

## RESEARCH ARTICLE

# ChromInst: A single cell sequencing technique to accomplish pre-implantation comprehensive chromosomal screening overnight

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**Data Availability Statement:** The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0001733. Due to the next-generation sequencing data involving Human Genetic Resources (HGR) information, according to the Regulation of the People's Republic of China on the Administration of Human Genetic Resources, we cannot share data as public data directly. If you want to apply for data access to controlled data, please go to CNGBdb Data Access(CDA) (<https://>

## Abstract

Next Generation Sequencing (NGS) is a powerful tool getting into the field of clinical examination. Its preliminary application in pre-implantation comprehensive chromosomal screening (PCCS) of assisted reproduction (test-tube baby) has shown encouraging outcomes that improves the success rate of *in vitro* fertilization. However, the conventional NGS library construction is time consuming. In addition with the whole genome amplification (WGA) procedure in prior, makes the single cell NGS assay hardly be accomplished within an adequately short turnover time in supporting fresh embryo implantation. In this work, we established a concise single cell sequencing protocol, ChromInst, in which the single cell WGA and NGS library construction were integrated into a two-step PCR procedure of ~ 2.5hours reaction time. We then validated the feasibility of ChromInst for overnight PCCS assay by examining 14 voluntary donated embryo biopsy samples in a single sequencing run of Miseq with merely 13M reads production. The good compatibility of ChromInst with the restriction of Illumina sequencing technique along with the good library yield uniformity resulted superior data usage efficiency and reads distribution evenness that ensures precisely distinguish of 6 normal embryos from 8 abnormal one with variable chromosomal aneuploidy. The superior succinctness and effectiveness of this protocol permits its utilization in other time limited single cell NGS applications.

## Introduction

With wide application of PCR, FISH and DNA chip technology, medical examination has moved into the age of molecular diagnosis in the past decade. Recently, high throughput sequencing methods had shown very much promising adding in this age. The constantly throughput increasing of Next Generation Sequencing (NGS) keeps lowering the unit cost of

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data generation that strongly promotes the application of NGS from an academic research tool toward a powerful clinical examination method. One of such pioneer applications of NGS in clinical setting is single cell sequencing based pre-implantation comprehensive chromosome screen (PCCS) during *in vitro* fertilization (IVF) procedure [1–7].

World widely, the human infertility rate had increased up to 10–15% [8–10]. The assisted reproduction, namely test-tube baby technology, is often the final resolution to against infertility in many cases. However, the success rate of conventional IVF procedure is merely 30–35% in average, and could be even lower to 5–8% in females over age 40 [11–16]. Scientific researches has revealed that embryo chromosomal abnormality is one of major causes of IVF failure [17–20]. It's estimated that chromosomal abnormalities exist in 40–60% embryos obtained by IVF, and this ratio is increasing with the age of the female [21–28]. Under this circumstance, it's substantially helpful for IVF success that performs preimplantation genetic testing for aneuploidy (PGT-A) in a PCCS manner, of which a few or even a single cell biopsy from *in vitro* cultured embryos are examined for genome wide chromosomal aneuploidy, then chooses the very embryo without chromosomal abnormalities to implant into the mother's womb [20, 25, 29–31].

A single cell genome counts for a few picogram DNA only. To obtain sufficient initiative materials for NGS library construction, the single cell genome has to be amplified (whole genome amplification, WGA) to millions fold first. The conventional NGS library construction is a time-consuming process including multiple steps of DNA fragmentation, blunt end generation, adapter ligation, PCR amplification etc. The additional WGA process prior to these steps makes single cell sequencing an even more time-consuming process. On the other hand, there is clinical necessity that takes embryo biopsy on the day 3 (or 5) post *in vitro* fertilization then implant a high-quality embryo into the mother's womb on day 4 (or 6). This requires the PGT-A procedure to be accomplished overnight. It's almost impossible to achieve this requirement by the conventional single cell sequencing procedure described above.

