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PAPER IN FOREFRONT

# Quantitative single-cell gene expression measurements of multiple genes in response to hypoxia treatment

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**Abstract** Cell-to-cell heterogeneity in gene transcription plays a central role in a variety of vital cell processes. To quantify gene expression heterogeneity patterns among cells and to determine their biological significance, methods to measure gene expression levels at the single-cell level are highly needed. We report an experimental technique based on the DNA-intercalating fluorescent dye SYBR green for quantitative expression level analysis of up to ten selected genes in single mammalian cells. The method features a two-step procedure consisting of a step to isolate RNA from a single mammalian cell, synthesize cDNA from it, and a qPCR step. We applied the method to cell populations exposed to hypoxia, quantifying expression levels of seven different genes spanning a wide dynamic range of expression in randomly picked single cells. In the experiment, 72 single Barrett's esophageal epithelial (CP-A) cells, 36 grown under normal physiological conditions (controls) and 36 exposed to hypoxia for 30 min, were randomly collected and used for measuring the expression levels of 28S rRNA, PRKAA1, GAPDH, Angptl4, MT3, PTGES, and VEGFA genes. The results demonstrate that the method is sensitive enough to measure alterations in gene expression at the single-cell level, clearly showing heterogeneity within a cell population. We present technical details of the method development and implementation, and experimental results obtained by use of the procedure. We expect the advantages of this technique will facilitate further developments and advances in the field of single-cell gene expression profiling on a nanotechnological scale, and eventually as a tool for future point-of-care medical applications.

**Keywords** Single-cell · Gene expression · Hypoxia treatment

## Introduction

Cell-to-cell heterogeneity in gene transcription plays a central role in a variety of vital cell processes, including differentiation [1, 2], stimulus response [3], survival [4, 5], and carcinogenesis [6]. Therefore, studies of the molecular mechanisms responsible for intercellular variability in gene expression levels at the single-cell level are expected not only to provide critical insights into core cellular processes but also to pave the way for new, more effective disease prevention and treatment strategies. The vast majority of currently existing experimental techniques for gene expression profiling are based on analysis of bulk samples containing $10^5$–$10^7$ or more cells. Inherent to samples of that size is the ensemble-averaging of the results over a large number of cells, hiding key information emanating from individual cells. Thus, bulk techniques are rendered unsuitable for intercellular heterogeneity studies. The emerging importance of cell population heterogeneity imposes a demand for reliable gene transcription profiling techniques specifically tailored for individual cells. Measuring gene expression at the single-cell level is challenging because of the small amounts of total available mRNA

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(~1 pg). The large dynamic range of expression levels among genes is another hurdle that must be overcome by developing highly sensitive, specific, and reproducible detection strategies. The most reliable quantitative approach suitable for single-cell studies is based on reverse transcription (RT) without pre-amplification followed by quantitative polymerase chain reaction (qPCR). Besides enabling highly quantitative measurement of mRNA copy numbers, RT-qPCR theoretically allows detection of a single copy of mRNA. Several experimental techniques based on RT-qPCR and specialized for quantitative gene expression profiling in individual cells have been reported in the literature [7–12]. Several of these techniques use a single-tube RT-qPCR approach [7, 9, 13], in which all steps, including cell lysis, cDNA synthesis by reverse transcription, and quantitative PCR, are performed in one tube. This reduces the probability of mRNA loss and possible contamination during the sample-handling process. Although advantageous in terms of sample conservation, these single-tube methods can generate only one measurement for each cell, making it impossible to distinguish biological variation from measurement variation. In addition, the single-tube operation limits the number of genes that can be detected per single cell to less than five [8, 13]. To overcome these hurdles, multiple-step qRT-PCR procedures were recently developed [8, 14]. One of these protocols is based on a reusable single-cell cDNA library immobilized on beads for measuring the expression of multiple cDNA targets (from several copies to several hundred thousand copies) in a single mammalian cell. The results showed that the measurement error of this method is less than 15.9% among replicates [8]. Another procedure has been successful in assessing ten mRNA transcripts from a single cell, and each with one technical replicate [12]. The procedure could potentially be extended to analyze twenty different mRNAs from a single cell by use of duplex PCR [14]. Despite some initial successes [8–10, 14], analyzing multiple genes from one single cell by RT-qPCR remains challenging because the total amount of cDNA must be divided into multiple portions, limiting the sensitivity of RT-qPCR product detection.

Molecular oxygen is required for energy production in aerobic organisms. A shortage of oxygen (hypoxia) creates significant stress in cells, to which they respond by several different molecular mechanisms, including reduction in energy demand, cell cycle arrest, production and secretion of survival and angiogenic factors, etc. Hypoxia plays a pivotal role in cancer, causing alterations in cellular metabolism, increased resistance to radiation and chemotherapy[15], and possibly increasing cells' metastatic potential [16]. Oxygen deprivation has been shown to cause alterations in stem cell proliferation, differentiation, and pluripotency [17]. Despite recent advances in understanding cells' responses to hypoxia, the underlying molecular mechanisms remain unclear. More specifically, there is a lack of studies at the single-cell level that could provide deeper insights into hypoxia-driven selection and survival among different cell populations.

To gain better understanding of the molecular mechanisms contributing to the hypoxia response pathways, we have developed and evaluated an experimental technique based on the DNA-intercalating fluorescent dye SYBR green. The developed method enables quantitative expression level analysis of up to ten genes of interest in single mammalian cells.

The technique is a modification of one initially developed for gene expression analysis in single bacterial cells [18] and has so far not been tested in mammalian cells. The choice of SYBR green for qPCR is based on its relative ease of use, low cost, and suitability for development of high throughput, lab-on-chip procedures for RT-qPCR in single mammalian cells. The procedure presented here enables detection of up to ten genes, each with three technical replicates, from a single cell, with high repeatability (i.e. low standard deviations of quantification cycle, $C_q$). In addition, the SYBR-based chemistry we used provides greater flexibility to measure more genes in the future than Taqman-based technology.

