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Extracellular phosphoprotein regulation is affected by culture system scale-down

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ABSTRACT

Background: Phosphorylated proteins are known to be present in multiple body fluids in normal conditions, and abnormally accumulated under some pathological conditions. The biological significance of their role in the extracellular space has started being elucidated only recently, for example in bone mineralization, neural development, and coagulation. Here, we address some criticalities of conventional culture systems for the study of the extracellular regulation of phosphorylation.

Methods: We make use of microfluidics to scale-down the culture volume to a size comparable to the interstitial spaces occurring in vivo. The phosphoprotein content of conditioned media was analyzed by a colorimetric assay that detects global phosphorylation.

Results: We found that miniaturization of the culture system increases phosphoprotein accumulation. Moreover, we demonstrated that in conventional culture systems dilution affects the extent of the phosphorylation reactions occurring within the extracellular space. On the other hand, in microfluidics the phosphorylation status was not affected by addition of adenosine triphosphate (ATP) and FAM20C Golgi Associated Secretory Pathway Kinase (FAM20C) ectokinase, as if their concentration was already not limiting for the phosphorylation reaction to occur.

Conclusions: The volume of the extracellular environment plays a role in the process of extracellular phosphorylation due to its effect on the concentration of substrates, enzymes and co-factors.

General significance: Thus, the biological role of extracellular phosphoregulation may be better appreciated within a microfluidic culture system.

1. Introduction

The first phosphorylated protein discovered, casein, was collected from the extracellular space, specifically in the mammary gland fluid [1]. Since then, research has mainly focused on the fundamental role of phosphorylation in intracellular signaling pathways [2]. In the last decade, however, attention to regulatory mechanisms related to the presence of phosphorylated proteins in the extracellular space has been increasing [3].

Phosphorylated proteins have been identified in multiple body fluids, such as blood, urine, and cerebrospinal fluid [3]. They have been found to play a role physiologically: in extracellular matrix [4–6] and biomineralization [7,8], in the central nervous system [9–11], in the immune system and in coagulation [12–14]. Moreover, they have been found to have higher concentration in inflammatory and pathological conditions, such as Alzheimer's disease [15,16] and cancer [17]. Thus, the relevance of the role of extracellular phosphorylated proteins is currently recognized at clinical level.

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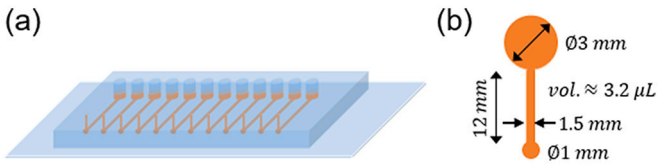

Fig. 1. Schematic representation of the microfluidic chip. (a) Microfluidic PDMS device for cell culture within 12 independent chambers. (b) Single microfluidic chamber top view.

Reversible phosphorylation can occur in the extracellular space by means of ectokinases and ectophosphatases [3]. Some of these are known intracellular enzymes that are also secreted. In vitro biochemical studies have recently identified few atypical kinases specifically acting in the secretory pathway and in the extracellular space [1]. The first of such kinases, responsible also for casein phosphorylation, is FAM20C Golgi Associated Secretory Pathway Kinase (FAM20C), which was identified only in 2012 [8].

Our hypothesis is that conventional culture systems have some limitations for extracellular phosphorylation studies, because the large volume of medium they contain dilutes the secreted molecules taking part in the extracellular phosphorylation processes. On the other hand, microfluidic systems are devices that handle few microliters of medium within micrometric channels [18]. When used for cell culture, they have been demonstrated to establish a different balance between cell-secreted endogenous factors and exogenous factors from the culture medium [19,20], with implications for biological response in multiple applications, such as human somatic cell reprogramming to pluripotency [21–23] and differentiation [24,25]. Given that in vivo the interstitial spaces in tissues have dimensions comparable to those of microfluidic systems, a thorough understanding on how extracellular phosphorylation is regulated and its functional implications in physiological and pathological conditions may be better captured in vitro at microscale.

In this work, we demonstrate, both theoretically and experimentally, that reducing the volume of cell culture to few microliters strongly affects the extracellular phosphorylated protein concentration and potentially their biological role. We show that miniaturization of cell culture affects both accumulation of secreted phosphoproteins and phosphorylation events occurring in the extracellular space.

