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Single-Molecule Analysis of Myocyte Differentiation Reveals Bimodal Lineage Commitment

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Abstract

Cell differentiation is the foundation for tissue development and regeneration, disease modeling, and cell-based therapies. Although the differentiation of cell populations has been extensively studied in many systems, much less is known about the distribution of decision making of single cells within these populations. To characterize the differentiation of single skeletal muscle cells, we used single-molecule mRNA fluorescence *in situ* hybridization (smFISH) to precisely quantify the expression levels of the master myogenic regulatory factors MyoD and myogenin in individual myoblasts. We identified distinct cell states characterized by the number of myogenin transcripts expressed by a cell, with myoblasts stochastically transitioning to a myogenin-high state during differentiation. We also used MyoD overexpression to force the transdifferentiation of C3H10T1/2 cells into an induced myoblast phenotype. These reprogrammed cells revealed the presence of a critical threshold of MyoD expression required to initiate myogenin expression. These results provide quantitative single-molecule data to support the model of switch-like cell decision making and lineage specification.

Keywords

Myogenesis; lineage commitment; single-cell analysis; cellular heterogeneity; genetic reprogramming

Introduction

Increasing the resolution of measurements of cell lineage commitment can enhance the general understanding of cellular decision making and improve the ability to guide cell behavior for regenerative therapies. In particular, single-cell and single-molecule

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Author Contributions

T.M.G. conducted all experiments. T.M.G. and C.A.G. designed experiments, analyzed data, and wrote the manuscript.

measurements can elucidate how cell populations are robust in their decision making, despite the intrinsically stochastic intracellular environment¹⁻³. Noise in gene expression within a cell population can significantly influence cell lineage commitment⁴ and genetic reprogramming^{5, 6}. Cells adapt to stochasticity by developing intricate gene regulatory networks that produce either switch-like or graded response to stimuli^{2, 7, 8}. The regulatory networks that control cell differentiation have been generally regarded as an irreversible switch, in contrast to a tunable rheostat⁹. However, reports of direct molecular measurement of mammalian cell switching between phenotypes are sparse. Monitoring cell differentiation with single-molecule methods can generate richer information of cell decision making and assist in the inference of the underlying gene regulatory networks¹⁰. This, in turn, can lead to the characterization of previously unknown hierarchies of gene regulation during differentiation^{5, 11}.

The gene regulatory network governing skeletal myoblast differentiation is particularly well-characterized. Therefore it is an ideal model system for analysis of heterogeneity in cell lineage commitment. The progenitor cells of adult muscle are the satellite cells that maintain a quiescent and nondividing state until stimulated to re-enter the cell cycle^{12, 13}. Once cells are activated to divide, the master transcription factor MyoD is upregulated¹⁴⁻¹⁶. MyoD is responsible for inducing genome-wide chromatin changes that poise the genome for myogenesis, as well as upregulating expression of myogenin, another MRF that drives terminal myogenesis¹⁷⁻²⁰. Myogenin expression is required for cells to fuse into myotubes and to form muscle fibers²¹. Additionally, activation of myogenin expression marks commitment to terminal myogenic differentiation, after which cells will upregulate a host of genes specific to mature myocytes²².

Although the general mechanism of myogenic differentiation is well-characterized, our understanding of single-cell behavior within this mechanism is much more rudimentary. Early experiments identified that a subpopulation of myoblast cell lines would not differentiate or activate myogenin, suggesting they may be “reserve cells” for later differentiation²³. More recently, single-cell RNA-seq revealed switch-like activation of key regulatory factors¹¹. In this study, we applied single-molecule mRNA fluorescence *in situ* hybridization (smFISH) to test the hypothesis that cell commitment to terminal myogenic differentiation is bimodal, with a particular focus on the activation of myogenin expression as the indicator of commitment to myogenic differentiation. smFISH provides a quantitative and sensitive method to measure gene expression in individual cells²⁴. Because this method visualizes individual mRNA transcripts, it provides exceptional sensitivity of gene expression levels and resolution of the spatial distribution of mRNA transcripts in single cells³. We observed a stochastic transition by myoblasts from their undifferentiated myogenin-low phenotype to a lineage-committed myogenin-high phenotype. By controlling the dose of exogenous MyoD expression, we demonstrated that activation of myogenin expression occurs only above a critical threshold of MyoD levels. Our findings provide direct molecular evidence that the upregulation of myogenin by MyoD, which drives cells to differentiate into a terminal myocyte phenotype, is a switch-like process. These observations provide strong molecular-level support for the current models of cellular decision making and lineage determination.