A commercial product (Takara) is able to accomplish single cell WGA simultaneously with the sequencing library construction. The integrated WGA-NGS library construction protocol significantly reduced the time consuming of single cell sequencing thus enable the potential of overnight PGT-A. However, in the Takara's sequencing library, artificial sequences containing only two types of nucleotide, G and T, were added to flank each DNA fragment to be sequenced. In an Illumina sequencing reaction, the sequencing primer has to extend and read through these artificial bases first then reach to target sequence (Fig 1). Determined by Illumina's sequencing principle, the signal emission from the first a few base positions of a sequencing read is to be used for cluster recognition to initiate a sequencing run, and the four nucleotide types (A, G, C and T) is preferred to be equally distributed in each of these base positions by default [32]. Therefore, if Takara's WGA-NGS library product is loaded alone on an Illumina sequencer, as lacking of C and A in the first a few sequencing positions, the cluster recognition will not be accomplished properly thus the sequencing run will end up with failure. The way to solve this problem is to add artificial random fragments, i.e. Phix (Illumina, San Diego, USA), into the sequencing library. In this manner, the library is sequenced in combined with the added in Phix fragments which provides randomness of the four nucleotides thus enables proper cluster recognition. The minimal amount of Phix fragments added in should not be less than 10% of total reads number [33], whereas these reads are certainly not to contribute to effective data.

An overnight PGT-A assay is typically a sequencing run required by one particular patient with embryo biopsy samples up to 15. It's estimated that minimally 500–1000 K effective reads would be needed to confidently reporting aneuploidy for any given chromosome in PGT-A (internal data, unpublished). Therefore, a sequencer with moderate throughput, such as



Table 1. The primer designs.

Designs	Sequences(5'-3')
Original MALBAC	GTGAGTGATGGTTGAGGTAGTGTGGAGNNNNNGGG
	GTGAGTGATGGTTGAGGTAGTGTGGAGNNNNNTTT
1	GCTCTCCGATCTNNNNNGGG
	GCTCTCCGATCTNNNNNTTT
2	GCTCTCCGATCTNNNNNGGG
	GCTCTCCGATCTNNNNNTTT
3	GCTCTCCGATCTNNNNNGGG
	GCTCTCCGATCTNNNNNTTT
4	GCTCTCCGATCTNNNNNGGG
	GCTCTCCGATCTNNNNNTTT
5	GCTCTCCGATCTNNNNNGGG
	GCTCTCCGATCTNNNNNTTT
exponential amplification primer- modified Illumina adapter sequence	Upstream: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	Downstream: CAAGCAGAAGACGGCATACGAGAT [Barcode]
	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

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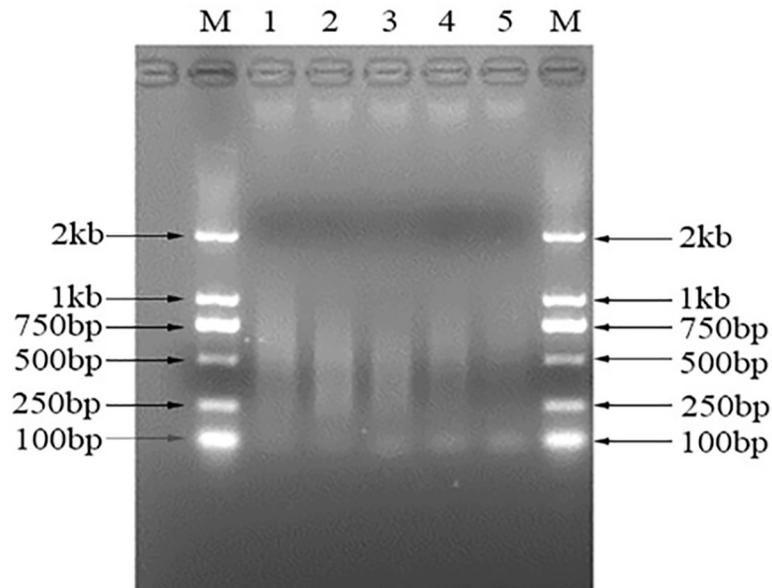
agarose gel (2%) electrophoresis were used to evaluate the efficiency and size range of WGA product (namely, sequencing library).