Our method features a two-step procedure consisting of RNA isolation from a single mammalian cell followed by cDNA synthesis and a qPCR step. The expression levels of multiple genes of interest can be quantified simultaneously in single mammalian cells. The primers were designed for selected gene targets known, on the basis of bulk-cell studies, to be involved in hypoxia response [19–25]. We evaluated the primers at both bulk-cell and single-cell levels. We applied the method to cell populations exposed to hypoxia and quantitatively measured the expression levels of ten genes spanning a wide dynamic range of expression in randomly picked, single cells. We present the technical details of method development and implementation, with experimental results obtained by use of the procedure. The results showed significant gene expression level heterogeneity among the analyzed cells for each of the target genes. Detailed interpretation of the observed heterogeneity among cells from an isogenic cell population and its biological significance requires more experimental data. However, the results presented here demonstrate that our method is sensitive enough to quantify cellular responses at the single-cell level and to reveal gene expression heterogeneity in a cell population. We expect the advantages of this technique will facilitate further developments and advances in single-cell gene-expression profiling.

## Methods and materials

### Cell culture

The Barrett's esophageal epithelial cell line CP-A was cultured using Gibco keratinocyte serum-free medium (SFM) cell growth medium (Invitrogen, Carlsbad, CA, USA), supplemented with hEGF (Peprotech, Rocky Hill, NJ, USA) at 2.5 μg/500 mL, BPE (bovine pituitary extract) at 25 mg/500 mL and penicillin–streptomycin solution (Invitrogen) at 100 units/100 μg/mL. Cells were grown at 37 °C under 5% $CO_2$. Before experimentation, cells were cultured in a 75-mL flask to approximately 80% confluency. Cells were washed with 1X PBS (Cellgro, Manassas, VA, USA) and detached from the flask with 0.05% (*v*/*v*) trypsin–EDTA (Invitrogen). The trypsinization was blocked by Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) (Invitrogen). After trypsinization, cells were centrifuged at 900 rpm for 3 min then resuspended in 1 mL cell-growth medium.

### Primer design and selection of gene target

Thirteen genes were chosen for RT-qPCR expression level analysis. 18S rRNA, 28S rRNA, ACTB, and GAPDH were selected as reference genes because they are highly expressed housekeeping genes in mammalian cells. The 28S rRNA gene is a reliable internal control for comparative transcription analyses under hypoxic conditions [26]. HSP70 and HSC70 genes were chosen because HSP70 mRNA undergoes dramatic changes under stress conditions, whereas HSC70 does not and can be used as a matched reference gene [27]. HIF1α [19], VEGFA [20], PRKAA1 [28], p300 [22], MT3 [23], Angptl4 [24] and PTGES [25] are involved in hypoxia response signaling pathways. Gene sequences were retrieved from GenBank. qPCR primers were designed using the Primer-BLAST, Primer 3 or PrimerExpress V2.0 software packages, or retrieved directly from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). For each gene, multiple primer pairs were designed on the basis of their coding regions with amplification product lengths between 100 and 700 bp and annealing temperatures mostly between 60 and 65 °C. The amplification efficiencies of the primers were evaluated at bulk-cell and single-cell levels. Optimized primer oligos for single-cell analysis of the target genes were obtained from Fisher Scientific (Pittsburgh, PA, USA). The selected genes and their corresponding primers are listed in Table 1.

### Cell staining and fluorescence activated cell sorting

Cells were stained by incubating in cell medium containing 10 μmol $L^{-1}$ Hoechst 33342 dye (Invitrogen, Eugene, OR, USA) at 37 °C for 30 min. After staining the cells were trypsinized using 0.05% (*v*/*v*) trypsin (Invitrogen, Carlsbad, CA, USA) solution and resuspended in keratinocyte SFM containing 10 μmol $L^{-1}$ Hoechst 33342. Cells were kept on ice before sorting with a BD FACSAria cell sorter (BD Biosciences, San Jose, CA, USA). Cells in G1 phase were used in gene expression assays.

### Hypoxia treatment

CP-A cells at 80% confluency were incubated in keratinocyte SFM cell-growth medium containing 2% (*v*/*v*) Oxyrase (Oxyrase, Mansfield, OH, USA) at 37 °C for up to 30 min (see below). Cells were subsequently trypsinized in 0.05% (*v*/*v*) trypsin solution containing 2% (*v*/*v*) Oxyrase at 37 °C for 9 min. The trypsinization was blocked by Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) (Invitrogen) containing 2% (*v*/*v*) Oxyrase. The oxygen concentration was determined by use of an optical sensor [29] calibrated with a Clark electrode. To determine the optimum Oxyrase treatment time, the expression levels of selected genes in bulk cells exposed to hypoxia for 10, 20, 30, 60, 180, and 360 min were measured.

### Single cell collection

Single cells were collected using a pick-and-place single-cell manipulation robot [30]. Single cells were aspirated and dispensed using a 40-μm diameter glass capillary micropipette under closed-loop microscopic vision-based feedback. After a selected cell was aligned with the micropipette orifice, the cell was aspirated by applying a negative pressure to the micropipette capillary, generating a drag force on the cell and pulling it into the capillary (Electronic Supplementary Material Video S1). The micropipette tip containing an aspirated cell was directed into the cap of a 1.5-mL microcentrifuge tube (VWR, West Chester, PA, USA) containing 200 μL keratinocyte SFM. The cell was dispensed by applying a positive pressure to the micropipette capillary, generating an ejection force on the cell. Fluidic aspiration and dispensing of the cells were accomplished with minimal shear force on the cells so as to not cause physical damage to the cell. A total of 36 hypoxia-treated single cells and 36 control single cells were collected and analyzed.

