Preliminary results of the control experiments

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The response of primary human epithelial cells to stringent virus amplification conditions refute the claims of existence of all viruses and of SARS-CoV-2.

Summary:

Exosomes are small extracellular vesicles that contain cargo RNA, DNA and cellular proteins. They are produced by all cell types, serve cell-cell communication and offer promising therapeutic possibilities.

To study RNA species and extracellular vesicles under harsh protocols routinely used in virology, healthy primary human epithelial cells were cultured over three passages with stress protocols for virus (virion) amplification. Despite the absence of virus inoculation, the cells developed severe cytopathic effects (CPE) leading to visible subtotal destruction and plaque formation in the cell lawn.

A blind inspection of cells under control and virus amplification conditions allowed the identification of the different morphologies with a hit rate of 100 %. Total RNA from cells and cell culture supernatants of three biological and two technical replicates per stress group was dissected by next-generation sequencing together with total RNA from the same optimally cultured cells. Sequence and extracellular vesicle analyses are ongoing.

Introduction:

Viruses from isolates, e.g. from bats, are propagated in cell cultures under harsh culture conditions by depriving them of much of their nutrition by reducing foetal calf serum (FCS) from 10% to 2% or 1% in Dulbecco's Modified Eagle's Medium (DMEM), in line with ATCC recommendations. Food deprivation is also routinely combined with high concentrations of Gibco's triple antibiotics (penicillin/streptomycin antibiotics with amphotericin B antifungal) and sequential "blind passaging" of cell culture supernatants onto the next cell culture.1 Morphologically, virion amplification leads to cytopathic effects (CPE), which ends in rounding of the cells, ballooning of the same and cellular degeneration, which is finally visible by plaque formation in a confluent cell culture.

Accordingly, viral particles enriched from these cell culture supernatants can be imaged by electron microscopy. To rule out the hypothesis that harsh stress conditions without viral inoculation may lead to the formation of exosomes2 that are virion-like, we subjected healthy primary human epithelial cells to routine viral amplification protocols. We then isolated total RNA from starved or control cells and supernatants using viral RNA isolation kits or routine TRIzol extraction and subjected the RNA next-generation sequencing.

Results:

Healthy, primary human epithelial cells were grown over four passages (P3-P6) under optimal culture conditions in defined epithelial control medium with 1x triple antibiotics (CM).

After the first passage, the cell pool was divided into four groups. After 3 days in CM, cultures were transferred either to fresh CM (CM, Control 1), DMEM/GlutaMAX with 10% FCS, 1x triple antibiotics (Control 2) or to stress medium (Starvation 1 & 2). During the first stress treatment, the stress medium contained DMEM, 1% FCS and 3x triple antibiotics.

The second and third passages were "blind" passages in which 50% of the culture supernatant from the last passage was transferred to the next passage in DMEM, 1% FCS and 3x triple antibiotics.

The second stress group was additionally treated with total yeast RNA (yRNA) at each passage for one hour before the addition of the stress medium (Starvation 2)

After transfer into DMEM with 10% FCS, the epithelial cells took on a flatter morphology than in CM and formed a continuous cell lawn, which can be attributed to the high calcium concentrations in DMEM. Otherwise, the cells continued to divide normally (Image 1A). In contrast, the cell lawns in the stress media shrank to small islands with reduced growth and incipient cell degeneration.

During the next two passages, the cells incubated with the supernatant of the stressed cells incubated with the supernatant of the previous passage,

CPE increased with cell-free areas reminiscent of Virion-induced plaques in the cell lawn, and more dead cells floating in the supernatant (Image 1B).

Confluent cultures under stress (Image 1C) stained with crystal violet (Image 1D) confirmed the distinct CPE. Pyknotic cells with condensed nuclei or ballooning cells were predominantly present in the Starvation 1 group, and areas with total cell destruction or plaques were also observed in Starvation 1, but predominantly in the Starvation 2 group.

The experiments were conducted in three biological replicates and two technical duplicates. All cultures were inspected blindly and the stressed cultures were easily recognised by drastic changes in morphology. After three passages, RNA was isolated from control 1 and the two stressed cell groups and supernatants using viral RNA kits or TRIzol and subjected to next-generation sequencing. The isolated total RNA quantity was most productive in control group 1 (Table 1) and was of perfect quality in all groups (data not shown). Additional supernatants were further used for extracellular particle analysis. The experiments are in progress.

