Subtle changes in host cell density cause a serious error in monitoring of the intracellular growth of *Chlamydia trachomatis* in a low-oxygen environment: proposal for a standardized culture method

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Abstract

We monitored *Chlamydia trachomatis* growth in HeLa cells cultured with either DMEM or RPMI medium containing 10% FCS under 2% or 21% O_2 conditions for 2 days. Bacterial numbers, host cell numbers, and fibrosis-related gene expression in the host cells were estimated by an inclusion forming unit assay, a cell counting assay, and a PCR array, respectively. In contrast to RPMI, bacterial growth under low oxygen conditions in DMEM rapidly decreased with increasing host cell density. The addition of supplements (glucose, glutamine, vitamin B12, D-biotin, non-essential amino acids, glutathione) to the media had no effect. The growth of host cells in DMEM under low oxygen conditions rapidly decreased, although the cells remained healthy morphologically. Furthermore, the downregulation of 17 genes was observed under low oxygen in DMEM. Whereas no effect on bacterial growth was observed when culturing in RPMI medium at low oxygen, and the downregulation of three genes (*CTGF*, *SERPINE1*, *JUN*) was observed following bacterial infection compared with the uninfected control cells. Thus, our findings indicate the need for carefully selected culture conditions when performing experiments with *C. trachomatis* under low-oxygen environments, and RPMI (rather than DMEM) is recommended when a low host cell density is to be used, proposing the major modification of cell culturing method of *C. trachomatis* in a low-oxygen environment.

Keywords: *Chlamydia trachomatis*, low-oxygen environment, host cell density, RPMI,

51 DMEM, CTGF

52

1. Introduction

Obligate intracellular bacterium *Chlamydia trachomatis* is the leading cause of bacterial sexually transmitted diseases worldwide, with an estimated 100 million chlamydial infections detected annually (Ziklo et al., 2016; World Health Organization 2011). Such infections are often asymptomatic in women (Imai et al., 2010; Haggerty et al., 2010; Qayum et al., 2000) and can therefore be left untreated, resulting in ascending infection with fibrosis, which is responsible for ductal obstruction, pelvic inflammatory disease, and infertility (Vodstrcil et al., 2015; Siracusano et al., 2014; Da Ros and Schmitt, 2008). It is well known that a low-oxygen environment is required for the induction of fibrosis (Menon et al., 2015), although whether infection is involved in the induction remains unknown. However, accumulating evidence suggests that a low-oxygen environment plays a central role in stimulating host cell signaling pathways involved in metabolism and inflammation, which are responsible for initiating fibrosis via stabilizing hypoxia-inducible factor 1α (HIF-1α), an oxygen sensor in mammalian cells (Semenza et al., 2010; Rupp et al., 2007; Kim et al., 2006). It is therefore reasonable to suggest that the intracellular growth of *C. trachomatis* may modulate the signal pathway governed by HIF-1α in human fallopian tube cells, facilitating fibrosis.

C. trachomatis possesses a unique developmental cycle, consisting of the elementary (EB) and reticulate body (RB) forms, differentiated from EB to RB (or re-differentiated from RB to EB) in inclusion bodies surrounded by the membrane vesicle (Rockey and Matsumoto, 2000). After infection, the maturation process in infected host cells is known to

require actin remodeling (Dunn and Valdivia, 2010; Jewett et al., 2006; Balañá et al., 2005), lipid metabolism (Stehr et al., 2013; Elwell and Engel, 2012; Saka and Valdivia, 2012), and inflammatory responses (Ziklo et al., 2016; Entrican et al., 2014; Cochrane et al., 2010). However, chlamydial studies in a low-oxygen environment are limited and difficulties are encountered in even assessing its intracellular growth. However, a few studies have shown that *C. trachomatis* adapts well to a low-oxygen environment, such as that found in the urogenital tract of women (Jerchel et al., 2014; Shima et al., 2011; Juul et al., 2007; Peters et al., 2005). Meanwhile, it is well recognized in low-oxygen environment that cellular metabolism such as glycolysis prompting ATP generation required for chlamydial growth can be up-regulated by stabilizing HIF-1α, which is a master transcriptional factor in the environment (Palmer and Clegg, 2014; Papandreou et al., 2006). Therefore, the cells left into culture under low-oxygen condition may rapidly consume substances in the cultures, inevitably responsible for deteriorating chlamydia growth into limited culture medium. Thus, accurate evaluation of chlamydial growth in a low-oxygen environment is a crucial issue for understanding chlamydial dynamics in the urogenital tract, although it has not been standardized among researchers.

In the present study, we carefully monitored *C. trachomatis* growth in immortal human epithelial HeLa cells and fibrosis-related host responses in a low-oxygen environment. We showed that in contrast to normal atmospheric conditions, subtle changes in host cell density can lead to serious errors in the monitoring of the intracellular growth of *C. trachomatis* in a low-oxygen environment, proposing the major modification of cell

culturing method of *C. trachomatis* in a low-oxygen environment.