### RNA isolation and reverse transcription

Each single cell in the cap of a 1.5-mL microcentrifuge tube was centrifuged at 4 °C and 17,000*g* for 20 min. Medium (160 μL) was taken out and 200 μL RNA lysis buffer from the ZR RNA MicroPrep Kit (Zymo Research, Orange, CA, USA) was added into the tube. The RNA extraction step was carried out using the ZR RNA Micro-

**Table 1** Genes and the corresponding primers

| Gene | GenBank access no. and description | Sequence | Amplicon size |
|---|---|---|---|
| 28S rRNA | NR_003287.2, *Homo sapiens* RNA, 28S ribosomal 1 (RN28S1) | F: CCGCTGCGGTGAGCCTTGAA<br>R: TCTCCGGGATCGGTCGCGTT | 312 |
| 18S rRNA | NR_003286.2, *Homo sapiens* RNA, 18S ribosomal 1 (RN18S1) | F: CCCGACCCGGGGAGGTAGTG<br>R: GCCGGGTGAGGTTTCCCGTG | 776 |
| ACTB | NM_001101.3, *Homo sapiens* actin, beta mRNA (ACTB) | F: CATGTACGTTGCTATCCAGGC<br>R: CTCCTTAATGTCACGCACGAT | 250 |
| GAPDH | NM_002046.3, *Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | F: TGTTGCCATCAATGACCCCTT<br>R: CTCCACGACGTACTCAGCG | 202 |
| HIF1A | NM_001530.3 and NM_181054.2, *Homo sapiens* hypoxia inducible factor 1, alpha subunit, transcript variants 1 and 2 (HIF1A) | F: CGTTCCTTCGATCAGTTGTGA<br>R: CTTCCATACGGTCTTTTGTG | 178 |
| VEGFA | NM_001171622-630, NM_001025366-370, NM_001025376, NM_001033756, *Homo sapiens* vascular endothelial growth factor A (VEGFA) transcript variants 1 to 8 | F: GCTACTGCCATCCAATCGAG<br>R: TGGTGATGTTGGACTCCTCA | 162 |
| p300 | NM_001429.3, *Homo sapiens* E1A binding protein p300 (EP300) | F: GCTTCAGACAAGTCTTGGCAT<br>R: GCCTGTGTCATTGGGCTTTTG | 198 |
| PRKAA1 | NM_006251.5, NM_206907.3, *Homo sapiens* protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1), transcript variants 1 and 2 | F: AACCATGATTGATGATGAAGCCT<br>R: GGTGTTTCAGCAACCAAGAATG | 261 |
| HSC70 | NM_153201.1, NM_006597.3, *Homo sapiens* heat shock 70 kDa protein 8 (HSPA8), transcript variants 1 and 2 | F: CGACCTGAACAAGAGCATCA<br>R: AAGATCTGCGTCTGCTTGGT | 342 |
| HSP70 | NM_021979.3, NM_005346.4, NM_005345.5, *Homo sapiens* heat shock 70 kDa protein 1A (HSPA1A), 1B (HSPA1B), 2(HSPA2) | F: TGTGGCTTCCTTCGTTATTGG<br>R: GCCAGCATCATTCACCACCAT | 213 |
| Angptl4 | NM_001039667.1 NM_139314.1, *Homo sapiens* angiopoietin-like 4 (ANGPTL4, ANGPTL2) | F: ACCTCCCGTTAGCCCCTG<br>R: CATGGTCTAGGTGCTTGTGGTC | 201 |
| MT3 | NM_005954.2, *Homo sapiens* metallothionein 3 | F: ATGGACCCTGAGACCTGCC<br>R: TTGCACACACAGTCCTTGGC | 155 |
| PTGES | NM_004878.4, *Homo sapiens* prostaglandin E synthase | F: TCAAGATGTACGTGGTGGCC<br>R: GAAAGGAGTAGACGAAGCCCAG | 201 |

Prep Kit following the manufacturer's instructions. A total volume of 6 μL RNA was eluted from the column matrix and immediately used in reverse transcription reactions. A total volume of 10 μL cDNA synthesis reaction mixture contained the reagents: 2 μL 5X VILO Reaction Mix (Invitrogen), 1 μL 10X SuperScript Enzyme Mix, including SuperScript III RT, RNaseOUT Recombinant Ribonuclease Inhibitor, and a proprietary "helper" protein (Invitrogen), 6 μL total RNA from a single cell, and 1 μL DEPC-treated water (Ambion, Austin, TX, USA). The tube contents were gently mixed and then cDNA synthesis was performed at 25 °C for 10 min, 42 °C for 60 min, followed by 85 °C for 5 min to inactivate the reverse-transcriptase. The cDNA obtained was stored at −20 °C until further use.

## qPCR

The Express SYBR GreenER qPCR SuperMix Kit (Invitrogen) was used for qPCR analysis. Each qPCR reaction for method development, primer testing, and optimization, and the hypoxia response experiment was performed using 5% (1/20th) of the cDNA obtained from a single cell. Reactions were conducted in 0.1-mL MicroAmp Fast 8-Tube Strips (Applied Biosystems, Foster City, CA, USA). Hypoxia treatment expression assays were conducted in adhesive-sealed, clear 384-well PCR plates (BioExpress, Kaysville, UT, USA). DEPC-treated water (30 μL) was added to 10 μL cDNA solution obtained from the reverse transcription step. The total reaction volume was 10 μL and comprised the reagents: 5 μL Express SYBR GreenER qPCR SuperMix Universal, 1 μL of each primer (4 μmol $L^{-1}$), 0.1 μL ROX reference dye (25 μmol $L^{-1}$), 2 μL cDNA solution (1/20th of a total of 40 μL cDNA solution obtained from a single cell) and 0.9 μL DEPC-treated water (Ambion). In negative control reactions, the 2 μL of cDNA solution was replaced with DEPC-treated water. The thermal cycling profile was set up as follows: one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 80 °C for 10 s with signal detection; melt-curve analysis at 60 °C for 1 min and the temperature increased in 0.3 °C increments to 95 °C, then

at 95 °C for 15 s. The method development experiments were run in a StepOne Real Time PCR System (Applied Biosystems). The gene expression profiling of hypoxia-treated cells was run in an Applied Biosystems 7900 Real-Time PCR System.

