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Nijmegen, 23 December 2019

Manuscript revision to JoVE

Dear Dr. Steindel,

With this letter we like to submit our revised manuscript to JoVE, entitled '*Characterization of an in vitro differentiation method of human primary keratinocytes by RNA-seq analysis*', which has a changed title according to referee's comments.

In this revision, we have tried our best to address your and referees' comments. However, we also need to point out that some of the comments seem to be more appropriate for an original protocol that is more flexible for changes and where home-made buffers and enzymes can be used. We believe that this was not the purpose of this manuscript, as previously discussed with the inviting editor. This protocol is to provide a guide on how to perform the described experiments, with necessary trick during the procedure. Please let us know if you have any further comment and suggestion, and we can adjust our manuscript.

Sincerely,

Jo Huiqing Zhou, PhD
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TITLE:

Characterization of In Vitro Differentiation of Human Primary Keratinocytes by RNA-Seq Analysis

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SUMMARY:

Presented here is a stepwise procedure for in vitro differentiation of human primary keratinocytes by contact inhibition followed by characterization at the molecular level by RNA-seq analysis.

ABSTRACT:

Human primary keratinocytes are often used as in vitro models for studies on epidermal differentiation and related diseases. Methods have been reported for in vitro differentiation of keratinocytes cultured in two-dimensional (2D) submerged manners using various induction conditions. Described here is a procedure for 2D in vitro keratinocyte differentiation method by contact inhibition and subsequent molecular characterization by RNA-seq. In brief, keratinocytes are grown in defined keratinocyte medium supplemented with growth factors until they are fully confluent. Differentiation is induced by close contacts between the keratinocytes and further stimulated by excluding growth factors in the medium. Using RNA-seq analyses, it is shown that both 1) differentiated keratinocytes exhibit distinct molecular signatures during differentiation and 2) the dynamic gene expression pattern largely resembles

cells during epidermal stratification. As for comparison to normal keratinocyte differentiation, the results show altered morphology and molecular signatures of keratinocytes carrying mutations of the transcription factor p63, which have differentiation defects. In conclusion, this protocol details the steps for 2D in vitro keratinocyte differentiation and its molecular characterization, with an emphasis on bioinformatic analysis of RNA-seq data. Because RNA extraction and RNA-seq procedures have been well-documented, it is not the focus of this protocol. The experimental procedure of in vitro keratinocyte differentiation and bioinformatic analysis pipeline can be used to study molecular events during epidermal differentiation in healthy and diseased keratinocytes.

INTRODUCTION:

Human primary keratinocytes derived from the human skin are often used as a cellular model to study the biology of the epidermis[1-4]. The stratification of the epidermis can be modeled by keratinocyte differentiation, either in a 2D submerged monolayer fashion or 3D air-lift organotypic model[2,3,5-7]. Although 3D models have become increasingly important to assess the epidermal structure and function, 2D differentiation models are still widely used, due to their convenience and the possibility to generate large numbers of cells for analyses.

Various conditions have been applied for inducing keratinocyte differentiation in 2D, including addition of serum, high concentration of calcium, lower temperature and inhibition of epidermal growth factor receptors[2,3]. Each of these methods has been validated by a number of keratinocyte differentiation marker genes and shown to be effective in assessing keratinocyte differentiation, including under pathological conditions. However, these induction conditions also show differences in their differentiation efficiency and kinetics when specific panels of marker genes are examined[2,3].

One of these methods involves keratinocyte contact inhibition and depletion of growth factors in the culture medium[8]. It has been shown that keratinocytes can differentiate spontaneously when cells reach full density. Excluding growth factors in the culture medium can further enhance differentiation. The method combining contact inhibition and depleting growth factors has been shown to generate differentiated keratinocytes with gene expression patterns similar to the normal stratified epidermis when using several epidermal markers[3], suggesting that this model is suitable for studying normal keratinocyte differentiation. Recently, two comprehensive gene expression analyses of keratinocyte differentiation using this model have been reported[9,10]. Researchers validated this model at the molecular level and showed that it can be used to study normal and diseased keratinocyte differentiation.

This protocol describes the procedure for the *in vitro* differentiation method and molecular analysis of differentiated cells using RNA-seq. It also illustrates characterization of the transcriptome of cells on differentiation day 0 (proliferation stage), day 2, day 4, and day 7 (early, middle, and late differentiation, respectively). It is shown that differentiated keratinocytes display gene expression patterns that largely resemble cells during epidermal stratification.

To examine whether this method can be used for studying skin pathology, we applied the same experimental and analysis pipeline to investigate keratinocytes carrying mutations of the transcription factor p63 that are derived from patients with ectrodactyly, ectodermal displasia and cleft lip/palate (EEC) syndrome[11,12]. This protocol focuses on the in vitro differentiation of keratinocytes as well as subsequent bioinformatic analysis of RNA-seq. Other steps in the complete procedure such as RNA extraction, RNA-seq sample preparation and library construction, are well documented and can be easily followed, especially when using many commonly used commercial kits. Therefore, these steps are only briefly described in the protocol. The data show that this pipeline is suitable for studying molecular events during epidermal differentiation in healthy and diseased keratinocytes.

PROTOCOL:

Skin biopsies were taken from the trunk of healthy volunteers or patients with TP63 mutations, to set up the primary keratinocyte culture. All procedures regarding establishing human primary keratinocytes were approved by the ethical committee of the Radboud University Nijmegen Medical Centre ("Commissie Mensgebonden Onderzoek Arnhem-Nijmegen"). Informed consent was obtained.

1. Human primary keratinocyte differentiation by contact inhibition

1.1. Prepare keratinocyte growth medium (KGM) from Keratinocyte Basal Medium (**Table of Materials**) when necessary.

1.2. Make 500 mL proliferation medium using pre-prepared stocks (KGM-pro; see **Table 1**).

1.3. Make 500 mL differentiation medium (KGM-dif; see **Table 2**).

NOTE: KGM with supplements can be kept at 4 °C for two weeks. For longer storage, it can be aliquoted and stored at -20°C.

1.4. Seed primary keratinocytes in the density of 5.0–20 x 10^3 cells/cm^2, depending on the cell proliferative capacity. Add sufficient KGM-pro medium to cover cells.

NOTE: 1) Details of regular cell culture within KGM medium can be found in the website of Lonza [13]. 2) The seeding density should be tested for each cell line. For example, a normal primary keratinocyte line can be seeded at a density of 5.0 x 10^3 cells/cm^2, whereas a line carrying mutations in the transcription factor p63 that is less proliferative should be seeded at a density of 20 x 10^3 cells/cm^2. 3) Depending on the experimental set up, several dishes or wells of cells should be seeded to allow collecting samples on different differentiation days in replicas.

1.5. Refresh cells with the KGM-pro medium for two days after seeding (seeding on day 0,

refreshing for the 1st time on day 3). Afterwards, refresh with KGM-pro medium every other day. Check cells regularly, at least every other day.

1.6. Induce cell differentiation when cells are more than 90% confluent by changing the medium to KGM-dif. The day of changing medium to KGM-dif is defined as differentiation day 0. Collect cells for further RNA analyses by first washing 2x with DPBS, afterwards adding in the lysis buffer (from an RNA extraction kit).

NOTE: With the abovementioned seeding density, cells should reach confluency within 7–10 days. Cells can be seeded with a higher initial density to reach confluency sooner. If cells are not proliferative, longer waiting may not give rise to fully confluent cells. A higher seeding density may be required.

1.7. Refresh cells with the KGM-dif medium every day and collect cells on differentiation day 2, 4. and 7 for further RNA extraction.

2. RNA extraction

2.1. Isolate total RNA. This can be performed using a commercial RNA isolation kit (such as Zymo Quick-RNA MicroPrep RNA), or using phenol[14]. However, an isolation kit containing a DNAse treatment is highly recommended for RNA-seq samples to prevent DNA contamination and phenol. An example of a commercial RNA-isolation kit is given in the **Table of Materials**.