2. Material and methods

2.1. Cell culture conditions and conditioned media collection

Human foreskin BJ fibroblasts, (ATCC CRL-2522) passage p8–18 (using the same passage within the same experiment), were seeded at 50 or 100 cell/mm^2 and cultured in Dulbecco's modified Eagle Medium (DMEM 11885, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific) for expansion in microfluidics or in 12-well plates. Cells were cultured at 37 °C and 5% CO_2 atmosphere, and routinely checked to verify absence of mycoplasma contamination by MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza) every 2 or 3 passages. For extracellular phosphorylation experiments, both in microfluidics and multi-well plates, expansion medium was replaced by DMEM supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich). Media that were analyzed for phosphorylation were conditioned for either 12 h or 24 h, before collection. Media from either 6 microfluidic chambers or 2 wells of a 12-well plate were pooled together to represent one biological replicate in the measurement. Two or three biological replicates were present in each experiment. The media were centrifuged at 200 *g* for 5 min to remove dead cells, then either analyzed immediately or frozen in −80 °C until analysis. Growth rate was assessed by cell counting after HOECHST 33342 (Cell Signaling) staining at defined time points. For live and dead assay, cells were washed with PBS; incubated with 4 μM ethidium homodimer-1 (stains dead cells, red), 4 μM calcein AM (stains live cells, green) and 4 μM HOECHST 33342 (stains cell nuclei, blue) for 45 min at room temperature; washed with PBS; and analyzed by fluorescence microscopy. Pictures were taken on a Leica DMI6000 B microscope.

2.2. Fabrication of microfluidic devices

Microfluidic platforms were fabricated according to standard photolithographic techniques and molded in polydimethylsiloxane (PDMS) as previously described (Fig. 1) [26]. Briefly, Sylgard 184 (Dow Corning), using a premixed 10:1 ratio prepolymer:curing agent solution, was cured on a 200-μm-thick patterned SU-2100 photoresist (MicroChem) in order to obtain a single PDMS mold with multiple independent channels, to be used as independent culture chambers of approx. 16 mm^2 of surface and 3.2 μL volume each. The PDMS mold was punched to produce inlets and outlets of the channels, and sealed on a microscope glass slide (Menzel-Glaser) by plasma treatment. Channels were rinsed with isopropanol and distilled water before autoclaving. To minimize dead volumes, during cell culture the medium in the channels was changed by manual pipetting every 12 h.

2.3. Measurement of phosphoproteins

The kit pIMAGO-biotin Phosphoprotein Detection Kit for Microplate Blot, avidin-HRP-based colorimetric detection (Sigma-Aldrich) [27,28] was used according to manufacturer's instructions for the detection of phosphoproteins in conditioned media. Standard curve samples were prepared using phosphorylated α-casein from bovine milk (C6780, Sigma-Aldrich). Dephosphorylated α-casein was also used as a negative control (C8032, Sigma-Aldrich). For perturbation experiments, 500 μM adenosine triphosphate (ATP, S1985 Selleck) and 10 μg/mL of recombinant human FAM20C protein (9265-FM-050, R&D) were added to the media, after collection, and let react at 37 °C for 3 h. For the assay, 0.5 to 2 μL of samples were mixed with binding buffer to 100-μL final volume. The plate was scanned with a Multimode Plate Reader EnSpire (PerkinElmer), and signals were recorded. Data are presented as mean ± standard deviation of at least two biological replicates. One way analysis of variance (ANOVA) test with Benjamini and Hochberg (BH) false discovery rate (FDR) correction was applied to identify statistically significant differences.

2.4. Reaction rate kinetics

The reaction rate, v, of a kinase phosphorylating an unphosphorylated protein was calculated assuming the reaction as a bi-substrate reaction with a Michaelis-Menten kinetics respect to either substrate. Parameters were obtained from FAM20C kinetic parameters [29]: $K_{M,\,A} = 1.5\mu M$, $K_{M,\,B} = 78\mu M$, with A representing casein and B representing ATP. K_{AB} was arbitrarily set equal to 1, and we verified that the main conclusions were not affected by this assumption. Simulation and figures were run on MATLAB R2020b (The MathWorks).

3. Results and discussion

3.1. Phosphoproteins in conditioned media within conventional culture systems

To obtain a general understanding in scaling phenomena occurring in the extracellular space, we chose as a benchmark a culture of human fibroblasts. These cells produce many extracellular matrix proteins, like collagens, which are known to be phosphorylated [4]. Moreover, fibroblasts also express FAM20C, one of the few known kinases operating in the Golgi secretory pathway and extracellularly (Human Protein Atlas Single Cell Type Atlas, www.proteinatlas.org/celltype [30]). To detect global protein phosphorylation we used a colorimetric assay based on a nanopolymer multi-functionalized with titanium ions for specific