2. Materials and methods

The epithelial cell line HeLa was purchased from ATCC (Manassas, VA, USA). The cell line was maintained at 37°C in 5% CO_2 in DMEM (Sigma) containing 10% heat-inactivated fetal calf serum (FCS), 10 μg/ml gentamycin, 10 μg/ml vancomycin, and 1 μg/ml amphotericin B (Sigma), according to a previously published protocol (Roblin et al, 2007). *C. trachomatis* D/UW3 Cx strain (VR-885) was purchased from ATCC, and propagated as described previously (Kubo et al., 2012). The number of infectious progeny for *C. trachomatis* was determined as inclusion forming units (IFU) by counting chlamydial inclusions formed in HeLa-2 cells using a fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-*Chlamydia* antibody specific for *Chlamydia* lipopolysaccharide (with Evans Blue) (Denka Seiken) (Kubo et al., 2012).

HeLa cells were adjusted to a concentration of 2×10^5 cells/well and simultaneously infected with *C. trachomatis* at a multiplicity of infection (MOI) of 2 by centrifugation for 450×*g* at room temperature. After washing to remove non-infecting bacteria, the cells at a concentration of 1 to 20×10^4 cells/well were incubated with DMEM (D6046) or RPMI (R8758) containing 10% FCS, gentamycin (10 μg/ml), vancomycin (10 μg/ml), and amphotericin B (0.5 μg/ml) for 2 days at 37°C under conditions of either 21% or 2% O_2. For some experiments, cells were cultured in DMEM plus supplements (vitamin B12,

D-biotin, non-essential amino acids, glutamine, and glucose) (See Table S1) or cycloheximide (final concentration 2 μg/ml). Cells were harvested and viability was determined by a cell counting assay (see below) and EB numbers were determined by an IFU assay. A low-oxygen environment (2% O_2) was created using a dedicated camber MIC-101 (Billups-Rothenberg). The mixed gas containing 2% and 21% O_2 used as a control consisted of 2% O_2, 5% CO_2, and 93% N_2 or 21% O_2, 5% CO_2, and 74% N_2, respectively. In addition, pH values in culture mediums were also monitored in some experiments with a hand-type's pH meter, twinpH (Horiba Ltd.); the values was measured less than 5 mim after removed from the camber to minimize affect of atmosphere.

To determine the morphology of chlamydial inclusions, cells stained with specific antibodies were observed using a conventional [IX71 (Olympus)] or a confocal laser microscope [LSM510 (Carl Zeiss Japan Group)] (Kubo et al., 2012). Cell Counting Kit-8 (Dojindo) was used to determine cell viability according to the protocol described in the manufacturer's instruction. Briefly, water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activity in cells to give a yellow colored formazan dye, which is soluble in tissue culture media. The intensity of the color is measured at $OD_{450\,nm}$ as an indicator of cell viability.

The Human Fibrosis RT^2 Profiler PCR Array kit equipped with 84 genes (Table S2) was used to determine the expression of fibrosis-related genes (Qiagen), according to the protocol described in the manufacturer's instructions. In brief, cells (20×10^4 cells/well in DMEM; 5×10^4 cells/well in RPMI) with or without *C. trachomatis* infection were

incubated for 24 h under 2% or 21% O_2 conditions. After incubation, the cells were collected and then total RNA was extracted using the High Pure RNA Isolation Kit (Roche). An RT reaction was performed on 500 ng of RNA and the synthesized cDNA was analyzed by Applied Biosystems StepOne Plus real-time PCR system to determine the changes in fibrosis-related genes. The values were expressed as relative fold changes compared with the control array. According to the protocol, more than or less than two-fold changes compared with the control were defined as meaningful changes. Each of the values was expressed as the average of two independent experiments.

Comparison of the total IFU numbers between oxygen conditions and between experimental groups was conducted using a Bonferroni/Dunn test and Student's *t* test, respectively. A p-value of <0.01 was considered significant.

3. Results

First, by assessing bacterial numbers using an IFU assay and confocal fluorescence microscopy observations, we compared the growth of *C. trachomatis* (MOI 2) in HeLa cells (5×10^4 cells/well of a 24-well plate) cultured either in DMEM or RPMI medium in a 21% or 2% oxygen environment (Fig. 1A). The growth of *C. trachomatis* was gradually dampened in DMEM compared with RPMI medium, in particular in the 2% oxygen environment 48 h after infection (Fig. 1B). However, even when cultured in DMEM, there was no significant difference in inclusion size between the 21% and 2% oxygen

158 environments (Fig. 1C). Furthermore, all of the cells appeared healthy morphologically,
159 although the growth of HeLa cells was slowed under hypoxic conditions compared with
160 conditions of 21% O_2 (Fig. S1). Thus, although bacterial growth initially appeared the same,
161 there was a significant difference in terms of bacterial numbers, with numbers being
162 decreased particularly in DMEM under low-oxygen conditions.