2.2. Measure RNA concentration with a spectrometer. Both DNA and RNA have an absorption peak at 260 nm.

NOTE: 1) The ratio between the absorbances at 260 nm and 280 nm is used to assess the purity of the RNA. A ratio of around 2.0 is generally accepted as 'pure' RNA. If the ratio is substantially lower than 2.0, the sample might be contaminated with contaminants that absorb at 280 nm such as proteins, phenols, or other substances. 2) The ratio between the absorbances at 260 nm and 230 nm measures nucleic acid purity. A ratio between 2.0 and 2.2 is expected. If the ratio is substantially lower, the sample might be contaminated with contaminants that absorb at 230 nm such as EDTA, ethanol, or other substances.

3. RNA quality check

3.1. Run RNA either on a bioanalyzer RNA Pico chip to validate RNA Integrity Number (RIN) quantitatively, or on a gel for a qualitative measure. In general, a RIN of at least eight is highly advisable. However, when extracting RNA from tissues the RIN may be lower.

NOTE: Optionally, quality control can be performed by qPCR on the RNA material. A protocol to perform these steps is included in the Appendix.

4. **RNA-seq library preparation**

NOTE: The RNA-seq library preparation is often performed with a commercial kit or under commercial settings. The described protocol is adapted from a commercial kit, KAPA RNA HyperPrep Kit with RiboErase (Illumina), with a brief description of all required steps: rRNA depletion with oligo hybridization to human ribosomal RNAs, RNA fragmentation, first-strand synthesis, second-strand synthesis and A-tailing, and cleanup after each step[15]. Other library preparation kits can also be used for this purpose. It is recommended to perform this step using a commercially available kit, as the quality of generated cDNA library is often more consistent. 2) The following steps are described for 1x library preparation. If preparing several samples, make master mixes with 10% extra volume.

4.1. **Oligo hybridization and rRNA depletion**

4.1.1. Prepare oligo hybridization master mix (total = 11 µL, with 4.4 µL of hybridization buffer, 4.4 µL of hybridization oligos, and 2.2 µL of RNase-free water) and depletion master mix (total = 5.5 µL, with 3.3 µL of depletion buffer and 2.2 µL of RNase H).

4.1.2. Set up the PCR reaction program on a thermocycler: 95 °C for 2 min; ramp down to 45 °C at -0.1 °C/s; 45 °C pause; 45 °C for 30 min; 4 °C forever.

NOTE: Consider starting with double the amount of RNA than necessary for amplification. In the first attempt, use one-half of the amount to continue with the following steps. This is to make sure that there is still material to repeat these steps with a different number of cycles (see step 4.7.2), if the cycles of amplification turn out to be insufficient or overamplified.

4.1.3. Use between 25 ng and 1 µg of total RNA in 10 µL of RNAse-free water, add 10 µL of oligo hybridization master mix. Place samples in the pre-programmed thermocycler and start the program.

4.1.4. When the program reaches the pause step at 45 °C, add 5 µL of depletion master mix to the 20 µL hybridization reaction without removing it from the thermocycler. Mix thoroughly by pipetting up and down several times.

4.1.5. Resume the thermocycler program to continue with the depletion step (45 °C for 30 min).

NOTE: 1) Put the beads for rRNA depletion (e.g., KAPA pure beads) at room temperature (RT) during the depletion step for the next part of the protocol. 2) Consider preparing the DNase digestion master mix (for section 4.2), since the reagents are needed directly after the rRNA depletion cleanup.

4.1.6. Perform a 2.2x bead-based KAPA Pure Beads cleanup: combine the RNA mix (25 µL) and KAPA Pure Beads (55 µL), by thoroughly resuspend the beads in the RNA mix by pipetting up and down several times.

4.1.7. Incubate at RT for 5 min to allow RNA binding to the beads, and place the tube(s) on a magnet rack to capture the beads until the liquid is clear, and carefully remove and discard 60 µL of supernatant.

4.1.8. Keeping the tube(s) on the magnet, wash 2x with 200 µL of 80% ethanol by incubating the beads with the ethanol for ≥30 s and discard the ethanol. Try to remove all residual ethanol without disturbing the beads after the second wash.

4.1.9. Dry the beads at RT for 3–5 min or until all ethanol has evaporated.

NOTE: Over-drying the beads may result in reduced yield.

4.2. **DNase digestion**

4.2.1. Prepare DNase digestion master mix (total = 22 µL, with 2.2 µL of DNase buffer, 2 µL of DNase, and 17.8 µL of RNase-free water) .

4.2.2. Resuspend the beads in DNAse digestion master mix (20 µL) by pipetting up and down several times. Incubate the tube(s) at RT for 3 min to elute the RNA off the beads.

4.2.3. Place the tube(s) on a magnet rack to capture the beads, until the liquid is clear, and carefully transfer 20 µL of supernatant into a clean tube.

4.2.4. Incubate the tube(s) with supernatant at 37 °C for 30 min.

NOTE: Consider preparing the RNA elution, fragmentation, and priming master mixes (for section 4.3), since the reagents are directly needed after the DNase digestion cleanup.

4.2.5. Perform a 2.2x bead-based cleanup by following 4.1.6–4.1.9.

4.3. **RNA elution, fragmentation, and priming**

4.3.1. Prepare the Fragment, Prime and Elute Buffer (1x) master mix with 11 µL of Fragment, Prime and Elute Buffer (2x) and 11 µL of RNase-free water.

4.3.2. Thoroughly resuspend the beads with purified, DNase-treated RNA in 22 µL of Fragmentation, Prime and Elute Buffer (1x) by pipetting up and down several times.

4.3.3. Incubate at room temperature for 3 min to elute RNA off the bead*s,* then place the tube(s) on a magnet to capture the beads until the liquid is clear.

4.3.4. Carefully transfer 20 µL of supernatant into a tube(s). Discard the tube(s) with beads.

NOTE: This is a safe stopping point, as samples can be stored at -20 °C for ≤24 h.

4.3.5. Place the tube(s) in a thermocycler and carry out the fragmentation and priming at 94 °C for 6 min. Resulting in approximate 200–300 bp long fragments.

NOTE: Incubate for 8 min at 94 °C for 100–200 bp fragments. When using partially degraded RNA, fragment between 1–6 min at 85 °C depending on the severity of degradation.

4.3.6. Place the tube(s) on ice and proceed immediately to first strand synthesis.

4.4. **First strand synthesis**

4.4.1. Prepare first strand synthesis master mix (total = 12 µL, with 11 µL of first strand synthesis buffer and 1 µL of KAPA script).

4.4.2. Set up the PCR reaction program on a thermocycler: 25 °C for 10 min; 42 °C for 15 min; 70 °C for 15 min; 4 °C forever.

4.4.3. On ice, combine 20 µL of fragmented primed RNA with 10 µL of first strand synthesis master mix. Keep the tube(s) on ice, mix thoroughly by gently pipetting the reaction up and down several times.

4.4.4. Place the tube(s) in the pre-programmed thermocycler and start the program.

4.5. **Second strand synthesis and A-tailing**

4.5.1. Prepare second strand synthesis and A-tailing master mix on ice (total = 33 µL, with 31 µL of second strand synthesis buffer and 2 µL of second strand synthesis & A-tailing enzyme mix).

4.5.2. Set up the PCR reaction program on a thermocycler: 16 °C for 30 min; 62 °C for 10 min; 4 °C forever.

4.5.3. Combine 30 µL of first strand synthesis product with 30 µL of second strand synthesis and A-tailing master mix, and mix thoroughly by pipetting up and down several times on ice.

4.5.4. Place the tube(s) in the pre-programmed thermocycler and start the program.

4.6. **Adapter ligation and post-ligation cleanup**

4.6.1. Dilute stock adapters (NEXTflex DNA barcodes, 25 µM) 3.57x in RNase-free water to 7 µM.

4.6.2. Prepare adapter ligation master mix (total = 50 µL, with 40 µL of ligation buffer and 10 µL of DNA ligase).

4.6.3. Combine 60 µL of second strand synthesis product, 45 µL of adapter ligation master mix, and 5 µL of diluted adapters. Mix thoroughly by pipetting up and down several times on ice.

