus vector injection. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. **: $p<0.01$ vs 3day in each groups. *: $p<0.05$ vs 3day in each groups. Con: Control (mCherry), n=10; Epo: Erythropoietin, n=10

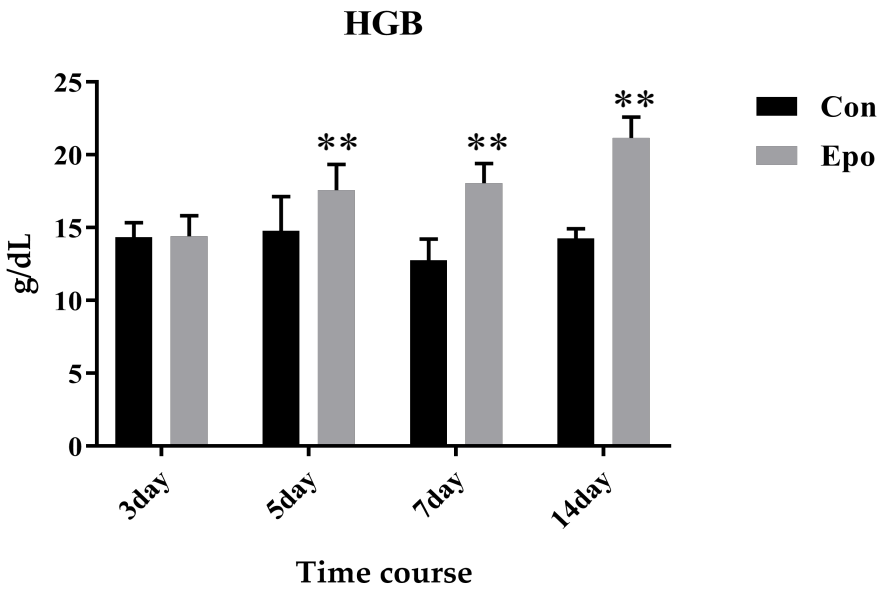

Figure 13. Changes in hemoglobin after adenovirus vector injection. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. **: $p<0.01$ vs 3day in each groups. Con: Control (mCherry), n=10; Epo: Erythropoietin, n=10

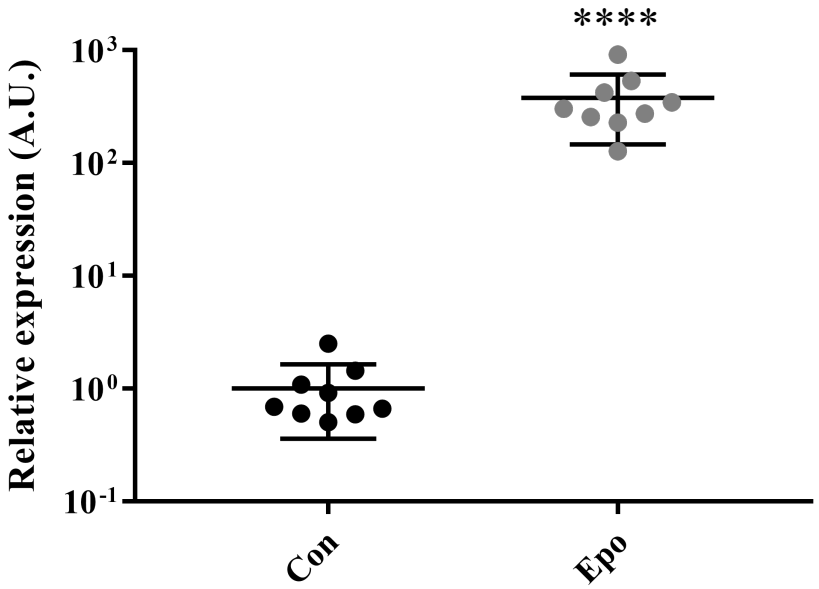

Figure 14. Gene expression level of Epo in liver. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. ****: $p<0.0001$ vs Con. Con: Control (mCherry), n=9; Epo: Erythropoietin, n=9