In order to push the limit on the number of genes whose expression levels could be quantified from a single mammalian cell, qPCR reactions were run on 1/45th of the cDNA obtained from individual cells. DEPC-treated water (80 μL) was added to the 10 μL of cDNA obtained from the reverse transcription step. Each reaction was conducted in a 0.1-mL PCR tube (Applied Biosystems). The reaction was set up in a total volume of 10 μL and contained the reagents: 5 μL Express SYBR GreenER qPCR SuperMix Universal, 1 μL of each primer (4 μmol $L^{-1}$), 0.1 μL ROX reference dye (25 μmol $L^{-1}$), 0.1 μL *Taq* DNA Polymerase (5 U $\mu L^{-1}$; Fermentas, Glen Burnie, MD, USA), 2 μL cDNA solution (1/45th of a total 90 μL of cDNA solution obtained from a single cell), and 1.8 μL DEPC-treated water. The thermal cycling profile was: 1 cycle at 95 °C for 10 min; 50 cycles at 95 °C for 15 s, 60 °C for 1 min, and 80 °C for 10 s with signal detection; melt-curve analysis at 60 °C for 1 min and the temperature increased in 0.3 °C increments to 95 °C then at 95 °C for 15 s. These experiments were run in a StepOne Real Time PCR System (Applied Biosystems). Data analysis was carried out using the StepOne software (Applied Biosystems). ANOVA *t*-test was used for statistical significance analysis.

## Results and discussion

### Two-step RT-qPCR analysis of single mammalian cells

We have developed a procedure for quantifying the expression levels of multiple genes in a single mammalian cell using SYBR green-based qPCR. The procedure comprises six steps:

1. fluorescence-activated cell sorting to obtain cells in a particular phase of the cell cycle, e.g. G1;
2. single-cell collection;
3. RNA extraction;
4. reverse transcription;
5. qPCR;
6. data analysis.

Adapting technology for RNA isolation and reverse transcription from single bacterial cells [18], we conceived, developed, and optimized a new procedure for the isolation, purification, and reverse transcription of the total RNA from a single mammalian cell. We used the ZR RNA MicroPrep kit (Zymo Research), followed by cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Because of picogram levels of available cDNA templates for qPCR in single-cell experiments, the probability of primer dimer formation increases. In this case, fluorescence signal originating from double-stranded primer dimers confounds quantification of the target gene amplification product. To eliminate such interference, we designed primer pairs whose amplification product lengths are between 100 bp and 700 bp. Therefore, amplification products can be distinguished from primer dimers, which are typically less than 100 bp, in both melt-curve analysis and agarose gel electrophoresis. Also, we modified the qPCR thermal cycling program to enable a signal-detection step (80 °C for 10 s) after the annealing/amplification step (60 °C for 1 min). The melting temperatures of primer dimers are usually less than 75 °C, whereas those of the target gene amplification products are more than 80 °C. Thus, when the fluorescence signal is detected at 80 °C, only the target gene amplification products will remain intact and the double-stranded primer dimers will dissociate. We used a total volume of 10 μL SYBR GreenER qPCR reagent kit for each qPCR reaction (as described in "Materials and methods"). The total amount of cDNA obtained from a single CP-A cell was divided into twenty equal portions and each portion was used for one qPCR reaction. Three technical replicates were run for each pair of primers to assess the sample-handling error. cDNA obtained from a bulk cell sample was diluted to a level corresponding to the amount of cDNA from 10 cells and used as the positive control. One negative control reaction, in which cDNA was replaced with DEPC-treated water, was run for each pair of primers. Experiments were conducted in a StepOne Real Time PCR System (Applied Biosystems). After qPCR, the contents of each PCR reaction were subjected to agarose gel electrophoresis and sequence analysis.

For method-validation purposes, we first conducted qPCR analyses of highly expressed genes (18S rRNA, 28S rRNA, ACTB, and GAPDH genes) in single cells. To increase primer binding specificity we conducted several iterations of primer optimization. So far, we have validated 13 pairs of primers targeting 13 different genes (Table 1). Specifically, 28S rRNA is a reliable internal control for comparative transcription analyses under hypoxic conditions [26]. Therefore, 28S rRNA was used as an internal reference for comparing gene expression levels within one cell and among multiple single cells. The choice of Hsp70 and Hsc70 genes is based on the fact that Hsp70 expression levels change significantly in response to stress whereas Hsc70 is a constitutively expressed cognate gene whose levels remain constant. Thus, Hsc70 can be used as a reference gene [27] for Hsp70. HIF1α [19], VEGFA [20], PRKAA1 [21], p300 [22], MT3 [23], Angptl4 [24], and PTGES [25] were chosen because of their roles in hypoxia response-signaling pathways. Using these primers, we

quantified the expression levels of genes of interest by RT-qPCR using 1/20th of the cDNA obtained from a single CP-A cell. Representative amplification plots for the 13 genes are shown in Fig. 1. The $C_q$ ratio of 28S rRNA to 18S rRNA has been found to be typically approximately 1 in mammalian cells [31]. Our results showed that the $C_q$ values for 28S rRNA and 18S rRNA are 19.73 and 20.71, respectively, indicating that the method yields intact mRNA and is reproducible and quantitative. Reactions with $C_q$ values lower than 37 and standard deviations of technical replicates smaller than 1% were regarded as successful. In most reactions, no-template negative controls (NTC) were not detected (Fig. 1). In some reactions residual NTC signals, most likely emanating from primer dimers, were detected at cycle numbers significantly higher than the product (at least 3 or 4 cycle difference). The spurious NTC signals were clearly distinguishable from those of the amplification products (cDNA) because of distinctly higher $C_q$ values (Fig. 1) and different melting peak temperatures (Electronic Supplementary Material Fig. S1). The amplification products from the reactions containing cDNA showed clear bands of the correct sizes in agarose gel electrophoresis (data not shown). The gel bands containing products of the right size were cut, and the product was purified for sequencing. Sequences were confirmed using BLAST annotation against the NCBI GenBank database.