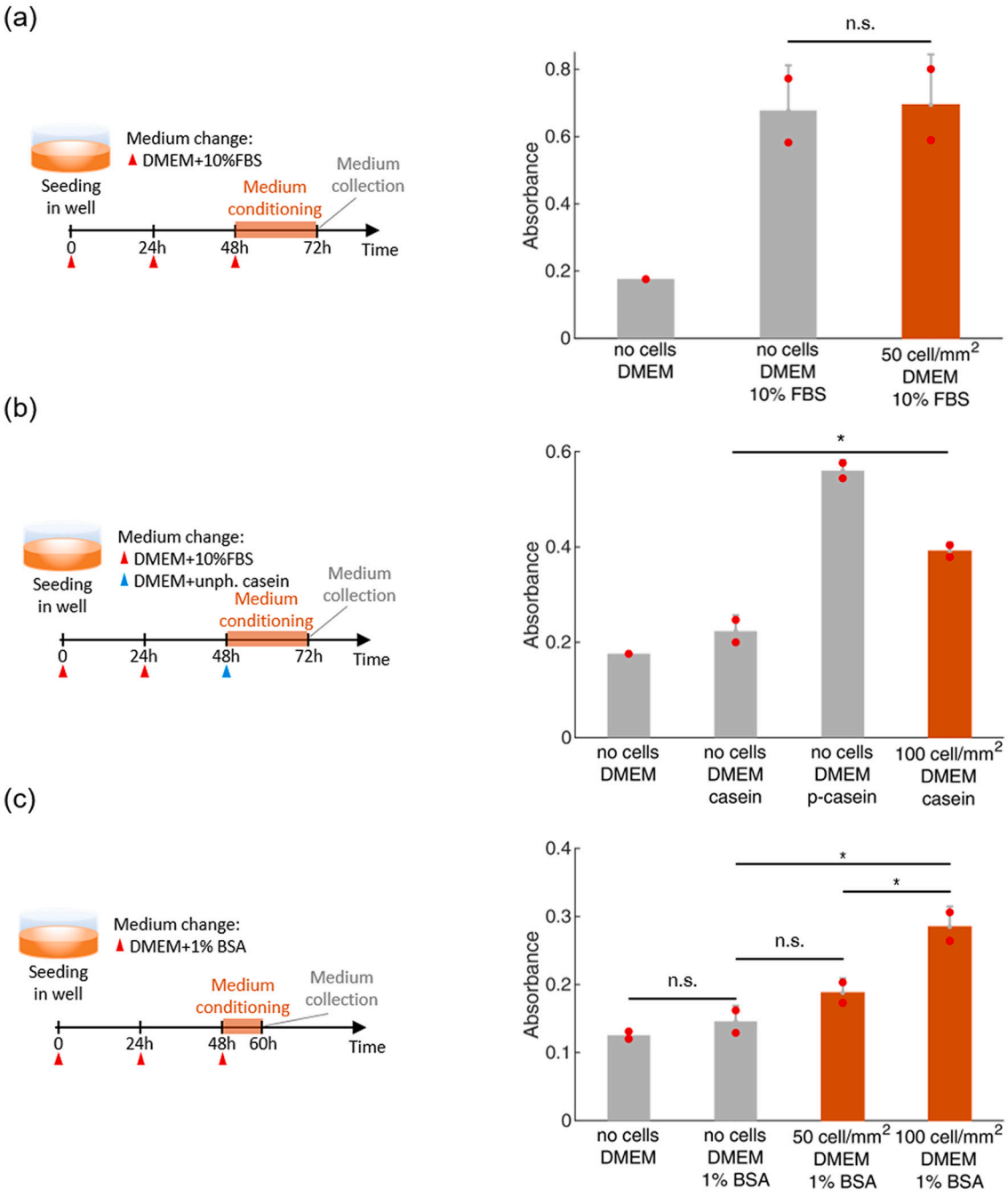

Fig. 2. Analysis of extracellular phosphorylated proteins from human fibroblasts cultured within a standard multi-well plate. (a) Phosphoprotein content of DMEM, DMEM supplemented with 10% FBS, and medium conditioned by fibroblasts seeded at 50 cell/mm^2. Medium was conditioned for 24 h, starting 48 h post-seeding. (b) Phosphoprotein content of DMEM, DMEM supplemented with 100 μg/mL unphosphorylated α-casein, DMEM supplemented with 100 μg/mL phosphorylated α-casein, and medium conditioned by fibroblasts seeded at 100 cell/mm^2. Medium was conditioned for 24 h, starting 48 h post-seeding. (c) Phosphoprotein content of DMEM, DMEM supplemented with 1% BSA, and medium conditioned by fibroblasts seeded at 50 or 100 cell/mm^2. Medium was conditioned for 12 h, starting 48 h post-seeding. The experimental design for each sub-figure is shown on the left. Error bars indicate mean ± st. dev. ($n = 2$). Red dots indicate single experimental data points. n.s.: non significant. *: Benjamini Hochberg (BH)-adjusted p-value<0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding to any phosphorylated protein [27,28].