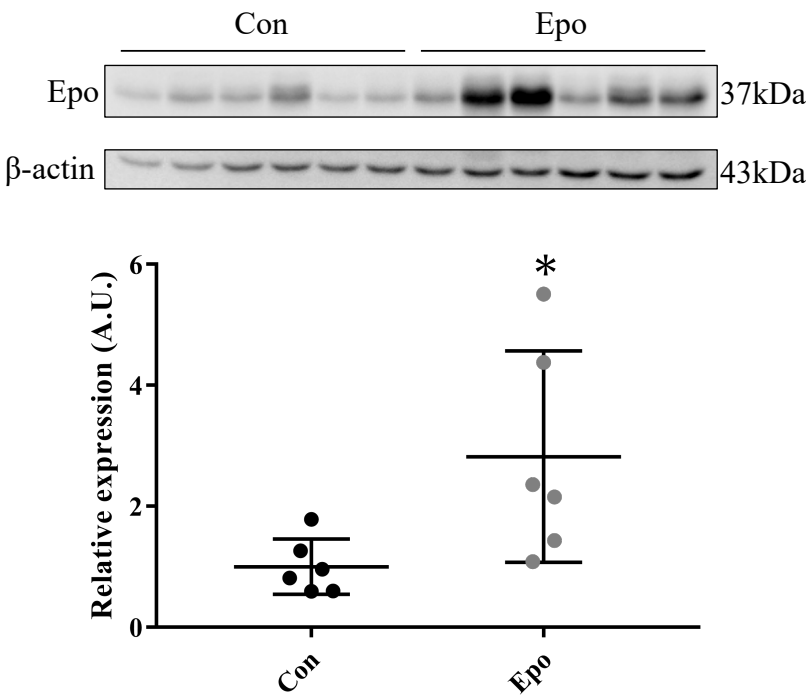

Figure 15. Protein expression level of Epo in liver. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. *p<0.05 vs Con. Con: Control (mCherry), n=6; Epo: Erythropoietin, n=6

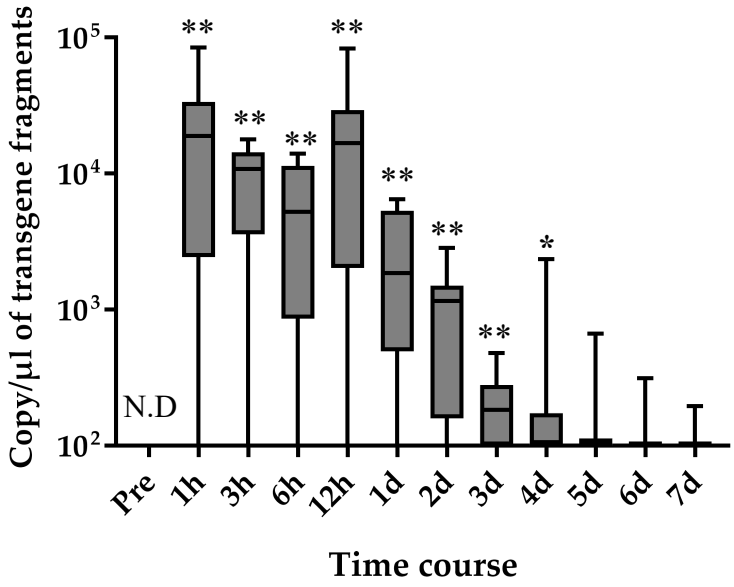

Figure 16. Chronological detection of hEPO gene fragments from blood sample. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. **: $p<0.01$, *:$p<0.05$ vs Pre. Boxes represent the 25th and 75th percentiles; whiskers represent the minimum and maximum values(n=15)

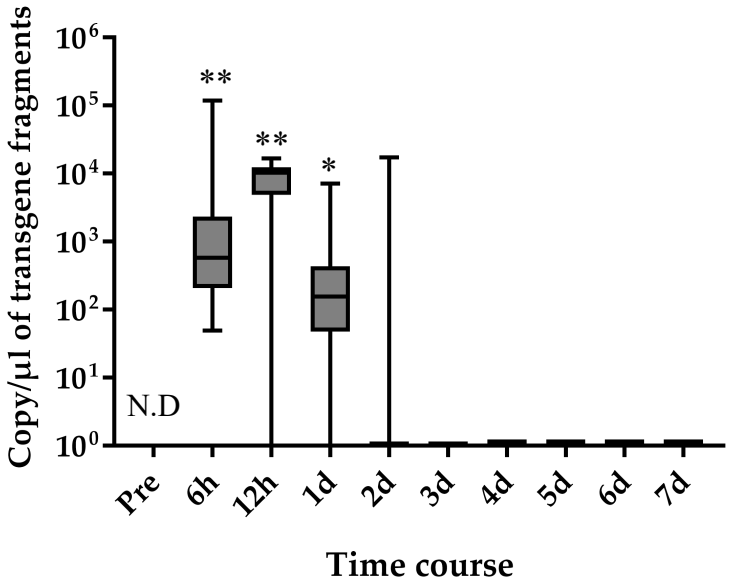

Figure 17. Chronological detection of hEPO gene fragments from stool sample. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. **: $p<0.01$, *:$p<0.05$ vs Pre. Boxes represent the 25th and 75th percentiles; whiskers represent the minimum and maximum values(n=15)