4.6.4. Incubate the tubes at 20 °C for 15 min and continue immediately with the first post ligation cleanup.

4.6.5. Perform a 0.63x bead-based cleanup by combining adapter-ligated DNA (110 µL) and KAPA pure beads (70 µL), then thoroughly resuspend the beads in the DNA mix by pipetting up and down several times.

4.6.6. Repeat steps 4.1.7–4.1.9.

4.6.7. Remove the tubes from the magnet. Thoroughly resuspend the beads in 50 µL of 10 mM Tris HCL (pH = 8.0–8.5) and incubate the beads at RT for 2 min.

4.6.8. Place the plate on the magnet and wait until the liquid is clear. Transfer 50 µL of the clear supernatant to a new tube.

NOTE: Safe stopping point for less than 24 h at 4 °C.

4.6.9. Perform a 0.7x bead-based cleanup by combining 50 µL of beads with purified adapter-ligated DNA with 35 µL of PEG/NaCL solution. Mix thoroughly by vortexing.

4.6.10. Perform cleanup steps 4.1.7–4.1.9. Thoroughly resuspend the beads in 20 µL of 10 mM Tris HCL (pH = 8.0–8.5), and incubate the beads at RT for 2 min.

4.6.11. Place the plate on the magnet; wait until the liquid is clear. Transfer 20 µL of the clear supernatant to a new tube and proceed to section 4.7.

NOTE: Safe stopping point for less than 1 week at 4 °C or less than 1 month at -20 °C.

4.7. **Library amplification and cleanup**

4.7.1. Prepare library amplification master mix (total = 33 µL, with 27.5 µL of 2x KAPA HiFi HotStart ReadyMix and 5.5 µL of 10x library amplification primer mix).

4.7.2. Set up the PCR reaction program on a thermocycler using the following parameters. 98 °C for 45 s; N cycles (see the note below) of: 98 °C for 15 s; 60 °C for 30 s; 72 °C for 30 s; 72 °C for 1 min; and 4 °C forever.

NOTE: The cycles for the library amplification depend on the input material. It can roughly be estimated as such: starting with RNA = 25–100 ng, N = 11–15 cycles; 100–250 ng, N = 9–12 cycles; 250–500 ng, N = 7–10 cycles.

4.7.3. Perform a 0.8x bead-based cleanup by combining amplified library DNA (50 µL) and KAPA pure beads (40 µL), then thoroughly resuspend the beads in the DNA mix by pipetting up and down several times.

4.7.4. Perform cleanup steps 4.1.7–4.1.9. Thoroughly resuspend the beads in 50 µL of 10 mM Tris HCL (pH = 8.0–8.5), and incubate the beads at RT for 2 min.

4.7.5. Transfer 50 µL of the clear supernatant to a new tube and proceed to the second library amplification cleanup.

4.7.6. Perform a 1x bead-based cleanup by combining amplified library DNA (50 µL) and KAPA pure beads (50 µL), then thoroughly resuspend the beads in the DNA mix by pipetting up and down several times.

4.7.7. Perform cleanup steps 4.1.7–4.1.9. Thoroughly resuspend the beads in 22 µL of 10 mM Tris HCL (pH = 8.0–8.5), and incubate the beads at RT for 2 min.

4.7.8. Transfer 20 µL of the clear supernatant to a new tube proceed to Qbit and bioanalyzer measurements.

4.8. **Concentration and fragmentation size**

4.8.1. Measure DNA concentrations of the samples. Using a highly sensitive fluorescent dye-based kit (e.g., Denovix Qbit, see **Table of Materials**), or if the concentration seems to be below 0.5 ng/µL, requantify using a more sensitive qPCR approach (e.g., Kappa Quantification, see **Table of Materials**).

4.8.2. Determine the fragment size of the library, using high sensitive electrophoresis (e.g., a bioanalyzer).

NOTE: Optionally, quality control by qPCR can be performed before sequencing (Appendix) using a diluted prepared library instead of cDNA.

4.9. **Sequencing**

Send the library for sequencing. For RNA-seq differential gene expression analysis, a sequencing depth of reads between 10–25 million per sample is generally sufficient.

5. **Data pre-processing**

5.1. **Quality check Fastq files**

5.1.1. Download and install Trimgalore[16] to remove low quality base pairs and empty reads within the Fastq files.

NOTE: 1) Most software mentioned here only works in Linux. If limited to a Windows computer, it is possible to run analyses on a Linux server using the software MobaXterm or Putty. Keep in mind that for genome indexing a lot of random-access memory (RAM) is required (around 64 GB). 2) Installing all required software into a conda environment is highly recommended. This will allow easy software installation and package management. For more information on conda, visit <https://docs.conda.io/en/Latest/>.

5.1.2. Create a directory (folder) for the data e.g. the folder 'RNA_seq_KC_diff'. Set this as the working directory by typing: "cd /home/user/RNA_seq_KC_diff/".

NOTE: For an overview of the folder structure, see 'folder_structure.txt' in the supplementary coding files.

5.1.3. Create several folders in the working directory with the specific names: 'Fastq', 'CRCh38_fasta', 'CRCh38', 'scripts', and 'Mapping'.

5.1.4. Move the fasta files of the sequenced data into the fastq folder. Alternatively, generate soft links using the command: "ln –s home/user/old_location_fastq/FastqFile home/user/RNAseq_KC_diff/Fastq/Fastqfile" to the Fastq files to save disk space.

5.1.5. Run Trim galore on the Fastq files, see the TrimGalore.txt file for the code to copy paste into bash. Change folder names and settings within the command where necessary.

5.2. **Mapping**

5.2.1. Download a genome to map the reads to, e.g., hg38 ensembl release 97 (unmasked version): ftp://ftp.ensembl.org/pub/release-97/fasta/homo_sapiens/dna/Homo_sapiens.GRCH38.primary_assembly.fa.gz ; and the corresponding gene annotation file: hg 38 gene annotation ensembl release 97, ftp://ftp.ensembl.org/pub/release-97/gtf/homo_sapiens/Homo_sapiens.GRCh38.97.gtf.gz. Transfer both the files to the CRCh38_fasta folder.

NOTE: Alternatively use a different version of the genome, for example, the UCSC version of hg38, if mapping to transcript IDs rather than gene IDs is preferred.

5.2.2. Install STAR 2.7.1[17].

5.2.3. Generate an in-house reference genome using STAR 2.7.1 by the generate genome script (see **Supplemental Coding Files**). Change the amount of threads to what the processer has (--runThreadN X). Change folder names and settings in this script where necessary. Run it by copy/pasting into bash.

5.2.4. Install samtools 1.9[18] for indexing the bam files and gzip to compress the wiggle files.

5.2.5. Align the Trim Galore validated sequencing reads to the human genome assembly hg38 (ensembl release 97) using STAR 2.7.1 followed by indexing using samtools and gzip. This should be performed using the script map_fastq.txt (see **Supplemental Coding Files**). Change the folder names and settings in this script where necessary. Run it by copying/pasting into bash.

NOTE: STAR mapping generates several output files, including a bam file, a wiggle file, and read counts table named *_ReadsPerGene.out.tab, which can directly be used concatenated by R to use as input for Deseq2.

5.3. **Genome browser visualization**

5.3.1. Install wigToBigWig from the UCSC genome browser tools to generate bigwig files with the big2bw script[19].

5.3.2. Transfer the files 'convertBigWigChroms.py' and 'CRCH38_ensembl2UCSC.txt' from the supplementary coding files to a folder 'scripts' in the working directory.

5.3.3. Make the convertBigWigChroms.py executable (using the command: 'chmod +x scripts/convertBigWigChroms.py' on a Linux machine).

5.3.4. Generate bigWig files from the Wiggle files, using the script wig2bw.txt (see **Supplemental Coding Files**). Run it by copy/pasting into bash.