In the second phase, single cells of *in vitro* cultured human lymphocytes (GM12878) were picked by mouth pipetting under stereo microscope, then used as biological samples. Illumina NGS sequencer MiSeq was used to evaluate the quality of sequencing library upon the integrated WAG-NGS library construction process. To apply multiple samples in a single sequencing run, unique barcode sequences were incorporated respectively into the primers of exponential amplification (of WGA) for each sample. After the WGA-NGS library construction, the libraries of each sample were mixed at equal volume and then purified with MagBead DNA Purification Kit (CoWin Biotech, Beijing, China) followed the manufacture's protocol. The DNA concentration of purified library mixture was determined by Qubit<sup>®</sup> 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) followed the manufacture's protocol. To determine the amount of library to be loaded into the sequencer, the molarity of the library was calculated from the Qubit results (in nanogram per microliter). The library was sequenced alone without adding of artificial random fragments (Phix). The MiSeq Reagent Kit v3 (theoretical Maximal 25 M reads production) was used and SE75 sequencing program was applied at early ending of 55bp following Illumina's sequencing protocol.

For data analysis, the sequencing raw data (in.bcl format) was demultiplexed and converted to the FASTQ format using a Perl script configure BclToFastq.pl in CASAVA(Consensus Assessment of Sequence and Variation, version 1.8.2) [35] package based on the sample sheet information. Illumina adaptors, low quality bases (bases with quality score less than 20) were removed from the FASTQ file using Trimmomatic [36] (version 0.35). High quality reads were mapped to human reference genome (hg19) [37] using BWA (Burrows-Wheeler Alignment Tool, version 0.7.12-r1039) with default parameters [38]. The mapped reads were sorted and converted to binary format.bam using SamtoBam.jar in Picard (version1.119) package [38].

In the third phase, trophoctoderm biopsy samples of *in vitro* cultured human embryo donated from an anonymous volunteer were examined to validate the application of the protocol established in clinical setting of overnight PCCS. The human embryo biopsies were collected on May 5, 2017 and testing and analysis were carried out from that day to May 6. We do not have access to information that can identify individual participants during or after data





**Fig 2. WGA-NGS library construction result from variable pre-amplification primer designs.** Fifty picograms human genomic DNA was used as amplification template. The amplification products are visualized on a 2% agarose gel as routine. M: DM2000 DNA Marker; Lane 1–5: the WGA products of design 1–5.

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adequate for comprehensive chromosomal aneuploidy screening (unpublished data). As the phase two results showed satisfied library uniformity among samples, we decided to perform the phase three test in a low read yield manner by using MiSeq reagent kit v2 that gives maximal reads production at 15M only, in examining the practicability of the protocol.

While the embryo biopsy samples were arrived in our laboratory at 6 pm, the WGA-NGS library construction was accomplished at 9:30 pm. After equal volume pooling, purification

**Table 2. QC data of 15 single cells in a single run of Miseq sequencing.**

sample	raw reads	GC%	high quality of raw	Mapping rate	Mapped of raw	Unique mapped of raw
1	1,514,213	40	96.04%	94.86%	91.1%	84.03%
2	1,616,350	40	95.79%	94.09%	90.14%	83.02%
3	1,837,612	40	96.07%	94.65%	90.93%	83.83%
4	1,495,434	40	96.12%	93.71%	90.07%	82.98%
5	2,381,030	40	96.32%	95%	91.51%	84.36%
6	1,944,712	40	96.28%	94.85%	91.32%	84.22%
7	1,992,210	40	96.27%	93.99%	90.48%	83.46%
8	2,174,977	40	96.7%	95.64%	92.48%	85.49%
9	1,700,875	40	96.46%	94.65%	91.31%	84.43%
10	1,567,411	40	96.06%	94.62%	90.9%	83.99%
11	1,505,962	40	96.18%	94.24%	90.64%	83.83%
12	2,110,626	40	96.24%	94.85%	91.28%	84.3%
13	1,481,780	41	96.09%	89.85%	86.34%	79.84%
14	1,888,895	40	96.14%	93.62%	90.01%	83.1%
15	1,389,200	40	95.88%	94.47%	90.58%	83.71%
CV <sup>a</sup>	16.9%	0.6%	0.2%	1.4%	1.5%	1.5%