163 In contrast to RPMI medium, DMEM has a low glucose concentration and lacks
164 certain components such as glutamine, non-essential amino acids, and some vitamins
165 (Table S1). We therefore assessed whether supplementation of these components into
166 DMEM could restore bacterial growth to the levels observed in RPMI medium under low
167 oxygen (2% O_2) conditions (Fig. 2A). However, regardless of supplementation, bacterial
168 growth did not recover to the levels observed under 21% O_2 conditions (Fig. 2B). Despite
169 this, it was noted that in contrast to supplementation of DMEM with vitamin B12, D-biotin,
170 and non-essential amino acids or glutamine alone, the addition of glucose, which is the
171 most effective energy source, had an impact on bacterial growth (Fig. 2C). This result
172 indicated that the lack of bacterial growth in DMEM under low oxygen was likely due to
173 the rapid starvation of glucose arresting host cell growth, presumably in turn resulting in a
174 decrease in bacterial intracellular growth because of the requirement for ATP energy from
175 the host cells (Rockey and Matsumoto, 2000). This implied that changing the host cell
176 density may have an impact on bacterial growth in HeLa cells in low oxygen environments.

177 To investigate the effect of host cell density changes, we compared the growth of *C.*
178 *trachomatis* when cultured with host cells at a range of seeding concentrations (from 1×10^4

179 to 20×10^4 cells/well in a 24-well plate) under conditions of 21% or 2% O_2 (Fig. 3A). In
180 contrast to growth in RPMI medium, bacterial growth in DMEM under low oxygen (2%
181 O_2) conditions was significantly decreased (by up to 10,000 times) with increasing host cell
182 density (Fig. 3B). Meanwhile, morphological observations and cell counts revealed that
183 even under low-oxygen conditions at high-cell density, the viability of host cells was
184 maintained during culturing despite the decrease in growth speed (Fig. 3C, Fig. S2). The
185 addition of cycloheximide, a protein synthesis inhibitor specific to mammalian cells that
186 inhibits cellular replication, recovered the inclusion size of the bacteria back to normal
187 levels (Fig. 3C, with cycloheximide), supporting host cell density as a critical factor for
188 bacterial intracellular growth under low-oxygen conditions. These results indicated that
189 unlike under normal oxygen conditions, subtle changes to host cell density under
190 low-oxygen conditions may be detrimental to the intracellular growth of *C. trachomatis*
191 particularly in DMEM, presumably causing serious errors potentially affecting host
192 responses; however, this has been overlooked when monitoring the intracellular growth of
193 *C. trachomatis* in low-oxygen environments. In addition, although pH values in cultures
194 were monitored during 48h after incubation, no significant changes was observed
195 regardless of either distinct medium or O_2 concentration (Fig. S3). The results indicated
196 effect of pH changes on *C. trachomatis* growth was minimal in either case.

197 As mentioned above, although several studies had shown decreased growth of *C.*
198 *trachomatis* in immortalized human epithelial cells (HeLa or HEp-2 cells) cultured in
199 DMEM under low-oxygen conditions (Jerchel et al., 2014; Shima et al., 2011; Juul et al.,

2007), subtle changes in host cell density seeding with *C. trachomatis* in these experiments may have been overlooked and may lead to serious errors in the monitoring the bacterial intracellular growth or the evaluation of gene expression changes in host cells. To investigate this, we employed a PCR array of 84 genes (Table S2) to evaluate the expression of genes associated with fibrosis in HeLa cells cultured in DMEM at high host cell density (20×10^4 cells/well) and RPMI medium at optimal host cell density (5×10^4 cells/well) under conditions of either 21% or 2% O_2 (Fig. 4A). Compared with gene expression under 21% O_2 conditions (HeLa cells: 20×10^4 cells/well) (without *C. trachomatis* infection), 17 genes were downregulated under 2% O_2 conditions (HeLa cells: 20×10^4 cells/well) (without *C. trachomatis* infection) in DMEM (Fig. 4B). However, no changes in gene expression were observed when cells were cultured in RPMI medium (HeLa cells: 5×10^4 cells/well) (with or without *C. trachomatis* infection) (Fig. 4C–E). These results clearly indicated the DMEM containing a high host cell density caused the artificial downregulation of host cell genes, resulting in a serious mistake in translation. By contrast, when cells were cultured in RPMI medium, no effect on bacterial growth at low oxygen was observed and we found that bacterial infection caused the downregulation of three genes, *CTGF, SERPINE1,* and *JUN,* all of which are critical factors regulating fibrosis, when compared with uninfected control cells (Fig. 4E). These data revealed that when performing *C. trachomatis* experiments under low-oxygen conditions, medium selection and host cell density are critical factors for the correct monitoring of chlamydial dynamics.

4. Discussion

C. trachomatis is the most common pathogen causing sexually transmitted infections worldwide (Qayum et al., 2013; World Health Organization 2011; Haggerty et al., 2010; Imai et al., 2010). The symptoms of infection are often minimal in females and therefore remain untreated, resulting in chronic inflammation responsible for pelvic inflammatory disease or infertility with fibrosis (Vodstrcil et al., 2015; Siracusano et al., 2014; Da Ros and Schmitt, 2008). *C. trachomatis* adapts well to a low-oxygen environment, such as that found in the urogenital tract of women (Entrican et al., 2014; Jerchel et al., 2014; Shima et al., 2011; Juul et al., 2007; Sommer et al., 2001). Therefore, accurate evaluation of chlamydial growth in a low-oxygen environment is a crucial issue for understanding chlamydial dynamics in the urogenital tract. Our findings showed that subtle changes in host cell density can cause serious errors in the monitoring of the intracellular growth of *C. trachomatis* in a low-oxygen environment.