5.3.5. Input the BigWig files on the UCSC genome browser or Integrative Genomics Viewer (IGV) for visualization.

6. RNA-seq data analysis

6.1. **Input sample data into Deseq2**

6.1.1. Download and install Rstudio (version 1.1.456) and R (version 3.6).

6.1.2. Install all the required R packages (see Supplemental coding files).

NOTE: For a detailed R-markdown file showing all programming code and output, see the attached files: "RNA_seq_kc_differentiation_wt.html" and "RNA_seq_kc_differentiation_patient.html". A general description of all steps is included below.

6.1.3. Generate a count table from the ReadsPerGene.out.tab files.

6.1.4. Write a sample data file, containing all filenames, the day of differentiation and other relevant sample data. For an example sample file, see "sample_data_example.csv" in the **Supplemental Coding Files**.

6.1.5. Use the count table and sample data to generate a Deseq2[20] object containing both the countable and sample data.

6.2. **Gene expression normalization, sample distance and PCA**

6.2.1. Normalize the count table in the Deseq2 object, using either Deseq2 rld, or vst normalization. Rld normalization is preferred, but with many samples, vst normalization is much faster.

6.2.2. Plot sample distance based on the normalized read count intensities using the "*dist*" function in R and subsequently performing "*hclust*" clustering based on sample distance. Plot the heatmap itself using the pheatmap function.

6.2.3. Generate a PCA plot of the normalized read count intensities using the plotPCA function of Deseq2.

NOTE: PCA serves as a tool in exploratory data analysis and can be used to visualize distance and relatedness between different samples.

6.3. **Differential/highly variable gene expression analysis**

6.3.1. Calculate either the differential genes using the Deseq2 result function. However, when assessing changes over several timepoints in which pairwise comparisons are not applicable, use highly variable genes. In this case, extract the top 500 highly variable genes via ordering on the variance between samples from different time points with the rowVars function.

NOTE: In our analysis, for the control samples, the top 500 highly variable genes (the genes with the highest standard deviation of their normalized intensity across days of differentiation) were used for further analysis. However, for the diseased vs control analysis differentially expressed genes between disease and healthy controls over time were calculated by Deseq2 using a multiple testing corrected p-value of 1×10^{-4} as a cutoff. Another possible filtering step is to exclude differential genes changing less than a certain fold change cutoff.

6.3.2. Perform kmeans clustering on the differential or highly variable genes to cluster them by different expression patterns.

6.3.3. Visualize differential or highly variable genes in a heatmap using the pheatmap package. The intensity plotted in the heatmap is the Deseq2 normalized intensity with the median value subtracted.

6.4. **Gene Ontology (GO) annotation enrichment analysis**

6.4.1. Generate a list of expressed background genes by taking all genes with more than 10 counts in a single sample.

6.4.2. Perform GO analysis using online tools such as GOrilla [21]. Use the lists of the differential/highly variable genes in clusters as "gene list", and the background genes as the background for comparison.

NOTE: More advanced R-users can use the package clusterProfiler[22] to automate Go-term enrichment analysis.

REPRESENTATIVE RESULTS:

Normal keratinocyte differentiation and RNA-seq analysis

In this experiment, keratinocyte lines derived from five individuals were used for differentiation and RNA-seq analyses. **Figure 1** summarizes the experimental procedure of differentiation and RNA-seq analysis results. An overview of in vitro differentiation procedures of normal keratinocytes and cell morphology changes during differentiation are illustrated in **Figure 1A**. Principle component analysis (PCA) showed that keratinocytes undergoing differentiation had connected but distinct overall gene expression profiles (**Figure 1B**). Highly variable genes were clustered by kmeans to visualize gene expression dynamics and patterns during differentiation (**Figure 1C**).

Each cluster of genes was represented by the keratinocyte differentiation hallmark genes (e.g., KRT5 for proliferation, KRT1 and KRT10 for early and mid-differentiation, and IVL/LOR/FLG for late differentiation). Gene ontology (GO) annotation analysis of highly variable gene clusters (**Figure 1C)** showed a clear difference in gene functions of these gene clusters (e.g., keratinization in the mid-stages of differentiation; and epidermal cell differentiation, keratinocyte differentiation, and peptide cross-linking in the late stages; **Figure 1D**). Protein expression of several differentiation markers were measured by western blotting (**Figure 1E**).

P63 mutant keratinocyte differentiation and RNA-seq analysis:

In the second experiment, cell morphology and gene expression differences were compared between keratinocytes from healthy controls and three lines derived from patients carrying p63 mutations (mutants R204W, R279H, and R304W). **Figure 2A** shows an overview of differentiation procedures and cell morphology changes. Mutant keratinocytes remained flat on the surface of the dish and did not become crowded or overlap growth as control keratinocytes on day 7.

In PCA analysis, the control cell lines clearly followed a differentiation pattern, as compared to **Figure 1B.** However, the pattern of gene expression of mutant cells during differentiation stays largely similar to those of proliferating/undifferentiated cells. Among the three mutant lines, differentiating R279 samples moved along PC1 and PC2 to some extent, indicating that its

differentiation was less impaired, compared to R204W and R304W.

In the clustering analysis (**Figure 2C**), genes downregulated in control cells (cluster 1) were partially downregulated in R204W and R279W, but their gene expression was not drastically changed in R304W. These genes likely play roles in cell proliferation, as shown by GO annotation **(Figure 2D**). In cluster 2 (**Figure 2C**), genes were first induced and subsequently downregulated in control cells. These genes are likely involved in keratinocyte differentiation, as epidermal differentiation and keratinization functions were highly enriched for this cluster genes (**Figure 2D**). These genes were not induced in R204W and R304W, whereas in R279H cells, these genes were induced but not downregulated as much as the control cells.

Genes in cluster 3 were only induced at the end of differentiation in control cells (**Figure 2D**). Consistent with this, these genes may have a role in the outer most layer of the epidermis, as they have been shown to be responsive to external stimuli and inflammation (**Figure 2D**). The expression pattern of these genes did not change much in all three mutant cell lines. The visible differences in gene expression patterns between control and mutant cells demonstrate that mutant cells cannot differentiate properly in these in vitro differentiation models.

FIGURE AND TABLE LEGENDS:

Figure 1: Keratinocyte differentiation and analysis. (A) Overview of the keratinocyte differentiation protocol and cell morphology. Scale bar = 100 μm. **(B)** Principle component analysis of differentiating control keratinocytes. **(C)** Heatmap of the top 500 highly variable genes during keratinocyte differentiation. Genes are clustered in three clusters using kmean clustering. Representative differentiation marker genes for each gene cluster are indicated at the side. **(D)** GO term enrichment analysis of overrepresented functions for genes in the highly variable gene clusters, as compared to a background of all expressed genes (counts of >10). GOrilla was used for the enrichment test. **(E)** Western blot of keratinocyte differentiation markers during differentiation.

Figure 2: Comparison of control and p63 mutant keratinocytes. (A) Overview of the keratinocyte differentiation protocol and cell morphology of control and p63 mutant keratinocytes. Scale = 100 μm. **(B)** Principle component analysis of differentiating control and patient (R204W, R279H & R304W) keratinocytes. **(C)** Heatmap of differential genes between control and mutant keratinocytes. Genes are clustered in three clusters using kmean clustering. Representative differentiation marker genes for each gene cluster are indicated. **(D)** GO term enrichment analysis of overrepresented functions for genes in the highly variable gene clusters, as compared to a background of all expressed genes (counts of >10). GOrilla was used for the enrichment test.

Table 1. KGM-pro medium supplements.

Table 2. KGM-diff medium supplements.

Supplemental Coding Files: Folder_structure.txt; Generate_genome.txt; Map_fastq.txt; RNA_seq_kc_differentiation_patient.html; RNA_seq_kc_differentiation_wt.html; Sample_data_example.csv; TrimGalore.txt; and Wig2bw.txt.

DISCUSSION:

This work describes a method for inducing human keratinocyte differentiation and subsequent characterization using RNA-seq analyses. In the current literature, many studies on human keratinocyte differentiation use a high calcium concentration (~2 mM) or serum as methods to induce differentiation[2,3,23]. A previous report carefully compared these three different methods [3] and showed that these methods can represent distinct biology of keratinocyte differentiation. In this same report, authors showed that differentiated cells induced by serum have high proliferative potential and express genes such as KRT16 and SKALP/ PI3 that are expressed in the psoriasis skin but not in normal epidermis, and that therefore serum-induced differentiation model can be used to study psoriasis.