<sup>a</sup>CV: coefficient of variation

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**Table 3. The timeline of overnight PGT-A procedure in this work.**

Steps	Duration (hr)	Hands on time (hr)
Reagent preparation upon sample receiving	0.5	0.5
WGA-NGS library construction	3	0.5
Library pooling, purification, quantification and loading into sequencer	2	2
Sequencing running	6	0
Data analysis	0.5	0.5
Total	12	3.5
Summary of major time saving measures	Integrate WGA and NGS library construction to a two-step PCR process of ~2.5 hr reaction time Library pooling at equal volume of each sample instead of conventional qPCR quantification A short length single-ended sequencing run of 55 bp	

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and quantification, the library mixture of 14 samples (in addition with positive and negative controls) was loaded onto the MiSeq sequencer at 11:30 pm. The sequencing run was accomplished at 5:30 AM on the next day and data analysis was accomplished at 6:00 AM. The timeline of the whole procedure and major measures to reduce time cost is summarized in the [Table 3](#).

The sequencing run was successfully accomplished with 13M reads at Q30 > 90% and an average read length of 55 bp. The average reads yield per samples was 871K, with maximal reads of 1.42M and minim reads of 541K ([Table 4](#)). Chromosomal CNVs were identified in 8 samples of the 14 embryos examined, while the other 6 samples were euploidy genome ([Fig 3](#)). No significant differences were observed in the paired comparison between the results above and the routine MALBAC-NGS based assay (data no shown).

## Discussion

In this work, we established a modified MALBAC protocol namely ChromInst, which combines the single-cell WGA and Illumina NGS library construction into an integrated procedure. In addition to time saving, the good compatibility of ChromInst with Illumina sequencing principle along with its good library yield uniformity provided superior data usage efficiency and reads distribution evenness. All these advantages allowed the feasibility of using ChromInst to perform overnight PCCS on multiple embryo biopsies in a sequencing run with relatively low throughput, such as by MiSeq reagent kit v2.

A collective evidence indicate that PGT-A in PCCS manner is beneficial to improve the success rate of IVF [[18](#), [20](#), [22](#), [23](#), [29](#), [45–47](#)], and NGS is a promising technique to perform PCCS [[1](#), [2](#), [5](#), [6](#), [48](#), [49](#)]. Nevertheless, the conventional NGS-PGT-A protocol takes days to accomplish the assay. As the optimal time for embryo biopsy is on the day 5 post *in vitro* fertilization and the timing for embryo implantation should be on the day 6 [[50](#)], in the conventional NGS based PGT-A, the post biopsy embryos have to be frozen in liquid nitrogen before the examination results turnover. The influence of embryo frozen is remaining in controversial [[51–55](#)]. A recent study published in the New England Journal of Medicine demonstrated that frozen embryo resulted higher IVF successful rate [[53](#)]. However, the same study also revealed that embryo frozen may cause higher chance of delivering overweight babies and three folds higher chance of gestational pre-eclampsia. Therefore, the benefit and risk balance between frozen and fresh embryo implantation is still to be further evaluated. At this moment, both

Table 4. QC data of 14 embryo biopsy sequencing.

sample	Raw reads	High quality GC%	High quality of raw	Mapping rate	Mapped of raw	Unique mapped reads	Unique mapped of raw
1	765,904	41	97.11%	97.17%	94.36%	621,999	81.21%
2	569,266	41	97.35%	97.43%	94.85%	457,252	80.32%
3	1,151,433	42	97.62%	97.87%	95.53%	954,220	82.87%
4	848,321	41	97.39%	96.82%	94.29%	680,302	80.19%
5	1,013,240	41	97.31%	97.32%	94.70%	823,884	81.31%
6	724,464	41	97.38%	97.03%	94.48%	585,789	80.86%
7	860,599	41	97.28%	97.61%	94.96%	709,675	82.46%
8	759,668	42	97.47%	97.05%	94.59%	619,175	81.51%
9	818,820	41	97.15%	97.67%	94.88%	676,557	82.63%
10	541,819	42	97.59%	97.60%	95.26%	439,336	81.09%
11	1,018,913	42	97.45%	97.78%	95.28%	837,823	82.23%
12	813,351	41	97.41%	97.25%	94.73%	657,646	80.86%
13	1,420,866	41	97.25%	90.75%	88.25%	1,086,243	76.45%
14	899,714	42	97.18%	96.31%	93.60%	732,360	81.40%
NC <sup>a</sup>	33,236	43	90.89%	57.68%	52.43%	15,001	45.13%
PC <sup>b</sup>	959,591	41	97.62%	95.13%	92.87%	764,751	79.70%
CV <sup>c</sup> (except NC)	25.1%	1.2%	0.2%	1.8%	1.9%	24.0%	1.9%