We initially tested global phosphorylation of conditioned media from fibroblast culture in conventional multi-well plates. Human fibroblasts are commonly cultured in a serum-containing medium, typically FBS. We found that FBS is itself a great source of phosphoproteins (Fig. 2a). After 24-h medium incubation with fibroblasts, conditioned medium was analyzed and compared to the non-conditioned one. Due to the high background signal from FBS, we could not detect significant differences (Fig. 2a).

We then performed the measurement using non-supplemented DMEM during the period of cell conditioning. As positive and negative controls, we used phosphorylated and unphosphorylated α-casein. A-casein is a protein, only produced by lactating mammary gland, that has many sites of phosphorylation and is commonly used as a phosphorylation control. Under these conditions and doubling the cell density, we could detect a clear accumulation of endogenous phosphoproteins in

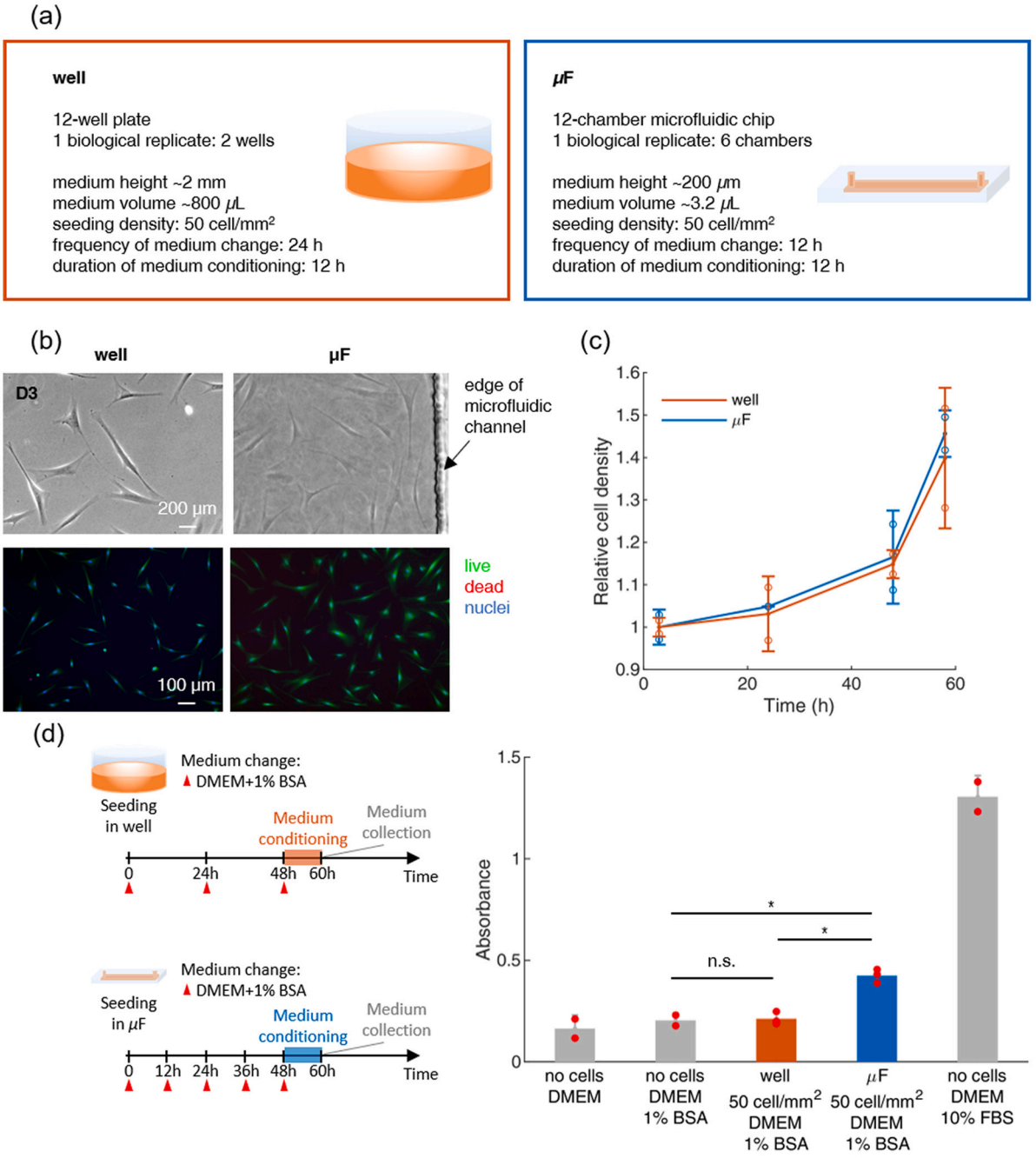

Fig. 3. Analysis of extracellular phosphorylated proteins from human fibroblasts cultured within a standard multi-well plate and in microfluidics. (a) Schematic representation and main features of the two culture systems compared in this study. (b) Phase contrast images and fluorescence images taken after live and dead assay staining to compare cell morphology and viability in the two culture systems at day 3 after cell seeding at 50 cell/mm^2 in DMEM supplemented with 1% BSA. (c) Growth curve of human fibroblasts seeded in well and in microfluidics at 50 cell/mm^2 and cultured in DMEM supplemented with 1% BSA. Data are normalized respect to the first time point at 3-h post-seeding. Error bars indicate mean ± st. dev. ($n = 2$). Circles indicate single experimental data points. (d) Phosphoprotein content of DMEM, DMEM supplemented with 1% BSA, media conditioned by fibroblasts seeded at 50 cell/mm^2 in well and in microfluidics, and DMEM supplemented with 10% FBS. Media were conditioned by cells for 12 h, starting 48 h post-seeding. The experimental design is shown on the left. Error bars indicate mean ± st. dev. (n = 2 for media only and $n = 3$ for conditioned media). Red dots indicate single experimental data points. n.s.: non significant. *: BH-corrected p-value<0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human fibroblast-conditioned medium (Fig. 2b).