**Fig. 1** Amplification plots of gene transcripts using validated primers. 1/20th of the total cDNA obtained from a single CP-A cell was used for each qPCR reaction shown. This includes three technical replicates and the no-template controls (NTC). Each panel shows real-time amplification signal curves obtained from a single cell. The $C_q$ values for 28S rRNA and 18S rRNA are 19.73 and 20.71, respectively, showing their abundance ratio of ~1. The $C_q$ values of 28S and 18S rRNAs are approximately 10 cycles lower than those of ACTB and GAPDH mRNAs. This is consistent with the fact that rRNA concentrations are 100 to 1000 times higher than mRNAs in cells, as found in bulk cell studies

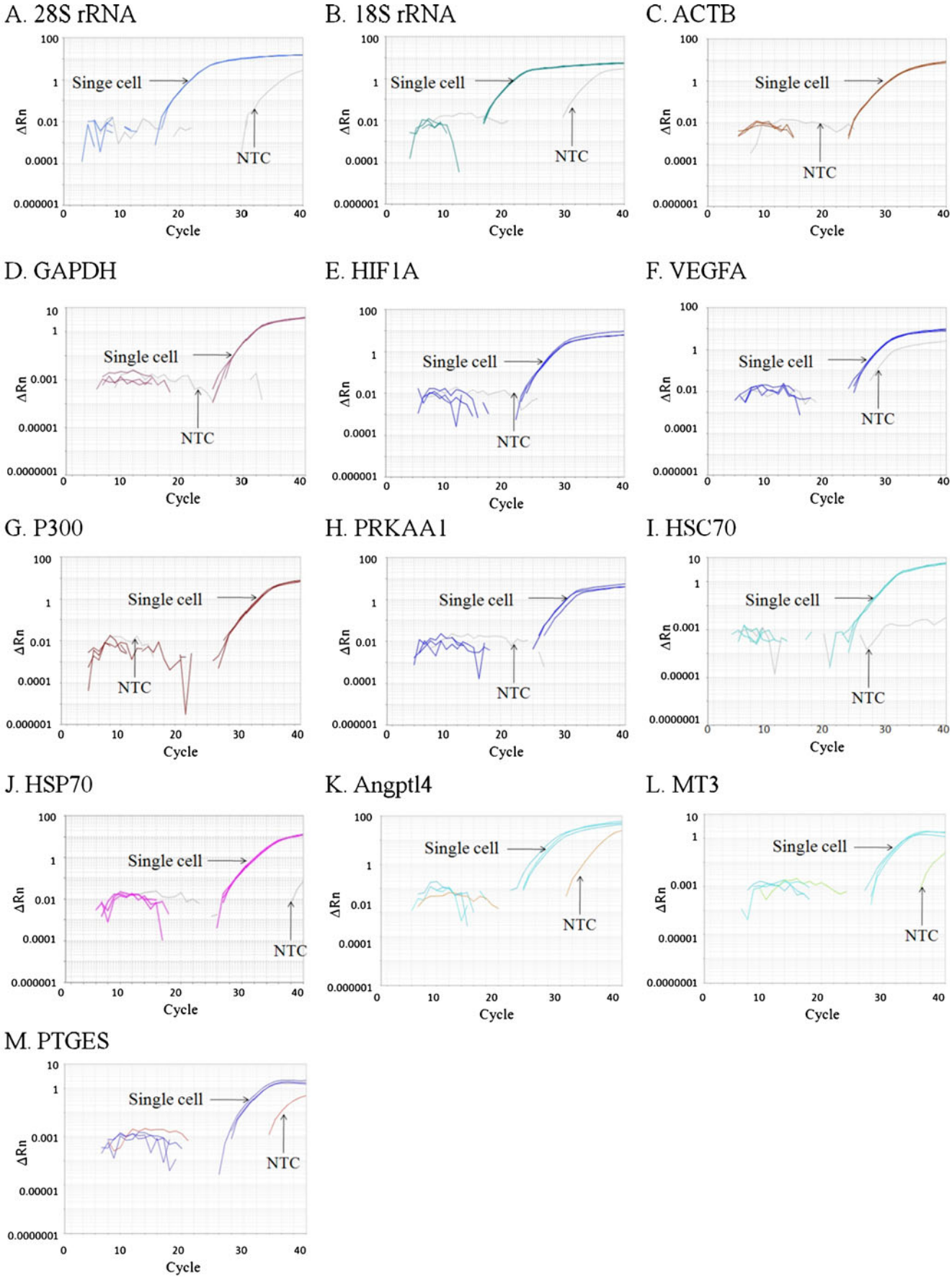
## Detection of up to ten genes from a single mammalian cell

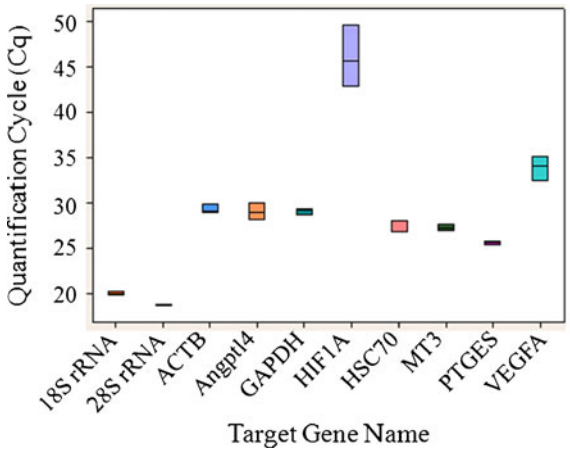
We have developed a method, which uses SYBR green-based RT-qPCR for detecting expression levels of up to ten different genes (18S rRNA, 28S rRNA, ACTB, Angptl4, GAPDH, HIF1A, HSC70, MT3, PTGES, VEGFA) in a single CP-A cell. These genes span a broad range of copy number in cells. A population of CP-A cells was exposed to hypoxia (as described in "Materials and methods"), and randomly selected, single cells were collected by use of a single-cell manipulation workstation. After RNA extraction and reverse transcription (described in "Materials and methods"), cDNA template obtained from a single CP-A cell was divided into 45 equal portions of 2-μL volume each. This dilution enabled up to 45 qPCR reactions to be performed on the cDNA obtained from a single cell, enabling triplicate analyses of multiple genes from the same cell. Each portion of the template was added to an SYBR GreenER qPCR reagent mixture, resulting in a total reaction volume of 10 μL. For each gene one RT-qPCR NTC was run in parallel. To increase the amplification level, 0.1 μL Taq DNA Polymerase (Fermentas) was added to each reaction mixture. As in the previous experiments, reactions for each of the genes were run in triplicate. To achieve sufficient amplification levels of low-abundance transcripts, the thermal cycling profile was modified by extending the amplification cycle number to 50. The reactions were run utilizing the StepOne Real Time PCR instrument.