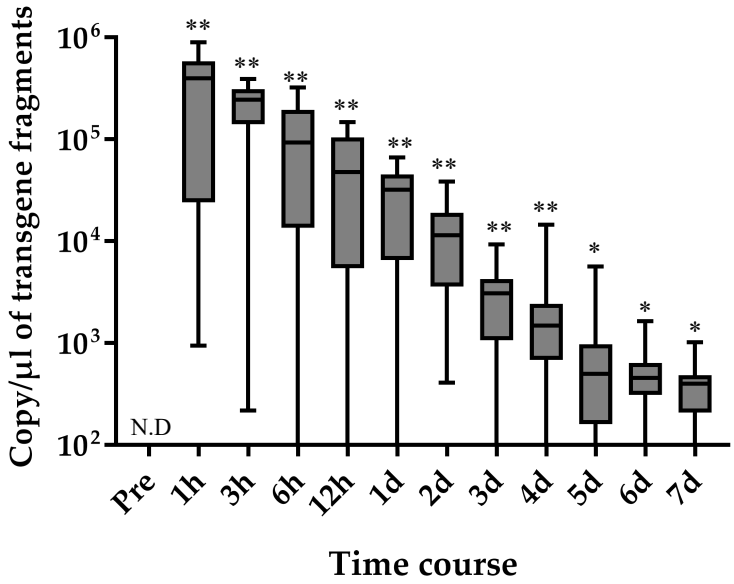

Figure 18. Chronological detection of hEPO gene fragments from blood sample using high volume template DNA. Adenovirus vector was injected from intravenous and virus volume was 2.2×10^{11}VP. **: $p<0.01$, *:$p<0.05$ vs Pre. Boxes represent the 25th and 75th percentiles; whiskers represent the minimum and maximum values(n=15)

4. Discussion

Study 2 clarified that the hEPO gene could be detected separately from genomic DNA. It was also suggested that Taqman qPCR could specifically detect transgene fragments. However, it involved naked plasmid transfer and did not confirm EPO gene or protein expression. In addition, I did not examine the changes in blood cell counts when hEPO was introduced. Therefore, in Study 3, I created a gene doping model using an adenovirus vector containing the hEPO gene and detected transgene fragments.

Construction of an EPO gene doping model using adenovirus vector containing the hEPO gene

EPO is an important factor in hematopoiesis [Debeljak & Sytkowski 2012]. It is normally produced in the kidneys, released into the bloodstream, and then binds to the EPO receptors in hematopoietic organs to promote hematopoiesis [Salamin et al. 2018]. EPO preparations are sometimes used in drug doping as well [Smeets 2009] because they enhance the aerobic capacity of athletes by increasing the red blood cell count [Haile et al. 2019]. When gene doping with EPO is performed, intravenous injection is more likely to be used than intramuscular injection because the high EPO expression in muscle can be examined by muscle biopsy. When adenovirus vectors are injected intravenously, most of them are concentrated in the liver [Engelhardt et al. 1993; Mastrangeli et al. 1993]. The liver is less likely to be ethically biopsied as compared to the muscle. Therefore, in the present study, I administered adenovirus vectors intravenously. However, as it is difficult for athletes to undergo muscle biopsy, I used blood as the sample using the concept of liquid biopsy as in Studies 1 and 2.