In additional measurements, we found that unphosphorylated α-casein is partially phosphorylated (Suppl. Fig. 1). Thus, next experiments will not make use of this negative control. Besides, to avoid contamination from serum-derived phosphoproteins in the conditioned medium, we switched to a serum-free medium from the time of seeding, using DMEM supplemented with BSA, a protein that does not contain phosphorylated sites according to previous low-throughput studies (www.phosphosite.org [31]) and to our data (Fig. 2c). Under these culture conditions, we could detect the presence of phosphorylated proteins in the conditioned medium for a sufficiently high cell density (Fig. 2c).

Overall, we confirmed that endogenous phosphorylated proteins accumulate in human fibroblast conditioned medium from conventional culture systems and that they are detectable using a colorimetric-based assay for global phosphoprotein quantification. Moreover, serum has

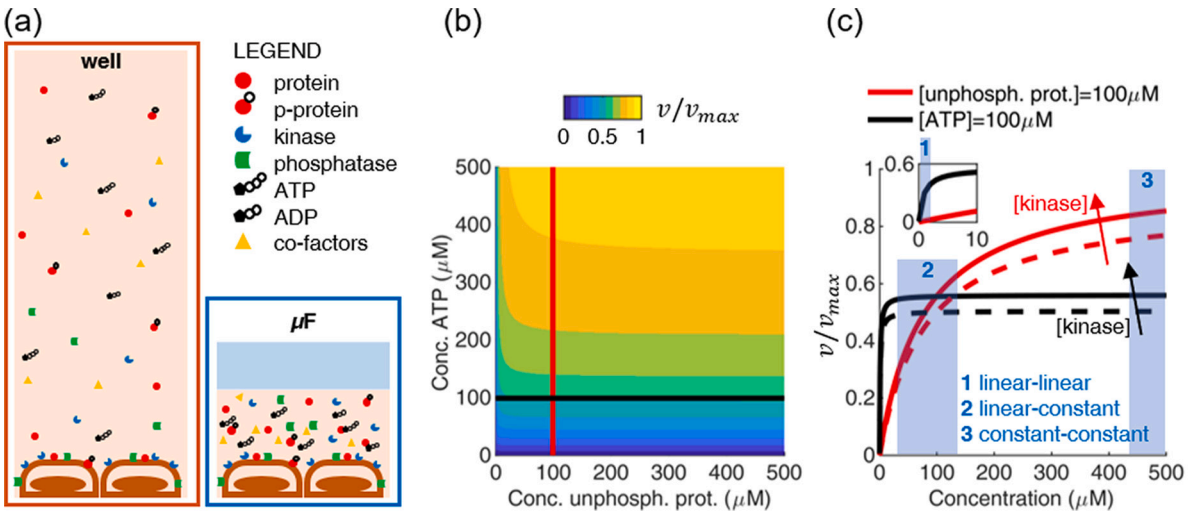

Fig. 4. Theoretical analysis of extracellular phosphorylation. (a) Schematic comparison between a conventional culture system and a microfluidic one. The same number of cell-secreted molecules are shown for the two systems to highlight how molecules relevant for extracellular phosphorylation-related processes are accumulated in the confined environment. Dimensions are not on scale. (b) Computational simulation of phosphorylation reaction rate for different concentrations of the reactants (unphosphorylated protein and ATP). Black and red lines indicate the concentrations plotted in (c). (c) Phosphorylation reaction rate, *v*, as a function of concentration of each reactant for a fixed concentration of the other one. Dashed lines indicate how curves change at lower kinase concentration. Inset shows an enlargement. Blue shaded areas approximately show the range of concentrations where reaction rate con be approximated as linear or constant respect to unphosphorylated protein and ATP, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

high phosphoprotein content, obscuring the detectability of endogenous signal and potentially the biological role of endogenous extracellular phosphoproteins.