Materials and Methods

Plasmid cloning

The self-inactivating lentiviral transfer vector TMPrtTA²⁵ was modified to remove the murine SEAP gene by MluI digest. A BstXI restriction site was removed from the multiple cloning site by inserting a point mutation. The IRES-Puro(r) sequence from Addgene plasmid 21313²⁶ was amplified by PCR and inserted into the modified TMPrtTA at the remaining BstXI site to create TPriP. An NsiI site and DNA sequence encoding the T2A ribosomal skipping peptide were added upstream of the coding sequence for DsRed-Express2 (Addgene plasmid 21770) by PCR, and the resultant amplicon was inserted into TPriP using the MluI and NheI restriction sites to produce TDPriP. The MyoD sequence from EMSV-MyoD²⁷ was amplified without the stop codon by PCR and inserted into TDPriP at the MluI and NsiI sites to produce T-MyoD-DPriP (Addgene plasmid #60624). In order to generate a longer mRNA sequence for the purposes of increased smFISH labeling, a stop codon was placed at the beginning of a LacZ coding sequence from pFRT/lacZeo (Invitrogen V6015) via PCR, which was inserted in place of the DsRed-Express2 sequence between the NsiI and NheI restriction sites to produce T-MyoD-ZPriP (Addgene plasmid #61441). All new plasmids have been deposited to the Addgene plasmid repository (plasmid #60623-4).

Cell culture

C2C12 cells were maintained in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Fetal Bovine Serum (FBS). Primary mouse myoblasts were maintained on collagen-coated plates in a growth medium consisting of F-10 medium supplemented with 20% FBS. Differentiation conditions for both of these sources of myoblasts involved substituting 2% Horse Serum (HS) in place of the FBS, and all cells were seeded at a density of 1,050 cells/cm² 24 hours prior to inducing differentiation. Primary myoblasts were differentiated on coverslips coated in 20 µg/mL ECL matrix (Millipore). C3H10T1/2 mouse multipotent mesenchymal progenitor cells were maintained in DMEM with 10% heat-inactivated FBS. HEK293T and NIH3T3 cells were maintained in a growth medium consisting of DMEM with 10% FBS.

Lentiviral transduction

The production of lentivirus and transduction of cells was performed based on a previously established protocol²⁸. Briefly, 2 million HEK293T cells were seeded in 10 cm tissue culture dishes. Each dish was transfected with 10 µg of the transfer vector T-MyoD-DPriP or T-MyoD-ZPriP with 8 µg psPAX2 and 3 µg pMD2G using calcium chloride precipitation. After 24 hours incubation, viral supernatant was harvested daily and purified by passing through a 0.45 µm filter. Viral supernatant was flash-frozen in liquid nitrogen and stored at -80 °C. C3H10T1/2 cells were transduced by adding the viral supernatant to the cell culture for 24 hours with 4 µg/mL polybrene. Transduced cells were selected with 2 µg/mL puromycin for at least 3 days. Expression of the MyoD transgene was induced by adding doxycycline to DMEM with 1% FBS in concentrations ranging from 10 ng/mL to 3,000 ng/mL.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA was extracted from cell samples using the RNEasy Plus Mini kit (QIAGEN). Reverse transcription was performed using the SuperScript VILO cDNA Synthesis kit (Life Technologies). Quantitative PCR was performed by mixing the cDNA and appropriate primers with the Ssofast EvaGreen supermix (Bio-Rad). PCR reactions were performed with a CFX96 Real-Time PCR Detection System (Bio-Rad). The primer sequences used were optimized to give 90-110% amplification efficiency per cycle (**Supplementary Table 1**). qRT-PCR results were quantified using the 2^{-C_t} method to indicate the change of expression of each gene relative to the GAPDH housekeeping gene²⁹.