In this study, all blood parameters were significantly higher after 7 days of intravenous injection than after 3 days (Figure 11, 12, 13). To determine the effects of adenovirus injection on the immune system, I injected the adenovirus vector containing the mCherry gene into the Con group, which may have confirmed the specific effects of EPO. The increase in blood cell parameters was consistent with previous studies using AAV containing the EPO gene [Lebherz et al. 2005; Rivera et al. 2005], suggesting successful hEPO gene delivery. However, some points were difficult to interpret. Initially,

the hemoglobin and red blood cell count were significantly elevated at different time points (Figure 12 and 13). Since hemoglobin is a pigment present in red blood cells, it is considered that it increases simultaneously with an increase in red blood cell count. Since in my preliminary study, hemoglobin level and red blood cell count increased simultaneously (data not shown), it is highly possible that this difference was due to measurement errors. Moreover, there was a significant increase in white blood cell count when the AdV-containing EPO was administered (Figure 11). Nagashima et al. (2018) have reported that recombinant human EPO (rHuEPO) decreases the number of B cells in the peripheral blood. On the contrary, one case study reported that rHuEPO increases the white blood cell count [Jinbo et al. 1992]. As described above, it was reported that the white blood cell count decreased and increased when rHuEPO was administered, and it was difficult to interpret the results of AdV-containing EPO in this study. It is possible that the rapid increase in EPO in the body stimulates the stem cells involved in hematopoiesis. In addition, it has been reported that an autoimmune reaction in response to the produced EPO occurs in monkeys [van der Gronde et al. 2013], and it is possible that the white blood cell count increases due to the immune reaction. The blood reference values for 10-week-old male ICR mice were 982×10^4 μL (red blood cell count), 15.4 g/dL (hemoglobin), and 63.8×10^2 μL (white blood cell count) [Japan SLC]; hence, there was a significant increase in all parameters within one week. Although the effects on the immune system of humans are unpredictable, an animal model was successfully established here.

Since blood cell parameters were increased, to confirm whether EPO overexpression takes place in the liver, I collected the liver samples 5 days after adenovirus vector injection, and confirmed that EPO gene and protein expression was significantly higher than that in the Con group (Figure 14 and 15). Subsequently, I used the liver as a secretory organ to produce EPO, which binds to EPO receptors in the hematopoietic organs via the blood and promotes hematopoiesis. Thus, I successfully constructed a gene doping model using an adenovirus vector containing the hEPO gene.

Detection of hEPO transgene fragment using adenovirus vector containing the hEPO gene

Since I constructed a gene doping model using hEPO, I chronologically detected the gene fragments using this model. After injecting the adenovirus vector, I was able to detect transgene fragments significantly until 4 days later in blood samples and until 1 day later in stool samples. In Study 2, I detected transgene fragments until 2 days after injection despite the naked plasmid; however, in the present study, I detected them until 1 day later. The exact reason for this is unclear; however, there is a possibility that the transgene differs with regard to its breakdown in the body. Alternatively, adenovirus injection could have triggered an immune response that resulted in degradation.

On the contrary, when I increased the template DNA concentration to 100 ng/μL, the detection period in the blood samples increased to 7 days. In Study 1 and Study 2, I performed PCR using low template concentrations, considering the possibility of PCR inhibition. However, the PCR could be performed even using 100 ng template, thereby increasing the detection sensitivity. I did not increase template volume of the reactions performed using stool samples in this study because I found some non-specific amplifications in Study 2.

5. Summary

In Study 3, I successfully constructed a gene doping model using adenovirus vectors with an increase in blood cell parameters. In addition, I successfully detected gene fragments from a gene doping model. Furthermore, I found that increasing the template DNA volume could extend the detection period without PCR inhibition.

VII. Whole discussion

1. New knowledge

The findings of this study were as follows: i) ddPCR can detect gene doping with high sensitivity, ii) among qPCR methods, Taqman qPCR can be used as a specific and an accurate detection method, particularly for detecting gene fragments by distinguishing the transgene from genomic DNA; iii) gene doping models using adenoviral vectors containing hEPO have a phenotype including hemopoiesis, and transgenes can be specifically detected; iv) gene doping can be detected from a drop of whole blood, which is a less invasive sample.

A possible detection system for gene doping is the one based on PCR; in this study, I evaluated the sensitivity of three different PCR methods to detect gene doping. As expected, ddPCR, qPCR, and sqPCR showed the highest detection sensitivity. ddPCR seems to be the most sensitive PCR technique and the most suitable for detecting gene doping, since it is capable of absolute quantification of the target gene fragment in the sample. However, due to its high sensitivity, it falsely detected a negative control, which should not have been detected, resulting in false positives. False positives should be avoided in doping tests, and positive and negative tests must be detected with a 100% probability. A similar event was observed with qPCR using the SYBR green method, in Study 2. The SYBR green method increases the possibility of false positives due to primer dimers or non-specific amplification. Since in the SYBR green method, fluorescence is emitted from the dye when it is elongated, both specific amplification and non-specific amplification are detected as signals. In contrast, the Taqman probe specifically binds, and when amplified, the probe is disconnected and fluoresces. Therefore, the Taqman probe method has high specificity, because only specific amplification occurs, and seems to be a reliable method for detecting genetic doping. In addition, although ddPCR is highly

sensitive, it is costly and time-consuming for a single detection. Therefore, the Taqman probe method is currently the most practical method for detecting gene doping targeting a single gene.