In contrast, the method of contact inhibition plus growth factor exclusion can induce KRT1 and KRT10, which are normally expressed in the epidermis and resemble normal keratinocyte differentiation. High calcium induction gives rise to a gene expression profile that is between serum induction and contact inhibition, as the least specific method. The analyses using RNA-seq analyses during differentiation confirmed that the method of contact inhibition plus growth factor exclusion results in differentiated keratinocytes with similar gene expression profiles as those expected for epidermal differentiation (e.g., KRT5 in proliferating cells, KRT1 and KRT10 in early differentiation induction, and LOR and FLG in late differentiation; **Figure 1C**).

These findings demonstrate that this differentiation technique is an easy-to-use and reliable method to study keratinocyte differentiation. Furthermore, this work on keratinocytes derived from p63 mutant keratinocytes also show that the method can be used to study affected keratinocyte differentiation under diseased conditions. In this in vitro differentiation approach, KGM purchased from Lonza is used, as this medium yields consistent results. In principle, other epidermal medium with similar composition such as keratinocyte serum-free medium (KSFM) from Thermo Fisher Scientific is also likely suitable, although this needs to be tested.

It should be noted that this method has some limitations. As it is based on contact inhibition, the confluency of cell density is required. In our experiments with p63 mutant keratinocytes, a higher initial seeding density has been necessary to ensure cells to reach confluency. In addition, when cells do not grow with the same speed, differentiation sometimes must be induced on different days. These considerations should be taken into account when setting up experiments.

In experimental settings where cells need to be induced at the same time, other differentiation methods should be considered, including addition of serum, high concentrations of calcium, and inhibition of epidermal growth factor receptors[2,3]. Nevertheless, all in vitro differentiation methods have pros and cons, as they probably represent partial differentiation induction signals that are present during in vivo skin development[2].

A comprehensive molecular analysis that compares these different methods will be highly informative and can instruct the choice of method most suitable for different studies on biological processes. In addition, validating changes measured at the transcriptomic level is highly advised, for example, via western blotting or proteomic analyses. Furthermore, in vitro data should be used with caution, and conclusions from these models should be validated in vivo, preferably in human skin development.

Basic principles of RNA extraction and RNA-seq library preparation have been well described previously[24-28]. In this protocol, the RNA extraction and RNA-seq library preparation procedures are based on workflows of commercially available kits. In principle, different methods for RNA extraction should not have a major influence on RNA-seq analyses if the RNA quality is good. In cases where RNA quality is poor (i.e., when RNA is extracted formalin-fixed paraffin-embedded tissues), RNA-seq can still be performed; however, the RNA fragmentation step should be adjusted. The RNA-seq library preparation covers from ribosomal RNA (rRNA) removal to the cDNA library construction for sequencing. The major procedures may be performed by using various kits, or using individual enzymes and homemade buffers[29-32]. It should be noted that, if RNA-seq analysis is performed using different basic principles (e.g., either ribosomal RNA depletion or polyA mRNA enrichment by hybridization to polyT oligos), the outcome of RNA-seq analyses may differ. Furthermore, the protocol utilizes ribosomal RNA removal by hybridization of DNA oligos to human, mouse, and rat rRNA. When working with different species, an alternative oligo set should be employed.

Sequencing of the generated library can be performed either on both ends of the fragments (paired end) or at one end of the fragment (single end). In general, paired end sequencing vastly improves mappability and provides more information regarding transcript variants. However, for a relatively simple differential gene analysis as described here, single end sequencing can also provide sufficient information.

For the important final step of data analysis, a relatively simple method for quality control of the fastq files is described, followed by mapping reads to the genome. Even though the provided bash code does not have ideal scalability, it has the advantage of transparency. The choice of software, which steps it performs, and version of the genome used during data preprocessing are all nontrivial steps that should be well-documented, which is essential for the repeatability.

For more advanced users, an automated pipeline can be used to perform the steps of fastQ file quality check, adapter trimming and mapping, e.g. the ARMOR snakemake workflow[33] or the 'RNA-seq' Snakemake workflow from the van Heeringen lab[34]. However, these completely automated pipelines are less transparent and more difficult to change. When using these tools, it is vital to understand the functions within these automated pipelines. Finally, the protocol includes RNA-seq data analysis with an emphasis on variable gene expression over time. Variable gene expression is preferred over pairwise differential testing when looking at processes with multiple timepoints, such as differentiation. In conclusion, this analysis pipeline

introduces relevant tools for bioinformatic analysis of RNAseq data. It contains tools that can thoroughly assess keratinocyte differentiation, both under normal and diseased conditions.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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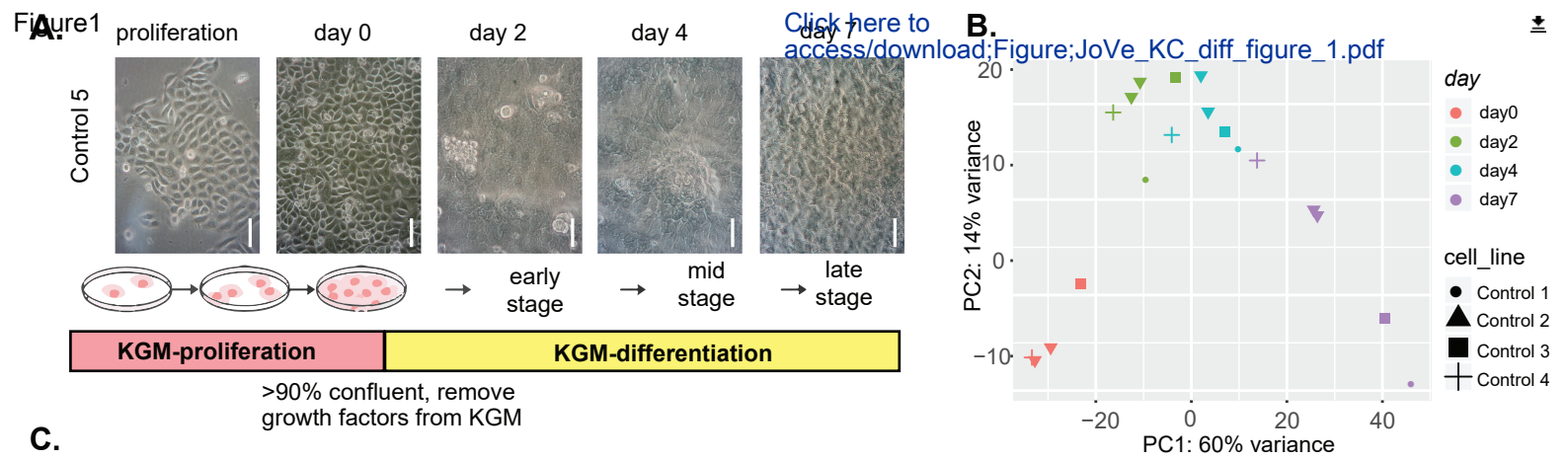
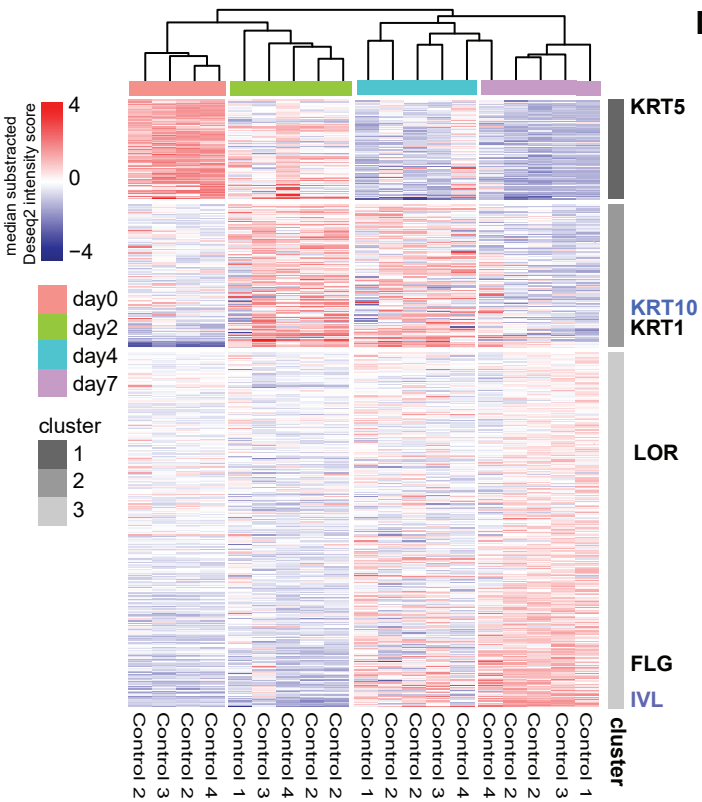
Figure1

Click here to access/download;Figure;JoVe_KC_diff_figure_1.pdf





D.