Single molecule mRNA fluorescence *in situ* hybridization

smFISH staining was based on previously published protocols³⁰. Probes were designed using the first version of the Stellaris Designer (Biosearch Technologies) to recognize the full MyoD-LacZ mRNA fusion. Probe sets were stored in 12.5 μ M stocks in TE buffer (**Supplementary Table 2**). Cells were grown on coated #1 glass coverslips and fixed in 3.7% formaldehyde in PBS for 10 minutes. The cultures were washed with PBS, then stored in 70% ethanol at 4 °C for at least an hour and for no longer than one week. To hybridize the probes to the target mRNA, the slides were washed once in a 10% formamide, 2x saline-sodium citrate (SSC) buffer. Hybridization buffer was made of 100 mg/mL dextran sulfate and 10% formamide in 2x SSC. smFISH probes were added to the hybridization buffer to a final concentration of 50 nM for the MyoD and LacZ probes conjugated to Quasar 670 and 250 nM for the myogenin probes conjugated to CAL Fluor Red 610. Cells were incubated in the hybridization buffer at 37 °C for at least four hours and washed twice in wash buffer. Finally, the cover slips were mounted on slides and imaged using a DeltaVision Elite microscope (Applied Precision). Deconvolution and image stitching was performed using the softWoRx Suite (Applied Precision). Images were analyzed using Imaris (Bitplane) and smFISH spots were identified and quantified using the Spot Detection feature of the program. The spot detection was performed by thresholding based on quality score and maximum spot intensity such that the values were set at a local minimum of the first derivative of the spot counts relative to the thresholding values²⁴. Multimodality of data was verified using the diptest package in R to perform Hartigan's Dip Test of Unimodality³¹.

Immunofluorescence staining

The primary antibodies used were anti-myogenin at a 1:50 dilution (F5D, Santa Cruz Biotech sc-12732) and anti-myosin heavy chain conjugated to Alexa Fluor (AF) 488 at a 1:200 dilution, (MF-20, Affymetrix 53-6503-80). The secondary antibody used was goat anti-mouse IgG conjugated to AF647 at a 1:1000 dilution (Invitrogen A21236). Nuclei were stained with DAPI. Cells were grown on glass coverslips coated with either gelatin or ECL. For myogenin staining, cells were fixed in methanol at -20 °C. For myosin heavy chain staining, cells were fixed in 5% formaldehyde and 8% acetic acid in 70% ethanol. Cells were incubated in a blocking buffer made up of 5% bovine serum albumin (BSA), 5% goat serum, and 0.1% Triton X-100 and incubated with primary antibodies for 3 hours at room temperature or at 4 °C overnight. Cover slips were then rinsed in PBS, incubated with the secondary antibody for 1 hour at room temperature, and then rinsed and mounted on glass

slides with Prolong Gold antifade reagent (Life Tech., P36934). Immunofluorescence images were quantified using a custom macro in ImageJ. Briefly, acquired images were auto-thresholded for both DAPI and myogenin staining. The Particle Analyzer tool in ImageJ was used to count the nuclei. A nucleus was counted as positive if the DAPI particle was identified over the Otsu threshold and the myogenin particle was identified visually. A nucleus was counted as positive for the MF20 stain if the DAPI particle had over a 90% overlap with a MinError threshold on the MF20 stain.

Statistical analysis

Least-squares linear regressions of the data were performed using the MATLAB Curve Fitting Toolbox (Mathworks). The Curve Fitting Toolbox was also used to produce 95% confidence intervals for the fit parameters, and the residual plots of linear regressions were checked to verify the validity of the linear fit. Additionally, Pearson correlation coefficients were calculated for the linear regression samples.

K-means clustering was performed in MATLAB. Briefly, the algorithm randomly assigns data points to clusters and iteratively optimizes clustering by re-assigning data points to the cluster with the nearest centroid to minimize the sum of the absolute distance between the data points, subsequently re-calculating the centroids of the clusters until an optimum has been reached.