In this study, I mimicked gene doping using adenoviruses and developed detection methods. However, not only adenoviruses but also adeno-associated viral vectors are used in gene therapy [Gaudet et al. 2013], and adeno-associated viruses may serve as a useful tool for gene doping with future technological advances, as they allow for long-term expression of transgenes in the body. However, gene therapy techniques using non-viral vectors such as naked plasmid and plasmid–PEI complexes, which combine plasmid and polyethylenimine (PEI; cationic polymer), have also been investigated in animals [Liufu et al. 2019; Samaddar et al. 2019; Ustinova et al. 2018]. As shown in Study 2, the naked plasmid degrades rapidly and is generally considered to be inefficient to induce gene expression by transfection. However, a previous study has reported that injecting a plasmid containing the firefly luciferase (Fluc) gene with PEI significantly increases gene transfer efficiency and enhances Fluc luminescence intensity (Figure 19) [Sugasawa et al. 2020]. The plasmid is more likely to be used for gene doping because it is safer than viral vectors; however, a detection system is also being investigated simultaneously and has been successfully used for long-term detection of transgene fragments (Figure 20) [Sugasawa et al. 2020]. In the present study, I attempted to detect only the regions of hEPO; however, previous studies have also successfully detected cytomegalovirus promoter (CMVp) region-specific sequences embedded in the skeleton of adenovirus vectors. Since CMVp sequence is not originally present in the human genome, it may be possible to use this region for supplementary detection.

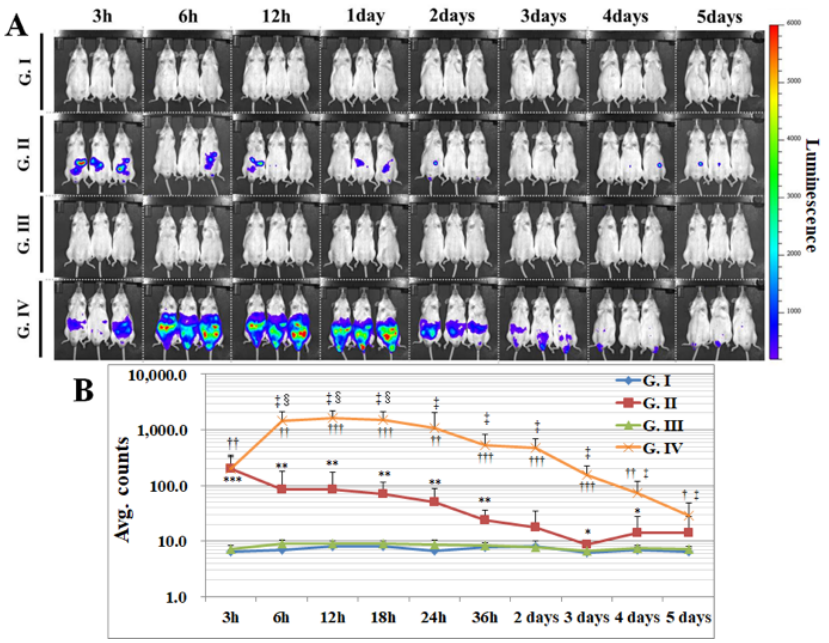

Figure 19. The gene delivery model mimicking gene doping using plasmid+PEI. (**A**): Chronic in vivo imaging of luminescence of representative mice after the injection of each solution: G1: DW + Buffer (N = 7), G2: DW + pFluc (N = 7), G3: PEI + Buffer (N = 7), and G4: PEI + pFluc (N = 7). The luminescence is shown as a color scale. (**B**): The chronological quantification values of intensity of the luminescence in each group and the data are shown as mean ± SD. The significant differences at each time point are shown for G1 vs. G2 as *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$; G3 vs. G4 as †: $p < 0.05$, ††: $p < 0.01$, and †††: $p < 0.001$; G2 vs. G4 as ‡: $p < 0.05$; and 3 h vs. 6, 12, or 18 h within G4 as §: $p < 0.05$.
Accepted by *Genes(Basel)*(Sugasawa and Aoki et al. 2020)