It was clear that during a 2-day culture, the viability of HeLa cells was morphologically maintained under low-oxygen conditions regardless of the bacterial infection. However, the speed of host cell growth decreased compared with cells cultured in a normal oxygen environment. Recent studies have revealed that HIF-1, which is a critical factor controlling cellular homeostasis in low-oxygen environments (Palmer and Clegg, 2014), reduces the induction of pyruvate dehydrogenase kinase-1 (PDK-1) in response to low-oxygen conditions (Kroening et al., 2009; Papandreou et al., 2006). Specifically,

PDK-1 phosphorylates and then inactivates pyruvate dehydrogenase E1α, which converts pyruvate to acetyl-coenzyme A, resulting in blocking the entry of pyruvate into the tricarboxylic acid cycle (Kroening et al., 2009; Papandreou et al., 2006). It has also been established that HIF-1 induction suppresses mitochondrial ROS production under hypoxic conditions, preventing cell death caused by apoptosis (Minet et al., 2000). Thus, although detrimental, the influence of low-oxygen on host cell viability is unexpectedly minimal, as the cells subtly adjust to the shortage in energy source (ATP) by slowing their growth. It is therefore inevitable that the bacterial growth rate would decrease in response to a subtle increase in the number of seeded cells as a result of the consumption of more ATP.

Studies to evaluate chlamydial dynamics in low-oxygen environments have concluded that *C. trachomatis* growth is similar to that under normal atmospheric conditions (Entrican et al., 2014; Jerchel et al., 2014; Shima et al., 2011; Juul et al., 2007; Sommer et al., 2001). Whether subtle changes in host cell density seeding and media type can influence chlamydial dynamics under low-oxygen conditions remains to be investigated. While host cells remain healthy even under low-oxygen conditions, chlamydial growth is gradually blocked when the number of seeded host cells is increased. Surprisingly, this decrease in bacterial growth rate was even more pronounced during culturing with DMEM compared with RPMI medium. In contrast to RPMI medium, DMEM has a low glucose concentration and lacks certain components such as glutamine, non-essential amino acids, and some vitamins (See Table S1). Supplementation of DMEM with these components partially rescued bacterial intracellular growth (although this was not statistically

significant), indicating that seeding host cell density is a crucial factor for propagating *C. trachomatis* under low-oxygen conditions.

As mentioned above, during the 2-day culture required for *C. trachomatis* maturation, the HeLa cells appeared healthy morphologically regardless of the oxygen conditions and the deterioration of bacterial growth at a host cell density of 5 to 20×10^4 cells per well (24-well plate) may therefore have gone unnoticed. However, regarding gene expression associated with fibrosis, a serious error was detected due to the downregulation of 17 genes only under conditions of 2% oxygen in DMEM. Thus, our findings emphasize the need to carefully select appropriate culture conditions for chlamydial experiments under low-oxygen conditions and we recommend the use of RPMI (rather than DMEM) media accompanied by a low host cell density.

It has been reported that accumulation of CTGF, a factor responsible for connective tissue growth (Kroening et al., 2009; Ito et al., 1999), occurred in low-oxygen tissues activating matrix proteases such as TIMPS, which in turn resulted in an increase in fibroblasts producing extracellular matrix proteins (Kroening et al., 2009; Ito et al., 1999). By performing PCR array experiments under optimal culture conditions using RPMI, we found that *C. trachomatis* infection caused the downregulation of three genes (*CTGF*, *SERPNE1*, and *JUN*). Since it is well known that cancer-associated fibroblasts play a key role in cancer progression by contributing to invasion, metastasis, and angiogenesis in a CTGF-dependent manner (Ren et al., 2015), the downregulation of these three genes may lead to the accumulation of *C. trachomatis*-infected cells in tissues such as the fallopian

284 tube, likely resulting in the exacerbation of inflammation. A previous study revealed that in
285 models of persistence under normal oxygen conditions, chlamydial infection induced
286 expression of the *CTGF*, *EGR-1*, and *ETV4* genes associated with fibrosis (Peters et al.,
287 2005), which supports our data.

288 Taken together, we conclude that in contrast to normal atmospheric conditions, subtle
289 changes in host cell density cause serious errors in the monitoring the intracellular growth
290 of *C. trachomatis* in low-oxygen environments, recommending the use of RPMI (rather
291 than DMEM) and low host cell densities in such experiments. Our findings highlight an
292 important issue that must be addressed when accurately monitoring the intracellular growth
293 of *C. trachomatis* in low-oxygen environments, proposing the major modification of cell
294 culturing method of *C. trachomatis* in a low-oxygen environment.

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297 **Abbreviations**

298 DMEM: Dulbecco's modified Eagle's medium; RPMI: Roswell Park Memorial Institute
299 medium; IFUs: inclusion-forming units; Glu: glucose; Gln: glutamine; GG: glucose +
300 glutamine; PCR: polymerase chain reaction; RT: reverse transcriptase

301

302 **Ethics**

303 The study reported in this manuscript did not involve any human participants, human data,
304 human tissue, data on specific individuals, or animal experiments.

305

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312 **Competing interests**

313 The authors declare that they have no competing interests.

314

315 **Author contributions**

316 Conceived and designed the project: HY. Analysis and confirmation: ST, JM, HY. Imaging:

SN. Critical editing: JM, TO, HY. Writing of the manuscript: HY. All authors read and approved the final manuscript.