3.2. Effect of microfluidic confinement

Microfluidic systems are characterized by a much smaller culture volume (few microliters) than conventional multi-well plates. Cell density scales with the surface of the culture system and, assuming cell secretion is the same on a per-cell basis, the total amount of secreted phosphoproteins per unit area of culture is the same in the two culture systems. However, the concentration of the phosphoproteins secreted depends not only on the quantity of secreted proteins but also on the volume where they are released. For the same unit area of culture, volume is proportional to medium height. In conclusion, the critical dimension that makes a difference between the two culture systems is the height of medium over the cells. The microfluidic chip used in this study has the geometric features displayed in Fig. 3a, with an approximate 10-fold height reduction compared to the conventional multi-well plate.

Next, we experimentally explored the role of microliter confinement to test our previous considerations. Due to metabolic limitations that may occur in the microfluidic system, medium changes in microfluidics were performed every 12 h, according to a protocol that makes possible long-term fibroblast expansion in microfluidics [22]. In conventional wells we performed medium changes every 24 h. First, we verified that the culture system and the adopted protocols did not affect cell morphology and viability (Fig. 3b and Suppl. Fig. 2), and the growth rate (Fig. 3c), which were comparable in the two systems. Then, we performed a 12-h medium conditioning using DMEM supplemented with BSA in microfluidics and in wells. We confirmed that BSA did not contribute to the phosphorylation signal and that the quantity of phosphoproteins in medium conditioned in wells was negligible compared to serum-supplemented medium (Fig. 3d). More importantly, we found that phosphoprotein signal was significantly detectable in microfluidic-conditioned medium and still significantly lower than the signal of serum-supplemented medium (Fig. 3d and Suppl. Fig. 3). By a calibration curve we estimated a concentration of phosphoprotein in microfluidics of approximately 3 μg/mL (Suppl. Fig. 3).

Overall, we experimentally confirmed our hypothesis of accumulation of phosphorylated proteins in conditioned media in microfluidics compared to conventional culture systems.

3.3. Biochemical considerations on phosphorylation occurring extracellularly

Extracellular phosphoproteins can have different origins, but at least some of them are phosphorylated within the extracellular space [3]. The reduced volume of microfluidic culture can play a role in terms of accumulation at multiple levels: concentrating secreted phosphoproteins directly, or concentrating the secreted enzymes, co-factors and substrates that will produce the phosphoproteins in the extracellular space (Fig. 4a). Here, we specifically focused on the latter cases.

The reversible phosphorylation of serine, threonine, and tyrosine residues in proteins is known to be catalyzed by protein kinases and phosphatases. A number of kinases and phosphatases has been described to perform their role outside the cell [1]. They can be membrane-bound facing the extracellular space, like Vertebrate Lonesome Kinase (VLK, aka PKDCC) or alkaline phosphatase, or they can be released as soluble proteins, for example the ectokinase FAM20C.

In the simplest cases, the enzymatic phospho-reactions follow a bisubstrate mechanism where ATP/adenosine diphosphate (ADP) plays as a donor/acceptor of a phosphate group:

$$protein + ATP \underset{phosphatase}{\overset{kinase}{\rightleftarrows}} p-protein + ADP$$

Besides, other co-factors can activate, enhance or inhibit enzyme activity, for example FAM20A Golgi Associated Secretory Pathway Pseudokinase (FAM20A) has been described as an enhancer of FAM20C catalytic activity [32]. Common co-factors are also divalent metal ions, like Mg^{2+} [8]. Medium composition commonly includes metal ion salts (for example DMEM contains $MgSO_4$ at 0.8 mM concentration), thus they are not expected to play as differential factors between microfluidic and conventional culture systems.