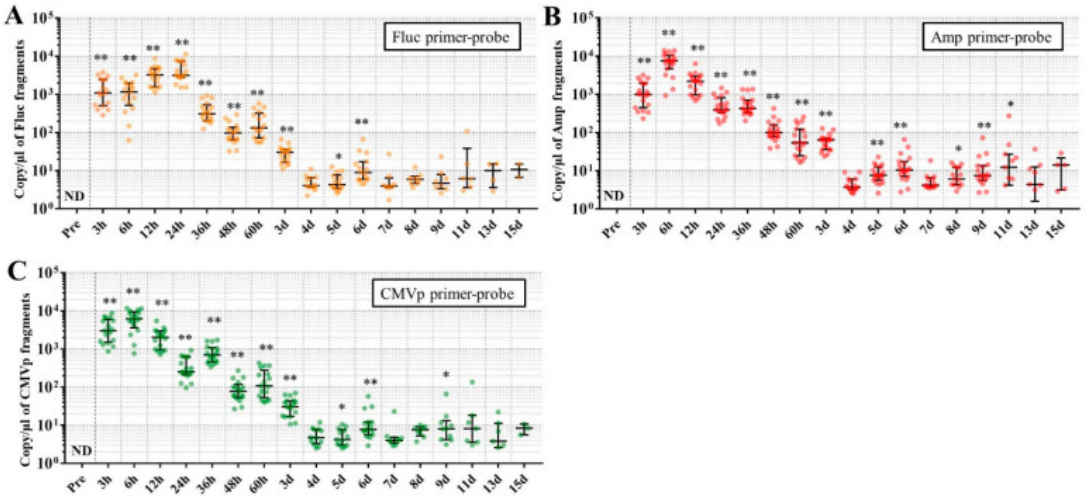

Figure 20. Long-term detection of multiple transgene fragments in the DNA from one drop of blood. (A): The results using Fluc primer-probe; (B): the results using Amp primer-probe; and (C): the results using CMVp primer-probe. The number of mice at each point is 17. The individual data are shown as a plot with bars indicating median and interquartile range. ND: not detected. *: $p < 0.05$ and **: $p < 0.01$ vs. the pre-values before the injection.
Accepted by *Genes(Basel)*(Sugasawa and Aoki et al. 2020)

2. Limitation and Perspective

Although I constructed a gene doping model and detected transgene fragments using it in the present study, there were several limitations.

First, I could not use the EPO gene doping model to examine the improvement in athletic performance. Previous studies have shown that increasing blood EPO concentrations by high-altitude training increases hemoglobin concentrations and red blood cell counts [Park et al. 2016]. It has also been reported that administering recombinant EPO protein to humans, as well as high-altitude training, concomitantly improve blood parameters and performance [Haile et al. 2019]. However, it was not clear whether this was a model for improving athletic performance, although the increase in blood parameters was captured in Study 3. In the future, researchers should aim to establish a gene doping model by examining the dose concentration and testing exercise capacity, which can also be used to optimize the conditions of the detection system and would allow us to create a sensitive detection system.

Second, I could only establish a detection system that targets a specific gene. Currently, several genes, including EPO, HIF-1, VEGF, IGF-1, and FST, can be targeted for gene doping, with varying effects. It is difficult to pinpoint the gene used for gene doping. Therefore, future studies are needed to construct a detection system that targets multiple genes. Till date, studies have examined multiplex qPCR assays that can detect multiple genes simultaneously, using horses and whole-genome sequencing using NGS to detect single nucleotide and structural variations [Tozaki et al. 2020, 2020]. In the future, a comprehensive detection system for gene doping should be constructed, using NGS, based on previously reported conditions. In addition, if a comprehensive gene doping detection system is established, it should be combined with the single-gene targeted detection method established here, to develop a robust method for detecting gene doping. In addition,

the measurement of drug-resistant and promoter regions in adenoviruses and plasmids, as described above using Taqman qPCR, might be a sensitive detection method until a comprehensive detection method is established.