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Conflicts of interest: none.

Supplementary data

Table S1. Comparison of the medium formulation between DMEM(D6046) and RPMI(R8758)

Table S2. List of the genes loaded onto the Human Fibrosis RT² Profiler PCR Array

Fig. S1. Comparison of the viability and morphological features of HeLa cells infected with *C. trachomatis* cultured under hypoxic (2% O_2) and normal (21% O_2) conditions. The bacteria were used to infect cells at a MOI of 2 (see the text). Cell viability was determined using a Cell-counting assay kit (see the text). Phase-contrast images were captured at 600×. White asterisks show representative inclusion bodies formed by *C. trachomatis* growth. Comparisons of the cell numbers between 2% and 21% O_2 conditions were conducted using a Student's *t* test. Black asterisks show statistical significance at $p<0.05$. Experiments were performed at least three times independently.

Fig. S2. Changes in the viability of HeLa cells grown under hypoxic (2% O_2) and normal (21% O_2) conditions depending on the number of cells seeded. Cell viability was determined using a Cell-counting assay kit (see the text). The experiments were performed in 96-well plates (not 24-well plates) as this was more time efficient. Cell numbers following seeding in 96-well plates were almost identical to those in 24-well plates. Comparison of the cell numbers between 2% and 21% O_2 conditions were conducted using

a Student's *t* test. Asterisks indicate statistical significance at $p<0.05$. Experiments were performed at least three times independently.

Fig. S3. Changes of pH values in culture of HeLa cells with or without *C. trachomatis* infection into DMEM and RPMI under hypoxic ($2\%O_2$) and normal ($21\%O_2$) conditions. The bacteria were infected to HeLa cells (5×10^4 cells) at MOI 0.2. Each of the values were expressed as average values plus SD into three experiments. Also, phase contrast images (magnification, ×400) show representative inclusion formations at 48h after infection (White arrows).

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Legends to figures

Fig 1. Dampening effect of DMEM on *C. trachomatis* growth in HeLa cells, particularly under low-oxygen conditions. Panel (A) shows the experimental design and time course. Panel (B) shows the comparison of IFU values between 2% and 21% O_2 conditions in DMEM or RPMI media. Statistical analysis was conducted by a Student's *t* test. Black stars indicate a significant difference ($p<0.05$) between the two oxygen conditions. Experiments were performed at least three times independently. Panel (C) shows representative images of the inclusion bodies formed in *C. trachomatis*-infected HeLa cells. White arrows show inclusion bodies. Scale bar: 5 μm.

Fig 2. Addition of supplements recovered bacterial growth in HeLa cells cultured in DMEM under low-oxygen conditions. Panel (A) shows the experimental design and time course. Panel (B) shows the comparison of IFU values between 2% and 21% O_2 conditions following the addition of supplements (glucose, glutamine, vitamin B12, D-biotin, non-essential amino acids, glutathione) lacking in DMEM but not RPMI medium. Statistical analysis was conducted by a Student's *t* test. Black stars indicate a significant difference ($p<0.05$) between the two oxygen conditions. Experiments were performed at least three times independently. Panel (C) shows a comparison of all sets of data. Statistical analysis was conducted by a Bonferroni/Dunn test. Blue color indicates a significant difference ($p<0.05$) between the two sets.

Fig. 3. In contrast to normal oxygen conditions, subtle changes in the host cell density were detrimental to the intracellular growth of *C. trachomatis* in a low-oxygen environment. Panel (A) shows the experimental design and time course. Panel (B) shows the comparison of IFU values between 2% and 21% O_2 conditions in DMEM or RPMI media depending on changes in the number of cells seeded. Statistical analysis was conducted by a Student's *t* test. Black stars indicate a significant difference ($p<0.05$) between two oxygen conditions. Panel (C) shows the phase-contrast images. White stars show inclusion bodies. Squares surrounded by dashed lines are enlarged versions of the images connected by arrows.

Fig. 4. PCR array data revealed that in contrast to RPMI medium, the artificial downregulation of 17 fibrosis-associated genes occurred in DMEM under low oxygen conditions. Human Fibrosis RT^2 Profiler PCR Array kit equipped with 84 genes (Table S2) was used to determine the expression of fibrosis-related genes (Qiagen), according to the protocol described in the manufacturer's instructions (see the text). Panel (A) shows the experimental design and time course. Panel (B) shows the changes in gene expression in the HeLa cells (20×10^4 cells/well) (without infection) under 2% O_2 conditions compared with 21% O_2 conditions in DMEM. Panel (C) shows the changes in gene expression in HeLa cells (5×10^4 cells/well) (with infection) under 2% O_2 conditions compared with 21% O_2 conditions in DMEM. Panel (D) shows the changes in gene expression in HeLa cells

(5×10^4 cells/well) (without infection) under 2% O_2 conditions compared with 21% O_2 conditions in RPMI medium. Panel (E) shows the changes in gene expression in HeLa cells (5×10^4 cells/well) (with infection) under 2% O_2 conditions compared with 21% O_2 conditions in RPMI medium. Genes names in red and green indicate upregulated and downregulated genes, respectively. Each of the values is expressed as the average of two independent experiments.