Using this method, we could simultaneously quantify the transcription levels of up to ten genes with $C_q$ values lower than 50 (Fig. 2). The standard deviations of the qPCR $C_q$ value of eight genes (18S rRNA, 28S rRNA, ACTB, GAPDH, Angptl4, MT3, PTGES and HSC70) were less than 1 (3%) among the technical replicates (Fig. 2). As expected, larger variation between the technical replicates was observed for low-abundance transcripts, for example HIF1A and VEGFA (3.4 (7%) and 1.3 (4%), respectively). It is most likely that this is because of picogram levels of available cDNA templates, which makes the amplification events at the beginning less probable and introduces more noise in $C_q$ values. No amplification signal was detected for the NTC reactions of HIF1A, VEGFA, and HSC70, and for NTC reactions of 18S rRNA, 28S rRNA, ACTB, GAPDH, Angptl4, MT3, and PTGES amplification signals were observed after a large number of cycles. However, the melting temperatures ($T_m$ value) were 0.4–10.3 °C different from the $T_m$ values of the corresponding products in cDNA sample reactions. Therefore, amplification products resulting from unavoidable minute contamination and/or random, non-specific amplification can be identified by their characteristic $T_m$ values. Both length and sequence of the amplification products were confirmed by agarose gel electrophoresis (Electronic Supplementary Material Fig. S2) and sequencing.

## Gene expression under hypoxia

One of our research objectives was to understand epithelial cells' responses to hypoxic stress. To address the role of intercellular gene expression heterogeneity under different physiological conditions, we studied gene expression levels in CP-A cells in response to hypoxia. We used Oxyrase, an oxygen-scavenging enzyme system, to produce hypoxic conditions in cell growth medium. Oxygen concentration in the cell medium was reduced to <0.01 ppm within 20 min after Oxyrase addition (Fig. 3). To determine the time course of changes in gene expression, we first analyzed bulk cell


A. 28S rRNA
Singe cell
NTC
B. 18S rRNA
Single cell
NTC
C. ACTB
Single cell
NTC
D. GAPDH
Single cell
NTC
E. HIF1A
Single cell
NTC
F. VEGFA
Single cell
NTC
G. P300
Single cell
NTC
H. PRKAA1
Single cell
NTC
I. HSC70
Single cell
NTC
J. HSP70
Single cell
NTC
K. Angptl4
Single cell
NTC
L. MT3
Single cell
NTC
M. PTGES
Single cell
NTC
ΔRn
Cycle




| Target Gene Name | $C_q$ Mean | $C_q$ SD | $C_q$ SD% |
|---|---|---|---|
| 18S rRNA | 19.96 | 0.20 | 0.98% |
| 28S rRNA | 18.71 | 0.06 | 0.30% |
| ACTB | 29.33 | 0.55 | 1.88% |
| Angptl4 | 29.08 | 0.90 | 3.09% |
| GAPDH | 29.09 | 0.38 | 1.31% |
| HIF1A | 46.09 | 3.37 | 7.30% |
| HSC70 | 27.65 | 0.66 | 2.40% |
| MT3 | 27.31 | 0.34 | 1.24% |
| PTGES5 | 25.62 | 0.16 | 0.64% |
| VEGFA | 33.83 | 1.32 | 3.90% |

**Fig. 2** Quantification of expression levels of ten genes in a single, hypoxia-treated CP-A cell. 1/45th of the total cDNA obtained from a single CP-A cell was used for each qPCR reaction. Each box includes quantification cycle ($C_q$) values from three technical replicates for one gene. Average $C_q$ values and standard deviations among three technical replicates are summarized in the table. The $C_q$ values for 28S and 18S rRNAs are approximately 10 cycles lower than those of the housekeeping genes ACTB and GAPDH (100–1000 times higher in copy number), and 7–17 cycles lower than those of the genes of interest (100–100,000 times higher in copy number)

samples (~$10^4$ cells per sample) 0, 10, 30, 60, 180, and 360 min after adding Oxyrase to the cell medium, and measured the expression levels of six genes (Angptl4, PTGES, MT3, PRKAA1, VEGFA, and GAPDH) known to be involved in hypoxia response, using 28S rRNA as the internal control. A 1:1000 dilution of the total synthesized cDNA was used for RT-qPCR. The results showed that mRNA levels of Angptl4, PRKKA1, and MT3 increased and that of GAPDH decreased in response to the Oxyrase treatment, whereas mRNA levels of VEGFA and PTGES were not significantly affected (Electronic Supplementary Material Fig. S3).

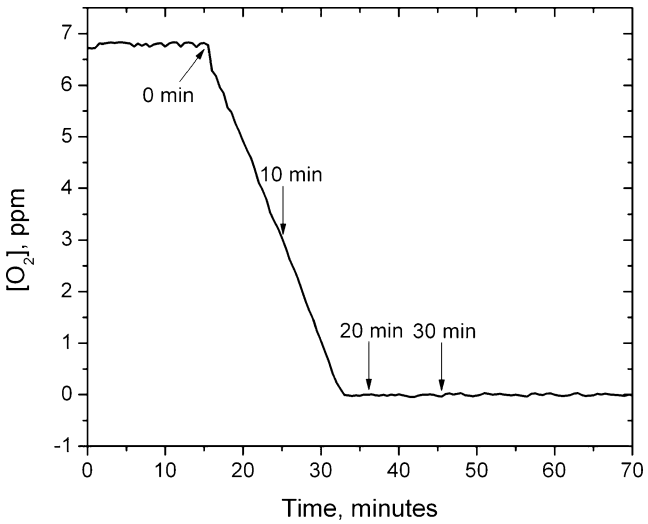



**Fig. 3** Oxygen depletion in the cell medium. Time course of the oxygen concentration in the cultivation medium before and after addition of 2.0% (v/v) Oxyrase. *Arrows* indicate the time points of gene expression profiling experiments. Oxyrase was added at time zero