Material and method:

Cell culture:

Commercial human primary passage 3 epithelial cells were thawed and seeded at 4,000 cells/cm2 in 75cm2 flasks for expansion at 37°C with 5% CO2 in defined epithelial low calcium medium (without FCS) and 1x triple antibiotic (Gibco) (control medium, CM). At >80% confluence, expansion cells were detached with 5mL accutase enzyme at 37°C for 10 minutes. Accutase was neutralised with 10mL CM, cells centrifuged at 400G for 5 min, resuspended in 1mL CM, and the living cells were counted by trypan blue staining in the Countess II instrument (ThermoFisher). The cells were sawn out for the experiment or parallel expansion rounds for subsequent experiments.For each experiment, four groups of healthy primary epithelial cells were seeded from the same expanded pool in CM at 4000 cells/cm2 in 25cm2 culture flasks and cultured to >50% confluence. Medium was then replaced with four experimental conditions; for control cells, fresh CM (Control 1) or commercial DMEM supplemented with GlutaMAX, 10% heat-inactivated FCS and 1x triple antibiotic (Control 2). Food was deprived by replacing CM with DMEM containing 1% FCS and triple antibiotics, which is essentially the same as virion amplification protocols1 (Starvation 1 & 2). The stressed group 2 Starvation was additionally treated with 10-µg total yeast RNA (yRNA) per culture flask for 1h and washed thoroughly with phosphate buffered saline (PBS) before changing medium group 1 & 2.

Subsequently, two "blind passages" were performed, in which 50% of the supernatant of starvation groups 1 and 2 was transferred to the next cell culture. The supernatants were cleaned of dead cells by centrifugation at 400G for 5 minutes. The control groups received 100% fresh medium. The experiments were repeated three times in duplicates. The length of culture under stress defined in the first biological replicate, was kept constant for all experiments. No change of medium was carried out during the stress period.

P4: Media change for control and stressed cells at approximately 50 confluency; control cells cultured to >80% confluency, stressed cells cultured for 5 days after media change.

P5: Media change in control and stressed cells >50 confluency, control cells cultured to >80% confluency, stressed cells cultured for 8 days after media change.

P6/RNA isolation: change of medium in control and stressed cells at approximately 50 confluency; control cells cultured to >80% confluency, stressed cells cultured for 5 days after media change.

P6/Crystal violet: media change for control and stressed cells at 100% confluency; stress induction for 3 days. A representative image of all cell cultures was taken daily at room temperature using a Nikon Eclipse TS100 microscope, Nikon 1J5, Nikon FT1 adapter and a 4x lens.

RNA extraction from epithelial cell cultures and Supernatants:

At the end of section 6, half of the total cellular RNA was isolated using the Promega miRNA kit (Promega, Z6211), which is recommended for small and long RNA samples, according to the manufacturer's protocol.

The other half of the total cellular RNA was isolated using the standard TRIzol protocol. The total RNA from the cell culture supernatant was isolated with the routinely used Qiagen viral RNA kit (Qiagen, 52904) according to the manufacturer's protocol. All RNA samples were treated with DNase.

The total RNA concentration and the 260/280 and 260/230 ratios were determined using a NanoDrop 2000 (ThermoFisher). The RNA amounts were highest in the samples cultured in CM and lowest in the stressed groups 1 and 2, while the supernatants had very low but similar RNA amounts (Table 1). 8.3 mg of high quality total RNA, assessed with the Bioanalyzer, from control group 1 and stressed groups 1 & 2 were sequenced with "next generation RNA sequencing".

Crystal violet colouring/staining:

In the final fragment, a second set of 25cm2 culture flasks was seeded with 8000 cells/cm2 (set 2) to visualise cytopathic effects. At 100 % confluence, these cells were exposed to one of the four media conditions. Three days after exposure, cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature and then stained with 1% Crystal Violet for an additional 30 minutes at room temperature before being before being washed thoroughly with tap water at room at room temperature.