Fig. 1

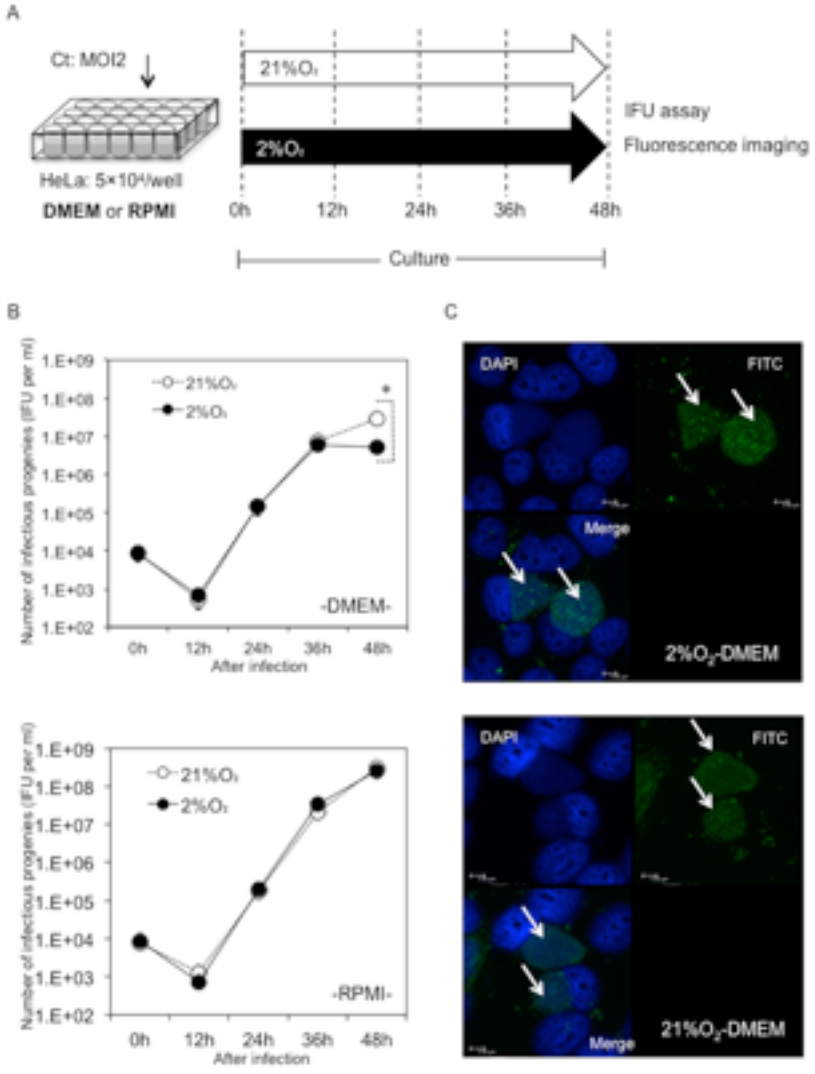

A
Ct: MOI2
21%O2
2%O2
IFU assay
Fluorescence imaging
HeLa: 5×10⁴/well
DMEM or RPMI
0h
12h
24h
36h
48h
Culture
B
C
Number of infectious progenies (IFU per ml)
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
21%O2
2%O2
*
-DMEM-
After infection
-RPMI-
DAPI
FITC
Merge
2%O2-DMEM
21%O2-DMEM