Results and Discussion

Myogenin activation predicts upregulation of markers of terminal differentiation

We used three systems to model myoblast differentiation. The first two included the differentiation of the immortalized C2C12 mouse skeletal myoblast cell line and primary mouse skeletal myoblasts by withdrawal of growth factor-containing serum (**Fig. 1A**). The final model consisted of forced overexpression of MyoD in the mouse C3H10T1/2 mesenchymal progenitor cell line. This strategy used a lentiviral vector encoding MyoD under the control of doxycycline such that the dose of differentiation stimulus could be tightly controlled (**Fig. 1B**). The C3H10T1/2 cell line is a multipotent line derived from whole mouse embryos and is able to produce contractile myotubes in response to MyoD expression³². Following differentiation by serum withdrawal or doxycycline induction, mRNA expression in the bulk cell population was assayed by qRT-PCR, including MyoD, myogenin, and the downstream myogenic markers MEF2C and desmin (**Fig. 1C**). All differentiation protocols led to an upregulation of myogenin as well as downstream markers. Because the C2C12 cells and primary myoblasts are already committed to the myoblast fate, MyoD levels were high at the initiation of the experiment and did not substantially change during differentiation^{23, 33}. In contrast, MyoD levels in the transduced C3H10T1/2 cells were quickly upregulated in response to doxycycline. To visualize myogenin protein levels in single cells, we used immunofluorescence staining of the C2C12 myoblasts and C3H10T1/2 cells at each day over seven days of differentiation (**Fig. 1D**). The frequency of myogenin-positive nuclei was as high as 40-50% of cells in both the C2C12 and C3H10T1/2 cell lines (**Fig. 1E-F**). Myogenin levels in C2C12 cells reached a maximum at day five. In contrast, the C3H10T1/2 cells reached a maximum myogenin level at days two and three,

and then decreased over the next four days. Myogenin is broadly accepted as a marker of terminal differentiation that initiates expression of markers of terminally-differentiated myocytes³⁴⁻³⁶. Consistent with this model, co-staining for the late marker myosin heavy chain and myogenin showed that approximately 80% of myogenin-positive nuclei were also positive for myosin heavy chain after seven days of differentiation (**Fig. 1G**). These observations support myogenin expression as an accurate marker of terminal myogenic commitment.

Myogenin expression in differentiating cells follows a bimodal population distribution

Although it is well-understood that myogenin is upregulated in differentiating muscle tissue and coincides with a commitment to terminal myogenic differentiation, this transition has not been studied in detail at the single-cell level. In particular, the degree of variability in the decision to activate myogenin between cells under otherwise uniform differentiation conditions is unknown. Furthermore, the extent to which this decision depends on expression levels of the upstream myogenic regulatory factor MyoD is unclear. Therefore we used smFISH to determine the exact copy numbers of MyoD and myogenin mRNAs in individual differentiating cells. Consistent with measurements of mRNA expression in myoblasts at the bulk level (**Fig. 1C**), we found that MyoD mRNA levels did not change significantly after serum withdrawal (**Fig. 2A-B**). In contrast, only background levels of MyoD mRNA signal were observed in the C3H10T1/2 cells, and these levels increased more than 10-fold after induction with 3,000 ng/ml doxycycline that saturated the induction of expression in the bulk population. This transition occurred with the same kinetics as the bulk qRT-PCR measurements (**Fig. 1C**), and was strikingly consistent with a uniform distribution across the cell population.

In response to differentiation signals, myogenin expression in most individual cells dramatically increased, including an approximately 100-fold increase in transcript levels (**Fig. 2A-B**). We observed similar trends of myogenin activation in all three models of myogenic differentiation, consisting of a mean expression of myogenin mRNA reaching a steady state level of approximately 1000 molecules per cell in five days. The C3H10T1/2 cells quickly upregulated myogenin expression in response to the MyoD stimulus, with 72% of cells expressing at least 100 myogenin transcripts after one day and 91% after three days, compared to background levels in the uninduced state of less than 10 transcripts per cell. The response in C3H10T1/2 cells was particularly homogeneous, as indicated by the rapid decrease in the variation of myogenin expression following the first day of dox induction (**Fig. 2C**). Conversely, the population distribution of myogenin expression in the immortalized and primary myoblasts took longer to stabilize. This is particularly interesting since these cells already express MyoD, and may be indicative of the time necessary for myoblast-specific decay of inhibitors of myogenin activation. A significant subset of C2C12 cells did not activate myogenin. These may be similar to the “reserve cells” observed in prior studies²³ or they may simply be cells that have lost differentiation potential. Notably, the frequency of myogenin-negative cells is smaller in the primary myoblasts compared to the C2C12 cell line.