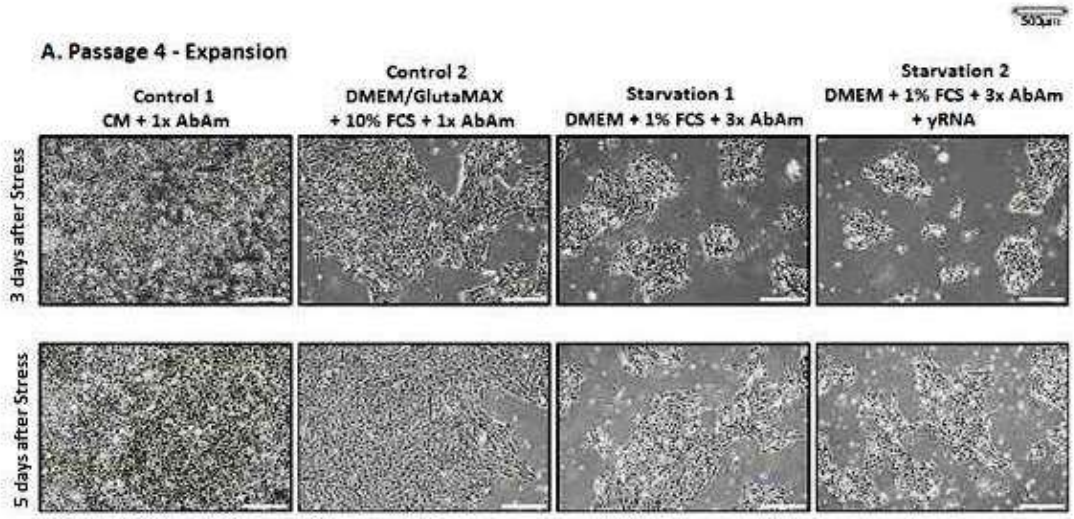
The stained cultures were photographed using the Nikon Eclipse TS100 brightfield microscope with a Nikon 1J5 camera, a Nikon FT1 adapter and a 4x or 20x lens.

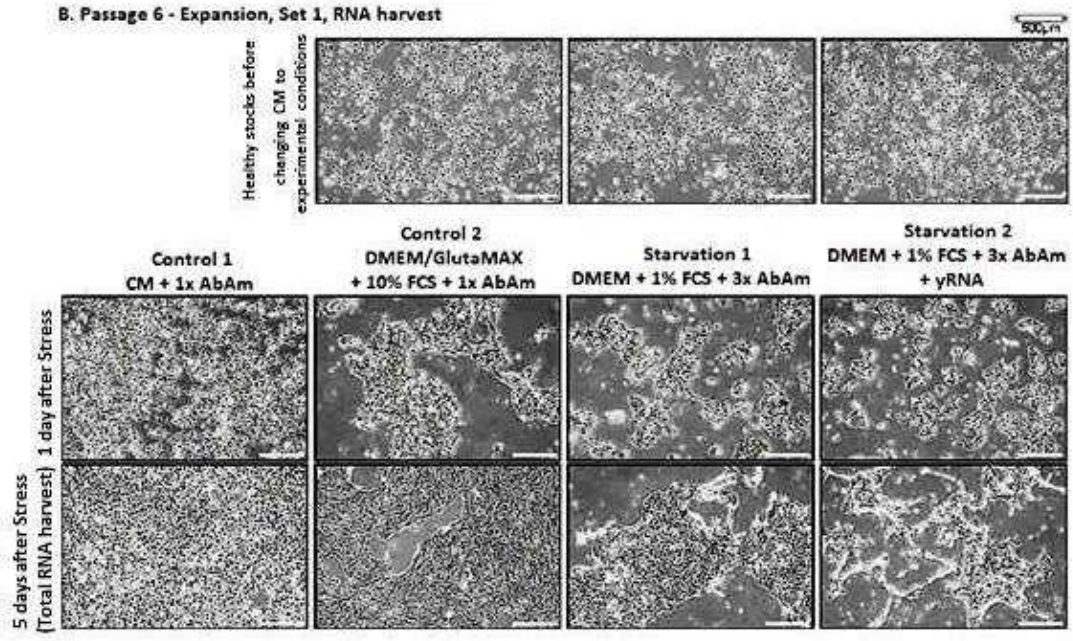
References:

1Ge, X. Y. et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature 503, 535-538, doi:10.1038/nature12711 (2013).