Fig. 2

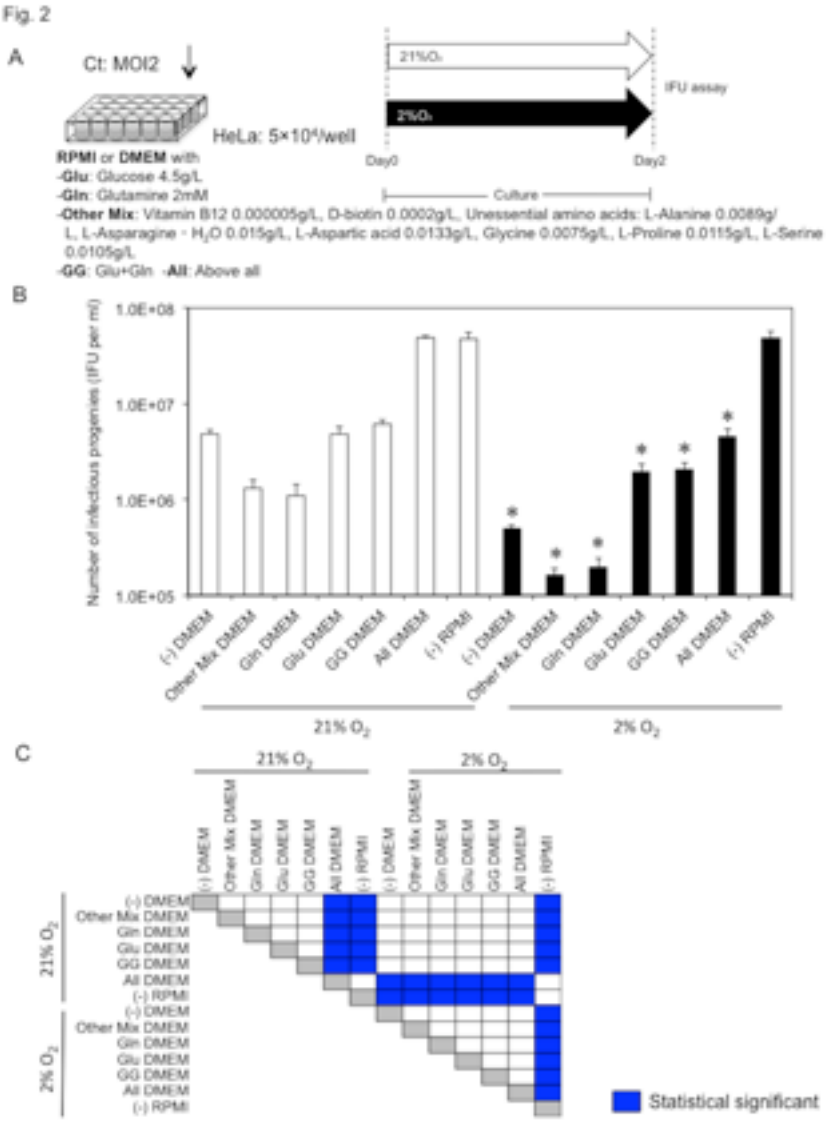

A
Ct: MOI2
HeLa: 5×10⁴/well
21%O₂
2%O₂
IFU assay
Day0
Day2
Culture
RPMI or DMEM with
-Glu: Glucose 4.5g/L
-Gln: Glutamine 2mM
-Other Mix: Vitamin B12 0.000005g/L, D-biotin 0.0002g/L, Unessential amino acids: L-Alanine 0.0089g/L, L-Asparagine・H2O 0.015g/L, L-Aspartic acid 0.0133g/L, Glycine 0.0075g/L, L-Proline 0.0115g/L, L-Serine 0.0105g/L
-GG: Glu+Gln -All: Above all
B
Number of infectious progenies (IFU per ml)
1.0E+08
1.0E+07
1.0E+06
1.0E+05
(-) DMEM
Other Mix DMEM
Gln DMEM
Glu DMEM
GG DMEM
All DMEM
(-) RPMI
21% O2
2% O2
C
21% O2
2% O2
Statistical significant