<sup>a</sup>NC: negative control, reagent only

<sup>b</sup>PC: positive control, 20 pg genomic DNA

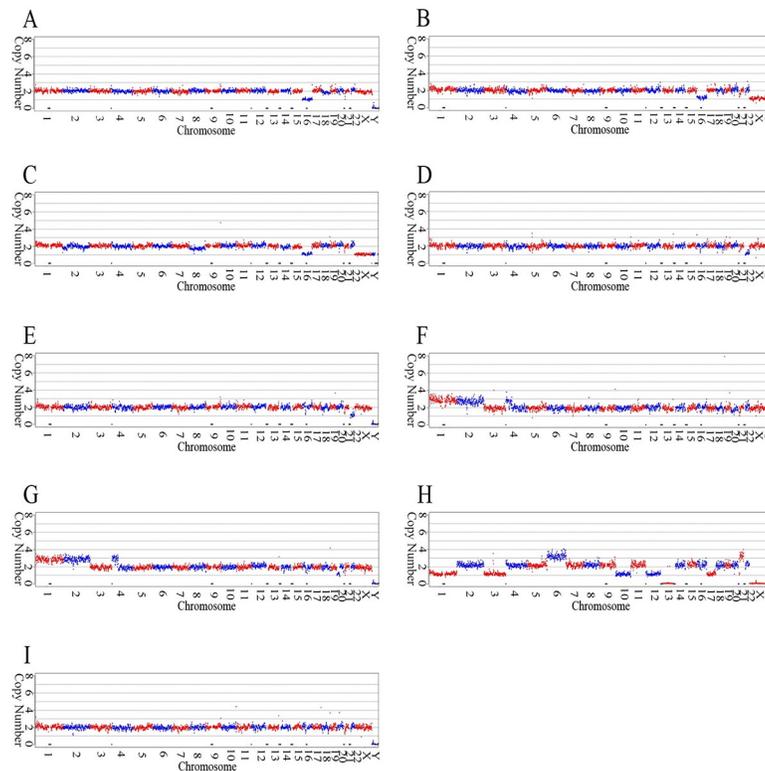
<sup>c</sup>CV: coefficient of variation

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frozen and fresh embryo implantation are applied in clinical practices. Furthermore, for a patient failed of pregnancy in the first attempt of conventional IVF procedure, her leftover embryos (have to be frozen) are worthy to PGT-A prior to the next try of implantation. Once defrosted, one should avoid the embryos from frozen again before implantation when at all possible. In this circumstance, the overnight PGT-A can be the only way to perform PCCS to improve the chance of IVF success in the second attempt of embryo implantation. As the result, it is necessary to establish an overnight PGT-A procedure that enables fresh embryo implantation in the reality.

The WGA amplification of ChromInst is based on that of MALBAC [34]. MALBAC is a relatively new WGA method which was invented in 2012. It's amplification uniformity and allele dropout (ADO) rate are superior to previous WGA methods, such as Degenerate Oligonucleotide Primer PCR (DOP-PCR) and Multiple Displacement Amplification (MDA) [56–58] for single cell genome amplification. Two major modifications of the ChromInst protocol were substituting the 27 bp constant part of MALBAC's pre-amplification primer sequence to the 13 bp (Table 1 Design 2) of the 3' part of Illumina's library construction adaptor and were extending the straight 5 "N" to 6 "N" (Table 1, Fig 1). These modifications kept the amplicon looping forming feature of MALBAC in the pre-amplification stage thus inherited the innate high uniformity feature in MALBAC WGA, while accomplishes the sequencing library construction simultaneously. As illustrated in the Fig 3, the WGA uniformity of ChromInst was sufficient for effectively detecting chromosomal aneuploidy from embryonic biopsy by NGS.