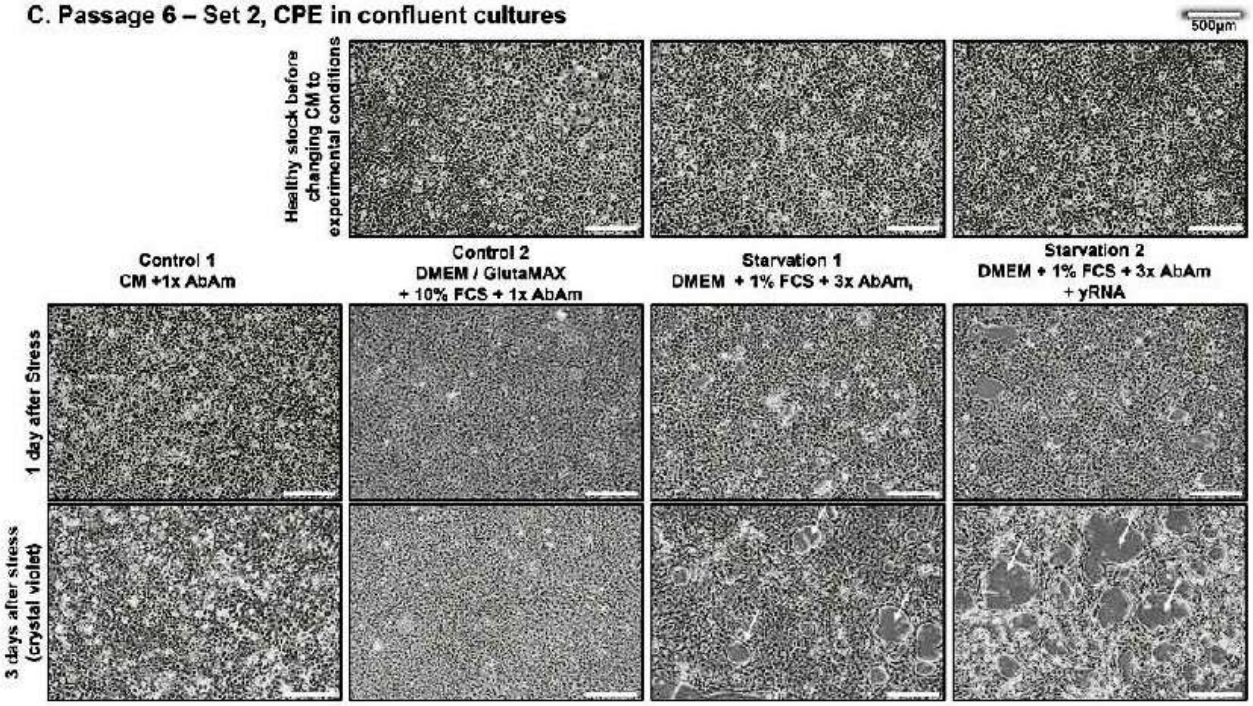
2Gurung, S., Perocheau, D., Touramanidou, L. & Baruteau, J. The exosome journey: from biogenesis to uptake and intracellular signalling. Cell Commun Signal 19, 47, doi:10.1186/s12964-021-00730-1 (2021).