Description	FDR q-value
DNA replication-dependent nucleosome assembly	1.09E-10
chromatin silencing at rDNA	2.26E-10
nucleosome organization	3.53E-10
protein-DNA complex assembly	3.36E-10

Description	FDR q-value
cornification	1.78E-11
keratinization	9.66E-10
programmed cell death	1.02E-06
response to biotic stimulus	1.51E-06
cell death	1.54E-06
type I interferon signaling pathway	2.13E-06

Description	FDR q-value
cornification	1.58E-20
epidermal cell differentiation	1.45E-19
keratinocyte differentiation	1.09E-17
peptide cross-linking	2.24E-15
epithelial cell differentiation	2.78E-12
epidermis development	4.39E-10
establishment of skin barrier	3.63E-09
regulation of water loss via skin	1.53E-08
cell death	0.00000016
programmed cell death	0.000000449
water homeostasis	0.00000104
keratinization	0.00000413

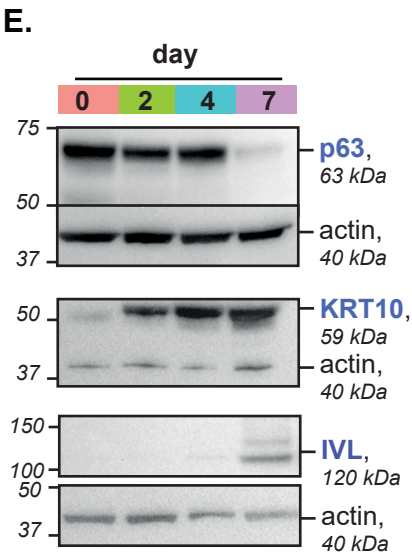
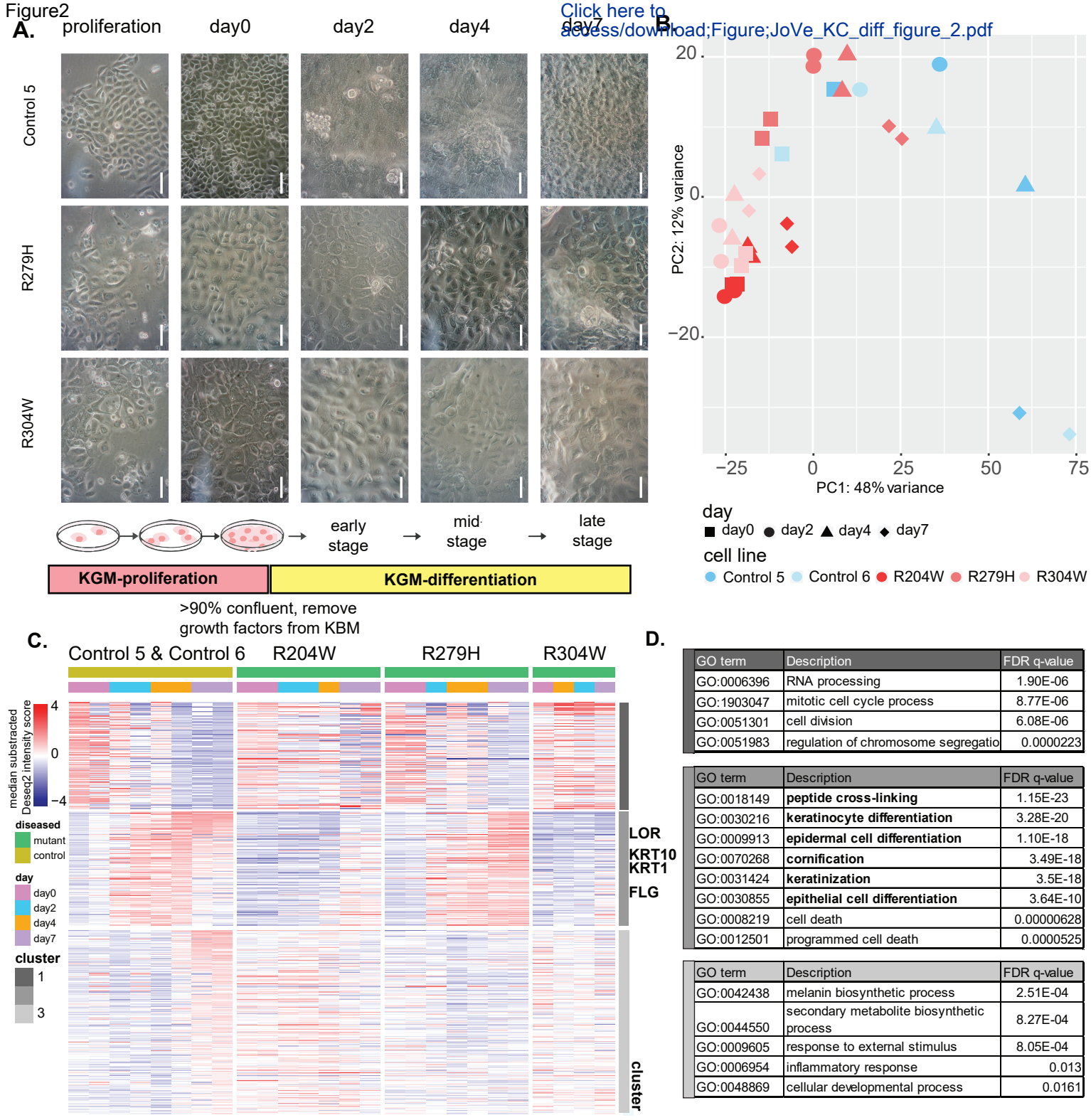


Figure2



D.

GO term	Description	FDR q-value
GO:0006396	RNA processing	1.90E-06
GO:1903047	mitotic cell cycle process	8.77E-06
GO:0051301	cell division	6.08E-06
GO:0051983	regulation of chromosome segregatio	0.0000223

GO term	Description	FDR q-value
GO:0018149	**peptide cross-linking**	1.15E-23
GO:0030216	**keratinocyte differentiation**	3.28E-20
GO:0009913	**epidermal cell differentiation**	1.10E-18
GO:0070268	**cornification**	3.49E-18
GO:0031424	**keratinization**	3.5E-18
GO:0030855	**epithelial cell differentiation**	3.64E-10
GO:0008219	cell death	0.00000628
GO:0012501	programmed cell death	0.0000525

GO term	Description	FDR q-value
GO:0042438	melanin biosynthetic process	2.51E-04
GO:0044550	secondary metabolite biosynthetic process	8.27E-04
GO:0009605	response to external stimulus	8.05E-04
GO:0006954	inflammatory response	0.013
GO:0048869	cellular developmental process	0.0161

Table 1

KGM component	Stock	Medium	Volume
KBM			500 mL
Pen/Strep	100,000 units/mL	100 units/mL	5 mL
BPE	~13 mg/mL	0.4%	2 mL
Ethanolamine	0.1 M	0.1 mM	500 µL
o-phosphoethanolamine	0.1 M	0.1 mM	500 µL
Hydrocortisone	0.5 mg/mL	0.5 µg/mL	500 µL
Insulin	5 mg/mL	5 µg/mL	500 µL
EGF	10 µg/mL	10 ng/mL	500 µL