We adopted an empirical approach to highlight some aspects on how

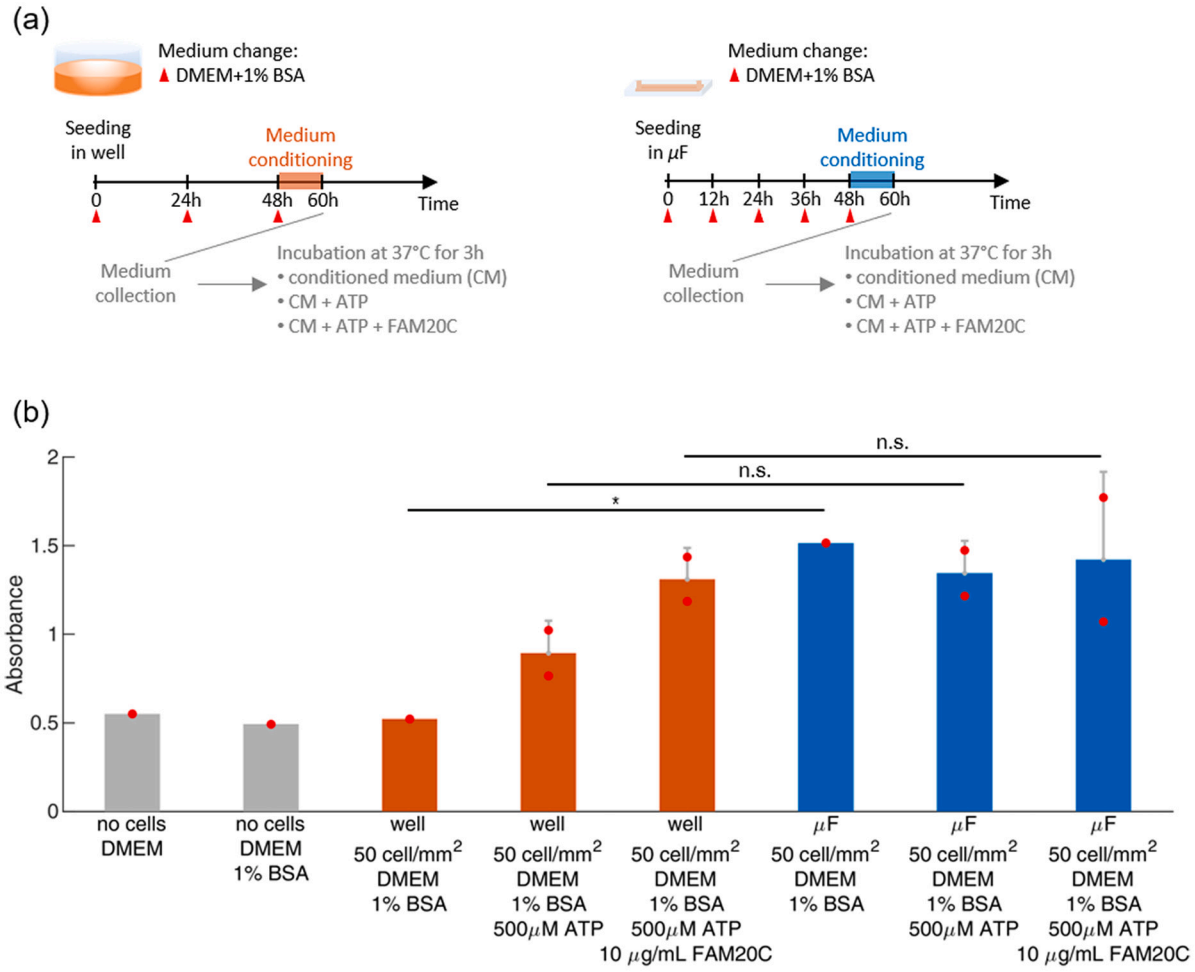

Fig. 5. Analysis of limiting factors for extracellular protein phosphorylation in human fibroblast cultures within a standard multi-well plate and in microfluidics. (a) Schematic experimental design. Media were conditioned by cells for 12 h, starting 48 h post-seeding. Fibroblasts were seeded at 50 cell/mm^2 in well and in microfluidics. Conditioned media were supplemented with 500 μM ATP and 10 μg/mL FAM20C after collection, and let react for 3 h at 37 °C before analysis. (b) Phosphoprotein content of DMEM, DMEM supplemented with 1% BSA, and conditioned media by fibroblasts. Error bars indicate mean ± st. dev. ($n = 2$). Red dots indicate single experimental data points. n.s.: non significant. *: BH-corrected p-value<0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microfluidic culture can affect extracellular phospho-regulated processes. In the next section, we will experimentally validate some of these principles.