Illustrations





C. Passage 6 – Set 2, CPE in confluent cultures



D. Passage 6 – Set 2, CPE in confluent cultures (Crystal Violet)

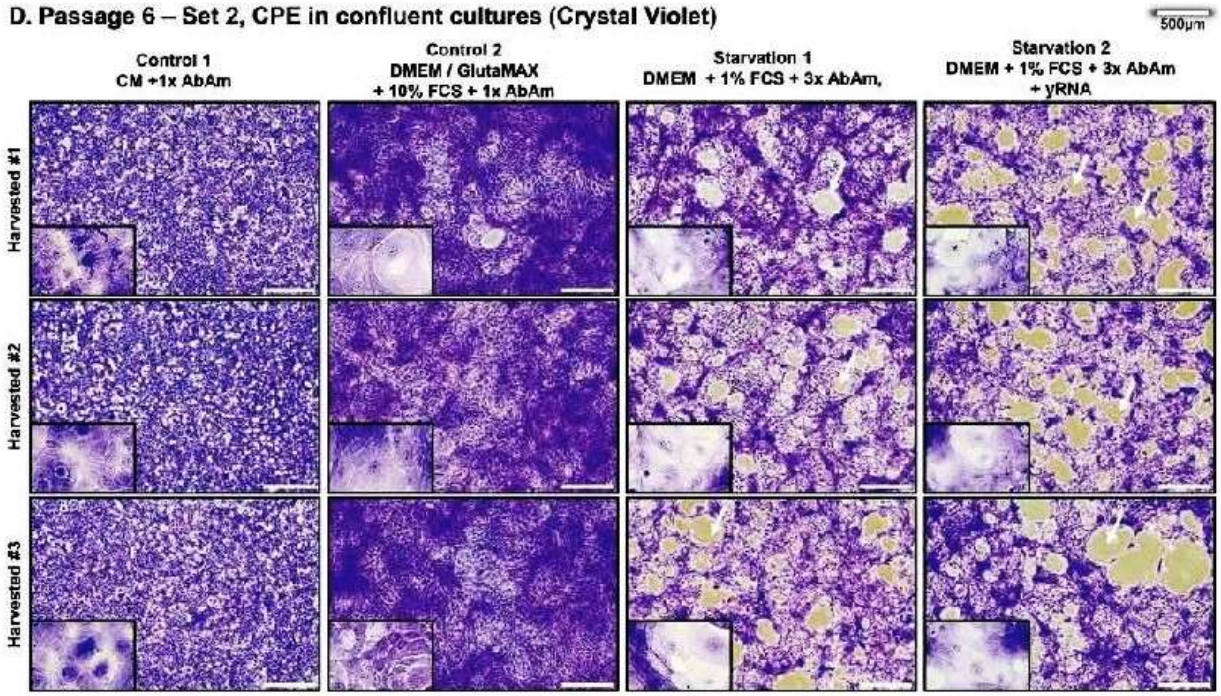


Illustration 1. Stress of Epithelial cells:

Representative microscopic images of the 4 experimental groups of epithelial cells at passage 4 and 6.
From left to right: healthy control cells with 1x triple antibiotics in control medium (CM) or DMEM/GlutaMAX with 10% FCS; stressed cells with 3x triple antibiotics and 1% FCS in DMEM.
Cells in the right panel were treated with total yeast RNA (yRNA) for 1h before media change.

(A), (B) Cells in expansion for the purpose of RNA isolation.
Note that CPE becomes more prominent over the three passages.
(B) Top row: Cells before medium change.
(C), (D) Confluent cells to visualise CPE;
(C) Top row: confluent cells before medium change.
(D) Cell cultures from 3 biological replicates, stained with crystal violet at the time of harvest.

Note that the cells in the two left panels form a continuous cell lawn while the cells in the two right Panels show a high number of plaques (arrows), which are compatible with significant cytopathic effects that increase from day 1 to day 5. Cultures treated with yeast RNA show a significantly higher number of larger plaques.

Excerpts: 20-times magnification; some rare pycnotic and ballooning cells were observed in control cultures; ballooning cells with empty cytoplasm are most common under stress conditions.
1. The cultures were inspected blindly daily by 2 experimenters with a hit rate of 100%. Bars; 500 µm.
All cultures: n=3 in duplicates.

Table 1: RNA Isolation

Groups #	Label	Sample Percentile	Harvesting Method	Total RNA in µg	RNA Vol. in µL
Control 1 CM + 1x AbAm	Control 1	100% Supernatant	Viral RNA Kit (Column)	0,27	30,00
		100% Cells	miRNA Kit+TRIzol	21,53	15,00
Control 2 DMEM + GlutaMAX + 10% FCS + 1x AbAm	Control 2	100% Supernatant	Viral RNA Kit (Column)	0,26	30,00
		100% Cells	miRNA Kit+TRIzol	14,78	15,00
Stress 1 DMEM + 1% FCS + 3x AbAm	Starvation 1	100% Supernatant	Viral RNA Kit (Column)	0,32	30,00
		100% Cells	mRNA Kit+TRIzol	8,32	15,00
Stress 2 DMEM + 1% FCS + 3x AbAm + Yeast tRNA	Starvation 2	100% Supernatant	Viral RNA Kit (Column)	0,27	30,00
		100% Cells	miRNA Kit+TRIzol	9,25	15,00