To determine whether there was a direct correlation in individual cells between levels of MyoD transcripts and activation of the myogenin gene, we plotted MyoD versus myogenin expression in each cell over the course of seven days of dox induction (**Fig. 3A**). Interestingly, for the subpopulation of cells that transitioned to the activated myogenin state with more than 100 mRNAs per cell by day 5, there was a strong positive correlation between MyoD levels and myogenin levels in all cell lines, with correlation coefficients 0.65 (**Fig. 3B**). This relationship emerged earlier in the C3H10T1/2 cells, consistent with faster differentiation kinetics. Fitting these MyoD- and myogenin-high cells to a linear regression showed that, in all cases, the slope of the line was significantly greater than zero ($\alpha < 0.05$, tested over multiple hypotheses using the Bonferroni correction) (**Fig. 3C**). The strong correlation and nonzero linear slope supports a model in which the level of myogenin expression is proportional to the level of MyoD expression in individual differentiated cells. This observation may be the result of a direct effect of MyoD transactivation of the myogenin promoter, or could also be caused by factors that were not measured here, such as global gene expression levels or cell size.

A critical amount of MyoD is required to transition to the myogenin-high cell state

The time course of MyoD reprogramming in C3H10T1/2 cells used a saturating amount of doxycycline to drive MyoD expression. However, these MyoD levels were greater, on average, than natural MyoD expression levels in the immortalized and primary myoblasts (**Fig. 2A**). To examine how varying amounts of MyoD expression influence myogenic differentiation, we induced the C3H10T1/2 cells with varying concentrations of doxycycline for three days. We observed a unimodal population-wide response in the induction of MyoD expression that was proportional to doxycycline concentration, with only few cells that did not respond (**Fig. 4A**). In contrast, at low concentrations of doxycycline, we observed a bimodal population distribution of myogenin expression levels, whereas at high doxycycline concentrations there were much fewer cells with low myogenin levels. The aggregate myogenin data produced a multimodal distribution (Hartigan's Dip Statistic = 0.567, $p < 1e-10$). To determine the level of MyoD expression required for commitment to myogenic differentiation, we plotted the number of myogenin transcripts as a function of the number of MyoD transcripts for all concentrations of doxycycline (**Fig. 4B**). We found that the majority of cells with activated myogenin expression (>100 transcripts/cell) also expressed at least 100 viral MyoD transcripts per cell. Furthermore, the cells with high levels of myogenin showed a strong correlation between MyoD and myogenin expression levels similar to what was observed at individual time points (**Fig. 3**). This suggests that there is a critical threshold of MyoD required to activate commitment to myogenic differentiation. To visualize the transition between these two states, we performed K-means clustering on the data (**Fig. 4C**), which strongly indicates there are two distinct states in this population.

We performed co-immunofluorescence staining with smFISH staining to simultaneously assess myogenin mRNA levels and protein expression (**Fig. 4D-F**). In general, most cells that have high myogenin mRNA levels by smFISH are also positive for myogenin protein by immunofluorescence staining. However, some cells have high myogenin mRNA levels but are negative for myogenin protein staining, particularly at day 7 in the MyoD-transduced

C3H10T1/2 cells. This lack of protein detection may indicate post-transcriptional regulation of the myogenin protein or simply insufficient sensitivity of immunostaining.