When multiple samples are simultaneously examined on a sequencer with limited reads yield capacity, the reads number uniformity over each sample in the sequencing run is important to ensure all samples covered by sufficient sequencing data. Normally, the good uniformity of inter-sample reads yield is achieved by qPCR quantification to the sequencing library of each sample, then pool the samples in equal molar manner. The qPCR is however not



**Fig 3. Chromosomal CNV examination of embryo biopsy samples.** Chromosomes are aligned along X axis from 1 to 22 with X and Y at the end. Chromosomal copy number was set as Y axis. Aneuploidy was identified from 8 of the 14 embryos examined, which are as shown in the panel A: Sample 1; B: Sample 4; C: Sample 5; D: Sample 6; E: Sample 7; F: Sample 8; G: Sample 9; H: Sample 13. Panel I shows a random example of normal diploid genome (Sample 11).

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generally available in the clinical laboratory setting and the extra time cost is highly undesirable for an overnight assay constrained by time. In our protocol presented, by merit of the reaction constancy of ChromInst, sequencing library of each sample was simply pooled at equal volume prior to load into the sequencer. And the resulted reads yield uniformity was fairly acceptable in term of either reads number on each sample or reads number CV among samples (Tables 2 and 4). Consequently, the ploidy of the 14 tested embryos, including 6 euploidy and 8 aneuploidy samples, were all well identified by merely 13M total reads yield from the sequencing run (Fig 3). The concise experimental protocol and satisfactory examination results demonstrated that the presented approaches are well practical to accomplish PCCS overnight in clinical laboratory setting.

In principle, the sequencing library construction of ChromInst belongs to the category of “random short fragmentation” that is incapable of detecting the chromosomal structural rearrangement, such as inversion and translocation. In Mate-Pair sequencing, self-cyclization of long DNA fragment is conducted prior to library construction, thus chromosomal structural rearrangement can be identified by sophisticated strategy in subsequent sequencing data analysis [59]. However, the integration of single cell WGA with Mate-Pair library construction remains a great challenge. We also observed that the ADO rate of ChromInst was not as good as in the original MALBAC (unpublished data), presumably due to the suboptimal design of the pre-amplification primers in comparison to the original MALBAC. For this reason, ChromInst may not be suitable in detecting SNPs or point mutations like other single-cell NGS applications.

In summary, a modified MALBAC protocol, ChromInst, was established in this work, which combines single cell WGA and Illumina sequencing library construction into an integrated procedure that reduces the time cost of WGA plus sequencing library construction to an approximately 2.5 hours reaction time. By maintaining the merit of high amplification uniformity of original MALBAC WGA and additional conciseness and superior data efficiency, ChromInst fulfills the clinical necessity of NGS based overnight PCCS. These advantages are also permitting the application of ChromInst in other pressing single-cell NGS applications.

## Supporting information

**S1 Raw images. The original image of Fig 2.**  
(PDF)

## Author Contributions

**Conceptualization:** Si-Jia Lu, Jun Ren.

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**Formal analysis:** Fang-Fang Gao, Li Chen, Shi-Ping Bo, Ya-Xin Yao.

**Funding acquisition:** Si-Jia Lu.

**Investigation:** Fang-Fang Gao, Li Chen.

**Methodology:** Fang-Fang Gao, Jun Ren.

**Project administration:** Fang-Fang Gao.

**Resources:** Si-Jia Lu.

**Supervision:** Jun Ren.

**Validation:** Zhong-Li Xu, Qing-Yu Ding.

**Visualization:** Peng Zhang.

**Writing – original draft:** Fang-Fang Gao.

**Writing – review & editing:** Jun Ren.

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