Fig. 3

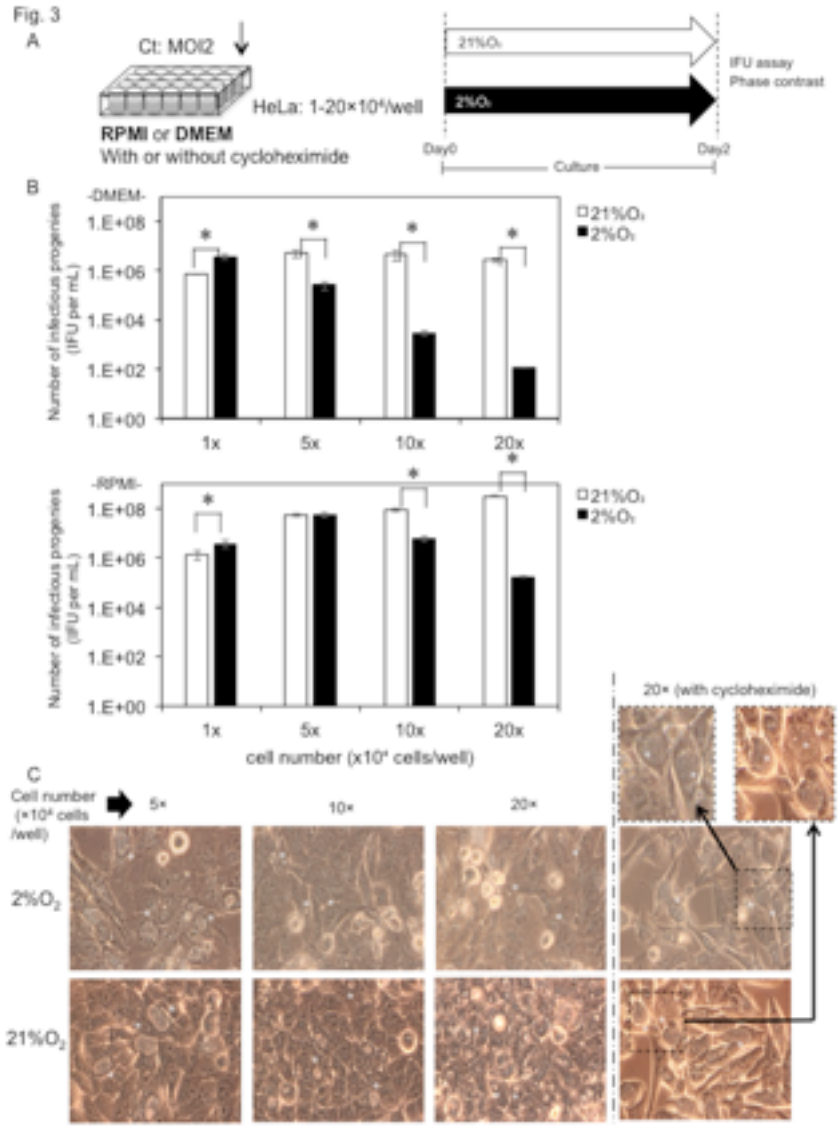
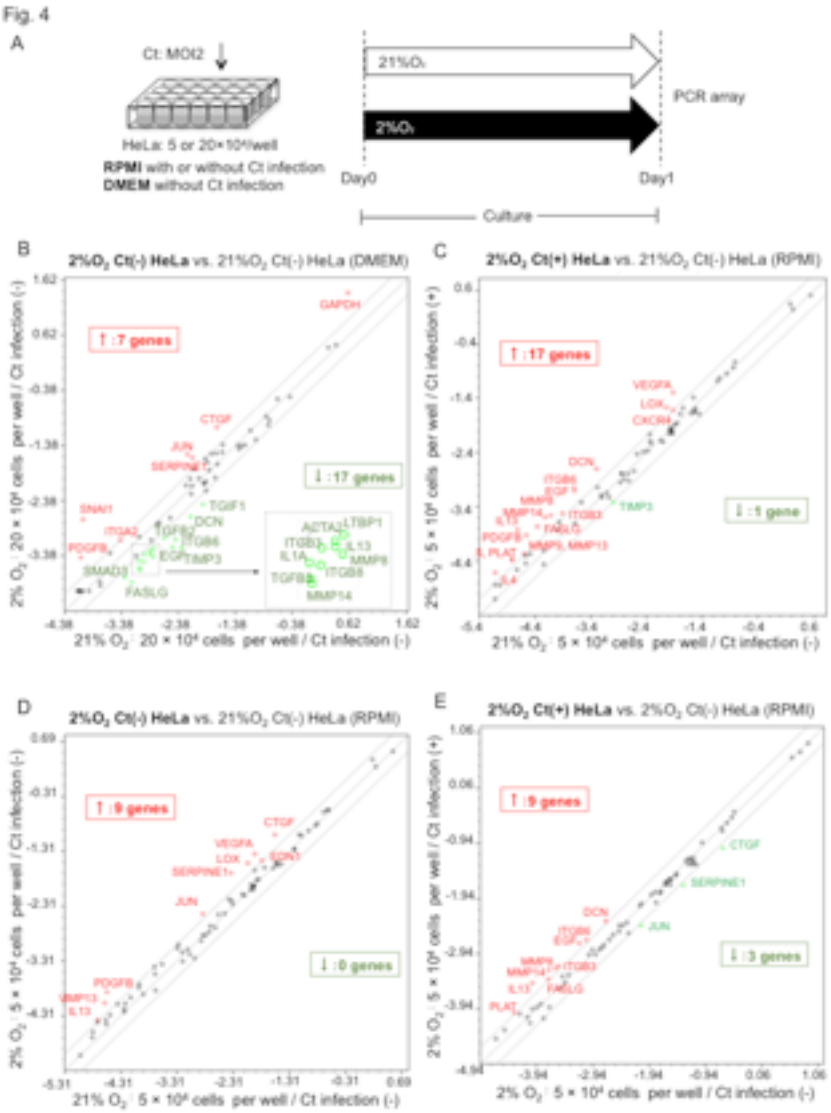

A
Ct: MOI2
HeLa: 1-20×10⁴/well
RPMI or DMEM
With or without cycloheximide
21%O₂
2%O₂
IFU assay
Phase contrast
Day0
Day2
Culture
B
-DMEM-
Number of infectious progenies (IFU per mL)
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
1x
5x
10x
20x
21%O₂
2%O₂
-RPMI-
cell number (x10⁴ cells/well)
C
Cell number (×10⁴ cells /well)
5×
10×
20×
20× (with cycloheximide)
2%O2
21%O2

Fig. 4


A
Ct: MOI2
HeLa: 5 or 20×10⁴/well
RPMI with or without Ct infection
DMEM without Ct infection
21%O₂
2%O₂
PCR array
Day0
Day1
Culture
B
2%O2 Ct(-) HeLa vs. 21%O2 Ct(-) HeLa (DMEM)
↑ : 7 genes
↓ : 17 genes
2% O2 : 20 × 10⁴ cells per well / Ct infection (-)
21% O2 : 20 × 10⁴ cells per well / Ct infection (-)
C
2%O2 Ct(+) HeLa vs. 21%O2 Ct(-) HeLa (RPMI)
↑ : 17 genes
↓ : 1 gene
2% O2 : 5 × 10⁴ cells per well / Ct infection (+)
21% O2 : 5 × 10⁴ cells per well / Ct infection (-)
D
2%O2 Ct(-) HeLa vs. 21%O2 Ct(-) HeLa (RPMI)
↑ : 9 genes
↓ : 0 genes
2% O2 : 5 × 10⁴ cells per well / Ct infection (-)
21% O2 : 5 × 10⁴ cells per well / Ct infection (-)
E
2%O2 Ct(+) HeLa vs. 2%O2 Ct(-) HeLa (RPMI)
↑ : 9 genes
↓ : 3 genes
2% O2 : 5 × 10⁴ cells per well / Ct infection (+)
2% O2 : 5 × 10⁴ cells per well / Ct infection (-)