The absence of significant changes in the expression levels of VEGFA and PTGES genes in bulk cell samples may be cell type-specific or a result of the relatively short hypoxia exposure (30 min). Among the genes studied, MT3 seems to be most sensitive to hypoxia, with 3 and 7-fold increases in expression level after 10 and 30 min treatments, respectively. At least twofold increases in transcription levels were observed for two other hypoxia-response genes, Angptl4 and VEGFA, in response to 30-min Oxyrase treatment. On the basis of the higher expression observed in bulk cell experiments after 30 min treatment with Oxyrase, we chose this treatment time for the single-cell analysis.

We randomly collected 72 single CP-A cells, 36 grown under normal physiological conditions (controls) and 36 exposed to hypoxia for 30 min, and measured the expression levels of seven selected genes in each cell. To limit cell-to-cell variability that may result from differences in cell cycle phase, we used cells in G1 phase sorted by means of fluorescence-activated cell sorting. In addition to six genes (PTGES [25], Angptl4 [24, 32–35], MT3 [23], GAPDH [36–38], PRKAA1 [21], and VEGFA [20, 37, 39]) known to be involved in the hypoxia response signaling pathway in various bulk cell-based studies, we also included the highly-expressed 28S rRNA as the reference gene. The number of cells with all seven target genes detected was higher in hypoxia-treated cells. For example, PTGES transcripts were detected in all 36 hypoxia-treated cells and in 27 control cells (Fig. 5A). We attribute this to higher mRNA copy numbers of these genes in cells exposed to hypoxia than in control cells. The 28S rRNA gene was detected in all 72 cells, with small cell-to-cell variations (standard deviations of $C_q$<±0.5).

The differences between $\Delta C_q$ values ($\Delta C_q = C_{q,\text{target gene}} - C_{q,28s}$ [40]) of the target genes as measured under normoxic

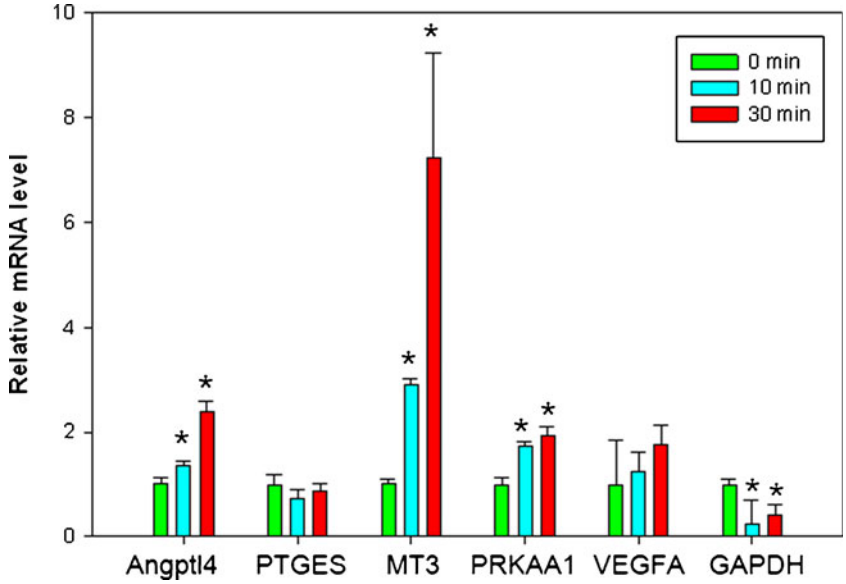



**Fig. 4** Gene expression levels of bulk cell samples under normoxic and hypoxic conditions. Results from three different experiments are shown. *Green bars* represent control cells that were not exposed to hypoxia. *Light blue bars* and *red bars* depict gene expression levels from cells exposed to hypoxia for 10 and 30 min, respectively. The changes of gene expression were calculated as $2^{-\Delta(\Delta C_q)}$, where $\Delta C_q = C_{q,\ target} - C_{q,\ 28s}$, and $\Delta(\Delta C_q) = \Delta C_{q,\ stimulated} - \Delta C_{q,\ control}$. *Asterisks* indicate statistical significance at $p<0.05$ on the basis of ANOVA *t*-test analysis