Table 2

KGM component	Stock	Medium	Volume
KBM			500 mL
Pen/Strep	100,000 units/mL	100 units/mL	5 mL
Ethanolamine	0.1 M	0.1 mM	500 µL
o-phosphoethanolamine	0.1 M	0.1 mM	500 µL

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/ Equipment
Bioanalyzer 2100
Bovine pituitary extract (BPE)
CFX96 Real-Time system
Dulbecco's Phosphate-Buffered Saline (DPBS)
Epidermal Growth Factor (EGF)
Ethanolamine >= 98%
High Sensitivity DNA chips
Hydrocortison
Insulin
iQ SYBR Green Kit
iScript cDNA synthesis
KAPA Library Quant Kit
KAPA RNA HyperPrep Kit with RiboErase
KGM Gold Keratinocyte Growth Medium BulletKit
Nanodrop
NEXTflex DNA barcodes -24
Penicillin-Streptomycin
RNA Pico Chip

Company	Catalog Number	Comments/Description
Agilent	G2929BA	
Lonza		Part of the bulletKit
Bio-Rad		qPCR machine
Sigma-Aldrich	D8537	
Lonza		Part of the bulletKit
Sigma-Aldrich	E9508	
Agilent	5067-4626	
Lonza		Part of the bulletKit
Lonza		Part of the bulletKit
BioRad	170-8886	
Bio rad	1708890	
Roche	07960255001	Low concentration mea
Roche	KK8540	RNAseq kit
Lonza	192060	
deNovix	DS-11 FX (model)	Nanodrop and Qbit for
Illumnia	NOVA-514103	6 bp long primers
Gibco	15140122	
Agilent	5067-1513	

n

asure kit

DNA and RNA measurements

Rebuttal

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the editor for this reminder and have done it accordingly.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

We have done so.

3. Please revise section 3 of the protocol to avoid textual overlap with previous publications.

We have done so.

4. Please provide at least 6 key words or phrases.

We have included 6 key words in the manuscript file.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Zymo Quick-RNA, NanoDrop, iScript, Illumina, NEXTflex, KAPA Pure, Experion, MilliQ, SYBR, etc.

We have checked the manuscript carefully, and removed the commercial names as much as possible. However, we like to point out that the purpose of this protocol is not to provide a stand-alone protocol that one can use with home-made buffers and enzymes. This protocol is to provide a guide on how to perform the described experiments, with necessary trick during the procedure. This was previously discussed with the inviting editor. We believe that, if we remove all commercial names, the protocol will not be sufficiently clear. Therefore, if editors have further comments or suggestions on how to revise this, we will like to hear and change accordingly.

Protocol:
1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have limited the protocol within the 10-page limit and the filmable content is limited to 2.6 pages.

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have checked this.

Specific Protocol steps:
1. 1.1: Where do the keratinocytes come from? If they come from an animal or clinical source, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee and/or the animal care guidelines of your institution, as appropriate.

We have included the original of the keratinocytes and an ethics statement.

2. 4: Please be more specific about how computational steps are done, in particular ones done on the command line.

All the steps have the entire code written out completely in the supplement coding files. We have written about this more explicitly.

3. 5: Presumable the Rmarkdown files are the .html files in the Supplemental Materials; please be explicit about this.

Yes, this is correct. We have rephrased the relevant parts to be more clear. Furthermore we have made some changes in the Rmarkdown file to remove a few lines of redundant code and improve the explanation at a few steps.

Figures and Tables:
1. Please remove the embedded tables from your manuscript. Tables should be uploaded as .xls/.xlsx formatted files, be cited in the text, and include legends after the Figure legends.
2. Figures: Please include a space in 'day 0' etc.

We have done these according to the instruction. However we also like to mention that some steps may become more troublesome to read and to follow, as the tables in the previous version make the steps and components of the reactions more clear.

Discussion:
1. Please expand the Discussion to 3-6 paragraphs and include information on critical steps of the protocol, modifications, and troubleshooting.

We have significantly expanded Discussion, making it to 4 paragraphs and discussing several critical steps of the protocols and points of attention.

References:
1. Please do not abbreviate journal titles.

We have done these according to the instruction.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

We have done these according to the instruction.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

This manuscript aims to provide a protocol for confluency-induced keratinocyte differentiation and RNASeq gene expression analysis. As the authors point out, it is well recognised that confluency induces differentiation. The keratinocyte differentiation protocol has sufficient detail but the basic protocol on the keratinocyte basal medium supplier's web site (Lonza) has far more detail and would be easier for non-specialists to follow.

We thank the referee for this suggestion. We have added a comment referring to the Lonza website for more information.

Parts of the protocol such as the RNAseq bioinformatics are quite well explained but there is little explanation of the theory behind RNA prep, library preparation etc. and no notes of "points of difficulty" or "points to watch". The reader is always referred to the instruction for the various kits the authors recommend.

The described protocol is indeed from a commercial kit, and based on our experience, it is straightforward to perform. We have added point of attention/difficulty in the description wherever appropriate.

As regards the data, RNAseq analysis will indeed prove that differentiation has occurred. On the other hand this should be confirmed by protein analyses, even just by western blotting. Moreover, it is very difficult to see and therefore compare the cells in the phase contrast pictures in Figure 1 and 2.
03

To address these comments, we have added pictures of western blots to show protein analyses. We have also changed the pictures in Figure 1 and 2 with the original higher resolution pictures.

Major Concerns:

I find many details missing from the protocols. For example, readers will need to know what absorbance wavelength is used for the nanodrop quantification. What about determining the quality of the RNA prep by reading 260/280 and 230/260 ratios? The RT-qPCR reaction has primers but lacks a probe and there is no explanation of how one might choose primers and probes. There is no mention of any controls, which are essential because the authors rightly choose the delta-delta Ct method for calculating differential expression. There is only brief mention of how to ensure quality control of the RNA, a major go/no go step for RNASeq library preparation, or quality of the final library preparation. There is no discussion of strategies for RNAseq, e.g. single end paired end.

We have added more details on RNA measurement and quality controls. We also added discussion on single- and paired-end RNA-seq.

The authors select reagents and kits from a specific supplier but do not explain the concentrations/contents of the enzymes/buffers/kits. This means that someone following the protocol without access to the specified Kapa kits, or if they chose to use the kits recommended by Illumina, would not be able to carry out the protocol.

As mentioned above to editor's comments, this described protocol is adapted from instructions of commercial kits. Indeed some information of the RNA extraction and RNA-seq kits is not clearly stated in the product information, which potentially causes one not to be able to perform the

analyses without the kits. However, these kits are common, and can be purchased world-wide. In our experience, these kits do give reliable results for RNA and RNA-seq quality. Here we like to emphasize that the purpose of this protocol is not to provide a stand-alone protocol that one can use with home-made buffers and enzymes. This was previously discussed with the inviting editor. The aim of this manuscript is to describe reliable and easy-to-follow protocols for one to perform keratinocyte differentiation experiment and molecular analysis, which is the focus of this manuscript. We have rephrase Introduction and Discussion to make this point clear. Nevertheless, we appreciate the point of this referee, and we also made notes on alternative methods, and discussed this point in Discussion.

Minor Concerns:
The protocol is highly repetitive in places. Repetitive sections could simply refer back to the first repeat.
There are repetitions of reagents in the reagent Table and several reagents are missing e.g. Denovix.

We have taken out the repetitive descriptions, and referred the repetitive steps.

Reviewer #2:
Manuscript Summary:
The manuscript describes analysis of human keratinocyte differentiation using RNA-seq. The protocol will be useful for wide audience. It needs some correction.

Major Concerns:

1) Authors name primary keratinocytes as"stem-like cells". This is not the case as keratinocytes inoculated into culture are at different differentiation stage. It is no doubt that the initial medium proposed by authors maintains high proliferation capacity of progenitor keratinocytes but nevertheless they are not all stem and even stem-like cells.