Most commonly, enzymatic reaction rates have a Michaelis-Menten-like form. This occurs also for bisubstrate reactions with respect to each substrate [33]. Thus, we can generally write the reaction rate, v, as:

$$\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_{M,A}}{v_{max}[A]} + \frac{K_{M,B}}{v_{max}[B]} + \frac{K_{AB}}{v_{max}[A][B]}$$

where v_{max} is the maximum rate of the reaction, K parameters are experimentally derived, and $[A]$ and $[B]$ represent the concentrations of the two substrates, such as unphosphorylated protein and ATP, or phosphoprotein and ADP. As an example, we plotted in Fig. 4b-c the reaction rate, using parameters drawn from biochemical studies of the extracellular kinase FAM20C [8]. From biochemical assays, FAM20C has much higher affinity for the protein substrate than for ATP. This means that the kinetic rate is linearly dependent on protein substrate only at low protein concentration and at higher concentrations it follows zeroth-order kinetics. On the contrary, the linear range of kinetics respect to ATP is wider and zeroth order is essentially not reachable at ATP concentrations that are physiologically relevant. Circulating plasma contains 100-nM ATP under normal in vivo conditions, and may increase to ~40 μM at wound sites due to the local release of intracellular ATP, and even more in pathological conditions [3]. As a consequence, the kinetics of phosphorylation seems to be overall dependent on ATP concentration, and ATP accumulation in microfluidics can increase the rate of up to 10 folds due to system geometry. ATP is also a relatively small molecule compared to proteins, with diffusion coefficient (~350 μm^2/s) two to ten times higher than proteins [34], thus it can be diluted much faster in a large-scale culture system.

Last, changing the kinase concentration typically changes the reaction rate, and may change the equilibrium between the phosphorylation and dephosphorylation reaction rates (Fig. 4c). Thus, also the accumulation of enzymes in microfluidics can have relevant impact on the relative rate of the two reactions, especially if, for example, a kinase is soluble and the corresponding phosphatase is membrane-bound and thus not differentially accumulated in the two culture systems.

3.4. *Extracellular phosphorylation is affected by microfluidic confinement*

To test some of the theoretical considerations discussed in the previous section, we performed perturbation experiments, with the aim of showing that microfluidic confinement affects phosphorylation reactions that occur in the extracellular space.

As previously discussed, ATP has been proposed as a limiting substrate for extracellular phosphorylation by FAM20C and potentially

other ectokinases [3]. Moreover, enzymes and substrates are differentially accumulated in microfluidics. We tested how adding ATP and FAM20C kinase into the collected media affects the concentration of phosphoproteins. Specifically, we added 500 μM ATP, with or without 10 μg/mL of FAM20C kinase, in conditioned media (after collection), and let the reaction occur at 37 °C for 3 h. According to previous in vitro biochemical studies, 2 h is a reasonable time for the reaction of extracellular phosphorylation to occur [8]. For this experiment we exacerbated the conditions using an ATP concentration above the physiological range and extending the reaction time. Results show that in microfluidics medium phosphoprotein content is unaffected by ATP and kinase addition, suggesting their concentrations are not limiting for the reaction to occur (Fig. 5 and Suppl. Fig. 4). On the contrary, medium collected from conventional wells progressively increased its phosphoprotein content after being incubated in presence of exogenous ATP and FAM20C (Fig. 5 and Suppl. Fig. 4). By a calibration curve we estimated a concentration of phosphoprotein in microfluidics of approximately 2 μg/mL (Suppl. Fig. 4).

In conclusion, we demonstrated that the balance between different players involved in the extracellular phosphorylation reaction is different between microfluidic and conventional culture systems. The results are consistent with a reduced phosphorylation taking place in well, due to a dilution of cell-secreted ATP and kinase.

4. Conclusions

We reported how the most commonly used cell culture conditions could overwrite the regulation of processes related to extracellular phosphorylation, due to high dilution of cell-secreted molecules and presence of a large quantity of exogenous phosphorylated proteins from serum. We showed how down-scaling the culture system to microfluidics has a relevant impact on extracellular phosphoprotein accumulation. Phosphorylated proteins are present in the extracellular space either because they are released already phosphorylated or because they are phosphorylated outside the cells. In either case, scale-down has an impact that we confirmed both theoretically and experimentally. First, we showed the global accumulation of phosphorylated proteins in microfluidics compared to the conventional culture systems. Then, we showed that system down-scaling also affects the phosphorylation events occurring extracellularly. In the specific case under consideration, a human fibroblast cell culture, we showed that ATP concentration and FAM20C kinase concentration are limiting factors for reaching in well the same level of global phosphorylation achieved within the microfluidic culture system. These limitations of ATP and FAM20C concentrations in well are not necessarily of general validity and potentially dependent on the specific biological system under study. However, they represent a meaningful example to demonstrate that system scaling is an important factor to consider when studying extracellular processes involving phosphoproteins.

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The authors declare no conflict of interest.

Data availability

The data that supports the findings of this study are available within the article and its supplementary material.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

See the supplementary material for additional measurements of cell morphology and viability, and phosphoprotein content. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2022.130165.

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