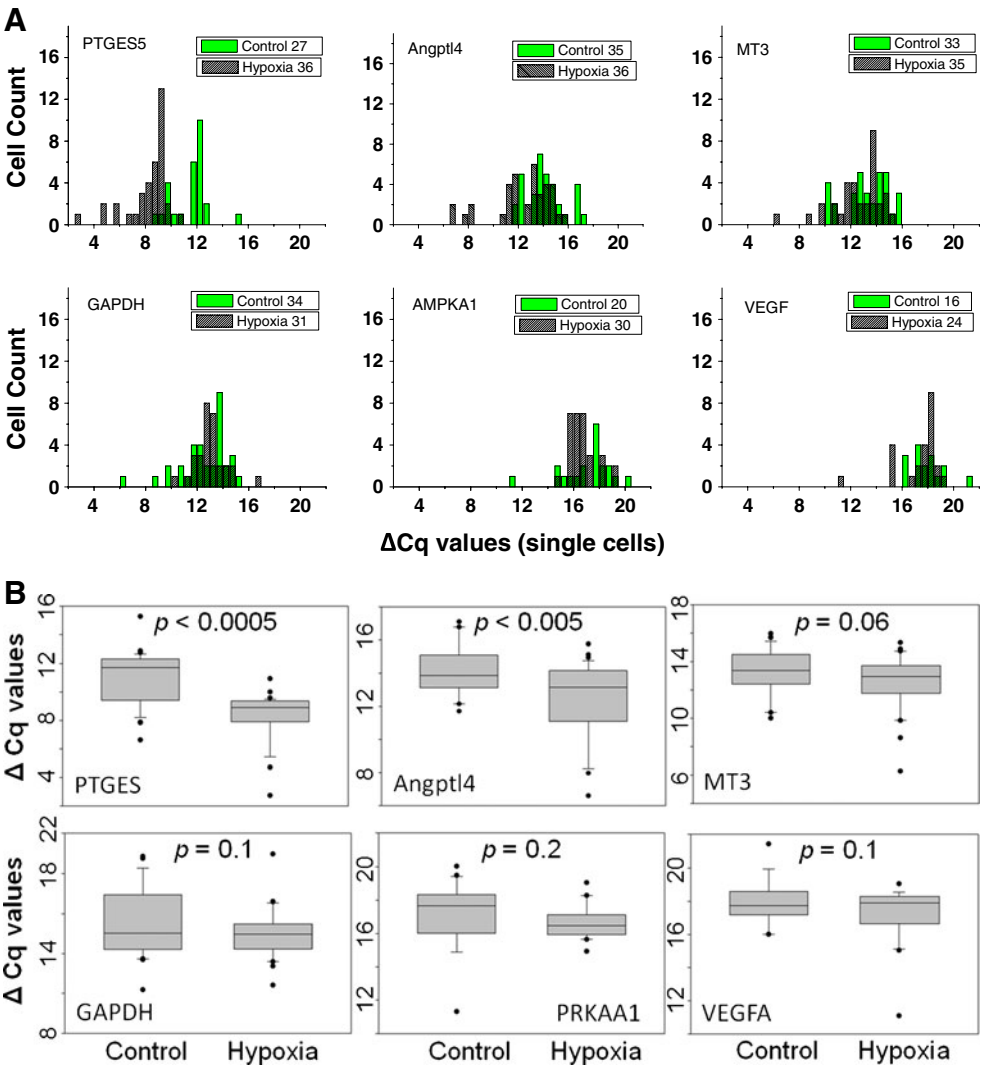



**Fig. 5** Single-cell gene-expression profiling in control and hypoxia-treated CP-A cells. (**A**) Histograms of gene expression levels in control (*green bars*) and hypoxia-treated (30 min, *hatched bars*) single CP-A cells in G1. (**B**) Box plots of single-cell gene-expression levels and *p*-values associated with differences between in untreated controls and hypoxia-treated CP-A cells

and hypoxic conditions were highly variable among cells, indicating significant cell-to-cell heterogeneity in the cell population. Distribution histograms of gene expression levels in control and hypoxia-treated CP-A cells indicate significant cell-to-cell heterogeneity in all six studied genes (Fig. 5A). PTGES and Angptl4 genes showed the largest differences in $\Delta C_q$ values between control and hypoxia-treated cells. The $\Delta C_q$ values for these genes were lower in the treated cells, indicating up-regulation of PTGES and Angptl4 in response to hypoxia. Statistical analysis of gene expression levels in hypoxia-treated vs. control single cells confirmed a significant reduction of $\Delta C_q$ values for PTGES ($p<0.0005$) and Angptl4 ($p<0.005$) genes, whereas changes in $\Delta C_q$ for the other four genes were not statistically significant (Fig. 5B). In some early bulk cell studies, expression levels of several of the target genes used in this study, for example the MT3 gene encoding a metal-binding protein metallothionein 3 and the PTGES gene encoding a prostaglandin E synthase, were dramatically increased under hypoxia [23]. The hypoxia treatment times used in these studies were 24 h [25], much longer than those used in our study (0.5 h) and, therefore, the findings are not directly comparable.

Interestingly, we found that the gene-expression patterns obtained from bulk-cell (Fig. 4) and single-cell samples (Fig. 5) differed in terms of response to hypoxia. Only two genes, Angptl4 and VEGFA, showed the same trend in both bulk and single-cell analyses: Angptl4 was up-regulated and VEGFA did not change significantly in response to hypoxia treatment. Whereas no significant changes at the single-cell level were observed for MT3 and PTGES, both genes were significantly up-regulated in bulk-cell samples, consistent with other bulk-cell studies [23, 25]. GAPDH was not significantly changed in single cells but significantly down-regulated in bulk cells whereas PTGES was significantly up-regulated in single cells whereas no significant change was observed in bulk cells. Recent studies on mRNA levels in individual cells suggest that cell-to-cell alterations in gene expression levels seem to be a result of variations at the bulk mRNA stability and/or translational level [41–47]. A recent study showed that single-cell gene expression has a log-normal distribution, reflecting true biological variability [48]. This finding indicates that average gene expression levels quantified in a population of cells may be substantially different from expression levels measured in individual cells from the same population [9]. This result also emphasizes the importance of developing and applying microfluidics-based instrumentation for high-throughput single-cell gene expression measurements with improved statistical power.

In summary, we have developed and applied a qPCR-based method for single-cell gene expression analysis, enabling measurement of multiple gene targets in a single mammalian cell. The method is based on separate RNA isolation, cDNA synthesis, and qPCR steps. Using this method, we quantified gene transcription levels in control and hypoxia-treated cells at both bulk and single-cell levels. The results show that quantitative analysis of gene expression of multiple genes can be achieved in single cells with good reproducibility and specificity. In addition, we observe significant gene-expression heterogeneity among the sorted cell population.

We plan to improve the procedure further by performing absolute mRNA abundance determination in single cells, using a gene sequence cloned into a plasmid as a reference to calculate absolute mRNA copy numbers. We will also use ACTB mRNA transcribed in vitro using T7 RNA Polymerase to validate the efficiency and reliability of the reverse transcription step of the procedure. Given the compatibility of this method with most commercially available RT-qPCR instrumentation and its relatively low cost, we expect it to be amenable to many applications focused on gene expression analysis in single cells, for example high-throughput, chip-based techniques [49], which will provide further insights into the cellular mechanisms involved in physiological and pathological processes at the single-cell level.

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