We have rephrased these terms.

2) The authors declare that differentiation of keratinocytes is induced by confluence. However, by fact, they change the proliferation medium fot differentiation one. The latter is devoid of EGF and other supplements and it stimulates differentiation regardless of the state of confluence. Therefore, it shoul be discussed in Introduction and Discussion. Additionally, "confluence" shoul be used carefully

We have rephrased these parts, and made clear that our protocol is based on a differentiation induced by ‘confluency plus growth factor exclusion/depletion’. In addition, we also discuss the pros and cons of this method, as compared to other methods, in Discussion.

Minor Concerns:
Line 528 It seems that "not" is missed in the sentence
Table of materials - "insuline" instead of "insulin"
The whole text needs proof reading

We have corrected the mistakes that we can find.

Reviewer #3:
Manuscript Summary:
The manuscript proposed by Zhou et al. describes a procedure for the analysis of human keratinocyte differentiation in an in vitro model induced by the removal of growth factors without the addition of calcium. The analysis of cell differentiation is performed by a RNA-seq approach allowing a complete

profiling of the transcriptome. A cellular model of human keratinocytes mutated on the CDKN2A gene (p16), known to induce a defect of differentiation, is used to validate the robustness of the method. The results are clear and concise and the procedure is well detailed; however, some questions remain.

Major Concerns:
1) The title puts forward the model of in vitro differentiation whereas this part represents only 10% of the methodological description. The remaining 90% describe the RNA-seq analysis pipeline. Would not it be better to invert the focus of the article, which is expected to be a methodological one?

We thank the referee for this suggestions. We have changed the title accordingly, focusing on RNA-seq analysis of the in vitro differentiation model.

2) No information is given on the origin of the cells. Did the authors isolate the keratinocytes themselves? If so, it would be important to describe the methodology. If not, please indicate the supplier and the cell passage used to carry out the experiments.
3) No ethical information is noticed. In the case of primary cells, it is mandatory to provide an official accreditation document for the use of human biological material for experimental purposes. If the cells have been purchased, the supplier must be authorized to issue this material.

We have included the information on the origin of the cells and an ethics statement.

4) The model of an in vitro differentiation by depletion of growth factors has been described in the literature for more than 10 years, as mentioned by the authors. Generally, this model uses the K-SFM culture medium (keratinocyte serum free media). Why did the authors use the KGM medium?

Both media are suitable and common for this type of differentiation. Here chose one of them to describe, as we have more experience with this medium, and it gives us more consistent results. We have added a comment about KSFM in Discussion.

5) The extraction of the RNA is carried out by a commercial kit and the quantification is carried out with nanodrop. No quality control of RNA is indicated. However, it seems essential to ensure the integrity of the material by determining the RNA integrity number (RIN) before establishing the libraries. Moreover, a nanodrop quantification is indicative of the concentration but remains very approximate and does not detect any contamination by genomic DNA. Has a qualitative analysis been performed?

We have included the information about RIN.

6) The state differentiation of keratinocytes would be more convincing by combining a proteomic analysis of one or more differentiation markers. For example, an immunocytofluorescent staining of cytokeratin 10, involucrin or transglutaminase would make it possible to affirm that the cells have been committed in a differentiated phenotype.

We have include western blots to show differentiation markers at the protein level.

Minor Concerns:
Lane 97: The cell density is 5.0 x 103 cells/cm² and not 5.0 cells/cm².

We have corrected this mistake.

Reviewer #4:

Manuscript Summary:
Authors describe the procedure of in vitro differentiation of human primary keratinocytes in 2D culture by means of two concomitant stimuli: contact inhibition of growth and exclusion of growth factors. They apply the procedure to keratinocytes isolated from healthy (control) patients and individuals carrying various mutation in p63 gene. They evaluate keratinocyte differentiation by analyzing the transcriptome at several time points using RNA-seq. The analysis delivers gene expression pattern indicative of an ongoing differentiation of normal keratinocytes, thereby validating the implemented model. An impaired gene expression pattern is observed for p63 mutants.

Major Concerns:

-In my opinion isolated primary keratinocytes cannot be considered as a model of epidermal stem cells or stem cell-like cells as stated in the Abstract/ introduction (line 48). Epidermal stem cells are rare and require special isolation methods. Cells isolated from biopsies represents a mixture of cells with various proliferation potential.

We have rephrased 'epidermal stem cells' to 'epidermal keratinocytes'.

- Did the Authors assess density of wt and p63mut cells at day 0? Judging from Fig 2A day 0 the wt keratinocytes are packed more tightly than p63 mutant keratinocytes. The same is true for day 7 (images of days 2 and 4 are of poor quality). Thus, in addition to the obvious impairment in proliferation rate and differentiation of mutant cells, lower starting cell density may contribute to the noted differences. High-calcium-induced differentiation might be a better option to use when comparing wt and p63 mutant cells. Could Authors refer to this point?

For both wt and mt cell differentiation, we induce differentiation when cells are confluent. For this, we seed mt cells in a higher density and always wait for cells to be fully confluent, and therefore differentiation may not be induced on the same day. Therefore different cell density is not an issue here. In our protocol, we prefer not to use high calcium induction, as it has been shown that this condition induces differentiation with a phenotype between the normal and psoriasis skin conditions, rather than normal differentiation, as we discuss in Discussion. But we have added pros and cons of different methods, and discussed the cell density.

-I am not sure whether the protocol for performing RT-qPCR should be included in this manuscript since Authors consider it only as an option to verify cDNA quality before sequencing (line 338) but do not perform such control and do not refer to the procedure either in the Results or Discussion. If, however, this part is to remain in the text, some information on reference gene(s) should be included.

We have now included more RT-qPCR information in Appendix, as we are restricted to the page limit.

Minor Concerns:

- In the second paragraph of Results the Authors refer to Fig.1 instead of Fig.2
-line 58: what does "keratinocyte inhibition" mean?
-line 97: 5.0 cells/cm2 should be corrected.
-In Table of materials several items are given twice; also, abbreviations: EGF and BPE, should be given along with the full name to match information in 1.1.1.
-The shades of red in 1B are not well discernible.

We have corrected these mistakes.

Appendix
qPCR RNA quality validation

1. Prepare cDNA from 0.5 - 1 µg of total RNA using the iScript cDNA synthesis kit as described in the manual (Table of material).

2. Set up RT-qPCR reactions with SYBR Green using 20 times H_2O-diluted cDNA. (total 25 µl, with 5ul 20x diluted cDNA, 12.5 µl 2x iQ SYBR Green Supermix, 5.5 µl MiliQ and 2 µl 5uM Forward & Reverse primers).

NOTE: RT-qPCR primers are designed using Primer3 to obtain exon spanning primers wherever possible using the following important criteria: product size, 50-150; primer size, Minimum 18, Optimal 20 Maximum 27; primer Tm, Minimum 58 Optimum 59, Maximum 60, Maximum Tm Difference 1; primer GC%, Minimum 40, Maximum 60; CG Clamp, 1.

3. Measure RT-qPCR reaction in a qPCR machine, using a 2/3 step + melt temperature protocol:
95°C for 3 minutes
39 cycles of : 95°C for 15 seconds; 59°C for 45 seconds
95°C for 1 minutes
5-degree increment curve: 65-95°C with 10 second increments.

4. Measure the Ct value of both a highly expressed housekeeping gene such as GAPD, and of a low expressed housekeeping gene such as GusB and potential transcription factors of interest (e.g. P63).

5. Calculate differences in the expression of each gene during differentiation using the 2ΔΔCt method[1].

NOTE: For good quality, RNA the GAPDH Ct should be 20 cycles or less. GAPDH its expression slightly varies during KC differentiation but should come up early. The Ct value of GusB should be around 30.

References:

1. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* **25** (4), 402-408, (2001).

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Supplemental Coding Files

folder_structure.txt

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generate_genome.txt

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Supplemental Coding Files

RNA_seq_kc_differentiation_wt.html