Finally, I could not conduct a detailed examination of the methods to prevent false positives. In this study, non-specific detection could be prevented by examining the PCR conditions. However, the prevention of false positives due to contamination, such as in Study 1, can only been prevented by using uncontaminated equipment and through experiment-by-experiment cleaning. False positives are also a major problem for athletes and need to be completely prevented. In the recent years, studies have been conducted using robots for detection without human intervention [Yachie et al. 2017]. Using a robot placed in a clean room with all movements programmed for detection, it is possible to prevent artificially occurring cross-contamination, and it can be used for testing many samples. Currently, the above-mentioned humanoid robot "Maholo" (Figure 21) is placed at the University of Tsukuba and being operated for establishing a gene doping detection method. In the future, when Maholo is operational and can support several detection protocols, it will be possible to build a perfect detection method with no false positives.

The model established in this study was used to establish a detection method for preventing gene doping. However, gene doping uses gene editing techniques to improve performance and is accepted as a gene therapy when used by patients. In this study, AdV-containing EPO was used to increase the blood cell parameters. The elevated WBC count needs to be regulated, but it could be a basic model for gene therapy in patients with anemia. In addition, if future studies focus on doping using genes that cause muscle hypertrophy, such as myostatin and follistatin, it can be used as a basic model for diseases such as muscular atrophy simultaneously. Thus, it is possible that the development of gene doping detection methods will lead to basic research on gene therapy in the future.

Figure 21. LabDroid ”Maholo”

VIII. Conclusion

Summary of this study is indicated in Figure 22. This study succeeded in constructing a gene doping model using an adenovirus vector containing the EPO gene and made it possible to detect transgene fragments from the model. Therefore, it is considered that gene doping based on adenovirus vector can be detected with high sensitivity by a detection method based on PCR.

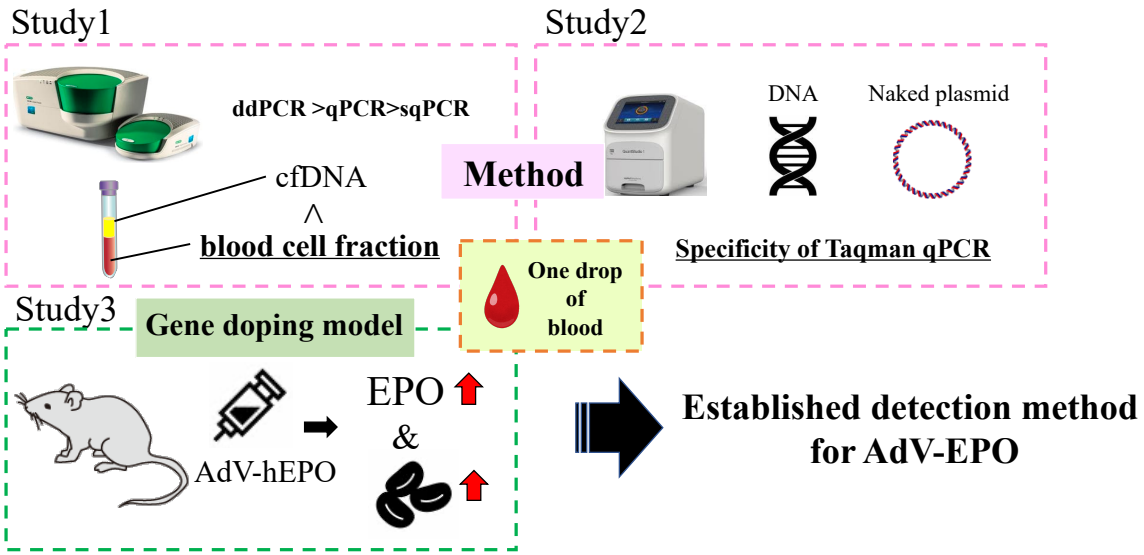

Figure 22. Summary of doctoral dissertation

IX. Acknowledgments

I would like to express my deepest gratitude to Professor Kazuhiro Takekoshi whose enormous support and insightful comments were invaluable during the course of my study. I am grateful to Professor Seiji Maeda, Professor Tohru Takemasa and Associate Professor Koichi Watanabe for carefully proofreading the manuscript. I am also indebted to Dr. Takehito Sugasawa and Dr. Yasuko Yoshida whose meticulous comments were an enormous help to me. I would like to thank everyone in Takekoshi Laboratory for cooperation in the experiment. In addition, I would like to thank Editage (www.editage.com) for English language editing. I acknowledge all the people who participated in this study. Finally, I would also like to express my gratitude to my family for their moral support and warm encouragements.

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