Collectively, these results support a model of commitment to myogenesis that occurs in a switch-like fashion at the single-cell level. This work also supports earlier studies showing myogenin expression is a strong early indicator of cells that commit to terminal myocyte differentiation¹⁷⁻²⁰. Cells generally exist in a state with few to no myogenin transcripts or a state of highly expressed myogenin, but they do not express intermediate levels at steady state, even when induced to expression intermediate levels of the upstream master myogenic regulatory factor MyoD. Therefore, myogenin upregulation does not occur proportionally to the expression of MyoD, but is induced uniformly above a critical threshold level of MyoD expression. For the multipotent C3H10T1/2 cell line, this level is achieved when cells have approximately 100 MyoD mRNA transcripts; however, this number may be dependent on the cell type in which MyoD is overexpressed. Interestingly, there is a positive relationship between MyoD and myogenin levels above this threshold. These results underscore the tight control of the gene regulatory network underlying myogenesis and highlight the ability of single-molecule detection technologies to provide novel insights into the dynamics of this network. This type of molecular analysis complements the study of other chemical and mechanical stimuli, such as growth factors, matrix elasticity, and surface topography, that control cell fate decisions³⁷⁻³⁹. Continued enhancement of the resolution at which cell decisions are studied can ultimately lead to improvements in therapies for myogenic disorders and well-defined models for lineage commitment and cell fate specification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Induction of myogenesis. (A) C2C12 or primary myoblasts are maintained under high serum conditions and are stimulated to differentiate by serum withdrawal. (B) MyoD is delivered to C3H10T1/2 cells using a tetracycline-inducible lentiviral vector. The phosphoglycerate kinase promoter (PGK) constitutively expresses a variant of the reverse tetracycline transactivator (rtTA2S-M2) as well as the puromycin resistance gene, separated by an internal ribosomal entry site (IRES). In the presence of doxycycline (dox), rtTA2S-M2 binds to the tetracycline response element (TRE) and activates expression of murine MyoD. This MyoD transcript includes a non-translated sequence for the LacZ gene following the stop codon (TGA). The LacZ sequence serves as an mRNA tag that enables visualization of the viral mRNA using smFISH. (C) Changes in gene expression of MyoD, myogenin, MEF2C, and desmin during myogenic differentiation. Gene expression was determined by qRT-PCR and normalized to GAPDH levels. (D) Representative image of immunofluorescence

staining for myogenin and myosin heavy chain (MHC) in C2C12 cells. Scale bar = 100 microns. (E-F) Percentage of nuclei that stain positive for myogenin in C2C12 myoblasts and C3H10T1/2 cells reprogrammed by MyoD. (G) Percentage of the myogenin-positive cells that are also positive for co-staining of the terminal marker of myogenic differentiation, myosin heavy chain (MyHC) after seven days.

Figure 2. Single-molecule quantification of MyoD and myogenin mRNA levels during differentiation. (A) Expression levels of MyoD and myogenin in individual cells over seven days of differentiation. Red lines indicate population average. (B) Representative images of smFISH staining (blue = DAPI, red = MyoD, green = myogenin). Scale bar = 10 μ m. (C) The coefficient of variation in myogenin expression levels across the cell population.

Figure 3.

The relationship between MyoD and myogenin expression in single cells. (A) The data from Figure 2 is plotted showing myogenin levels as a function of MyoD levels in single cells. Dashed lines in the day 5 and 7 plots illustrate a threshold of 100 mRNA molecules per cell. (B) The data in (A) is plotted showing only the cells with MyoD and myogenin levels greater than 100 mRNA molecules/cell. Gray lines show a linear regression fit to the data for this cell subpopulation. (C) Slopes of the linear regressions obtained in B. Error bars indicate the 95% confidence intervals. The values are all significantly greater than a slope of zero ($\alpha < 0.05$, Bonferroni correction).

Figure 4.

Correlation of myogenin upregulation to MyoD levels. A) MyoD and myogenin mRNA levels in the transduced C3H10T1/2 cells are quantified in single cells three days after MyoD induction with various concentrations of doxycycline. Red dashes indicate population mean. B) Same data as in (A), plotted as the relationship between MyoD and myogenin. C) Same as in (B), but recolored based on which cluster each cell is assigned to by K-means clustering. The centroids of each cluster are marked with an X. D) Representative images of immunofluorescence and smFISH co-staining in MyoD-reprogrammed C3H10T1/2 cells.

Red = anti-myogenin protein stain, green = myogenin smFISH mRNA stain, blue = DAPI nuclear stain. E) Immunofluorescence and smFISH co-staining for myogenin protein and mRNA, respectively, at zero, three, and seven days after 1000 ng/mL doxycycline induction. Cells that had visible anti-myogenin protein stain were categorized as “IF+”. F) Same as in (E), but for C2C12 cells induced to differentiate by serum withdrawal.

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