Modulation of Mammalian Cell Behavior by Nanoporous Glass

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The introduction of novel bioactive materials to manipulate living cell behavior is a crucial topic for biomedical research and tissue engineering. Biomaterials or surface patterns that boost specific cell functions can enable innovative new products in cell culture and diagnostics. This study investigates the influence of the intrinsically nano-patterned surface of nanoporous glass membranes on the behavior of mammalian cells. Three different cell lines and primary human mesenchymal stem cells (hMSCs) proliferate readily on nanoporous glass membranes with mean pore sizes between 10 and 124 nm. In both proliferation and mRNA expression experiments, L929 fibroblasts show a distinct trend toward mean pore sizes >80 nm. For primary hMSCs, excellent proliferation is observed on all nanoporous surfaces. hMSCs on samples with 17 nm pore size display increased expression of COL10, COL2A1, and SOX9, especially during the first two weeks of culture. In the upside down culture, SK-MEL-28 cells on nanoporous glass resist the gravitational force and proliferate well in contrast to cells on flat references. The effect of paclitaxel treatment of MDA-MB-321 breast cancer cells is already visible after 48 h on nanoporous membranes and strongly pronounced in comparison to reference samples, underlining the material’s potential for functional drug screening.

1. Introduction

The behavior of living cells in the complex 3D environment of tissues and organs inside the human body vastly differs from in vitro cell culture on simple 2D culture surfaces that are state of the art in medical products and the pharmaceutical industry. This imposes a vast need for novel surfaces that influence cellular behavior in a defined manner to trigger or support specific cell functions.[1] For example, the efficient clinical translation of promising experimental studies on stem cells is still impeded by the lack of culture substrates for their robust expansion and differentiation.[2–4] In drug development, there is a great demand for functional assays with novel culture surfaces for the investigation of drug effects on different cellular functions like adhesion, migration or spreading behavior.[5] Elucidating these effects will facilitate the early identification of drug candidates for specific applications, reduce the number of substances that finally go into animal testing and clinical studies and eventually, increase the safety of the product and the benefit for the patient.

One of the most promising approaches to design such next-generation biomaterials is adding a defined surface topography or porosity in the micron- or nanometer scale.[6] Topographical features at these scales are on dimensions of subcellular structures and have been identified to be a modulator of various intracellular processes like cytoskeletal ordering, gene expression and transport mechanisms.[7–9] When being exposed to a new substrate, a cell explores the surface with actin containing protrusions of the cell membrane called filopodia, inducing mechanical interaction. In the case of positive interaction, the cell increases its contact with the new substrate by actin polymerization toward the material surface, leading to novel focal adhesions. The cell-substrate interaction can depend on the material type, the functional surface groups or proteins, or on topographic stimuli. In the growing field of topographic surface research for cell culture, various kinds of topographies are being examined, e. g. pillars, gratings, grooves, ridges or pores. For porous substrates, it has been shown that the cell-substrate interaction can be controlled by alterations of the pore size or the pore distribution. As an example, seeding hepatoblastoma cells on nanoporous alumina membranes with a pore diameter of ≈60, ≈85, or ≈200 nm results in an increase in proliferation with increasing pore diameter.[10]
Fibroblasts show increased adhesion and cell proliferation on nanoporous surfaces, opening up opportunities to intentionally impede the proliferation of certain cell types. All of these examples display the potential of porous substrates as future biomaterials for cell culture and tissue engineering.

Another material group for the fabrication of porous cell culture substrates is nanoporous glass. Glass is a material class that has been routinely used in biology and biomedical research since the first days of cell culture.[11] Thus, identifying distinct effects of glassy materials on living cells might lead to applications more quickly due to shortened approval processes compared to completely novel material compositions. Glasses from the ternary glass system Na$_2$O–B$_2$O$_3$–SiO$_2$ show a big miscibility gap and completely novel material compositions. Glasses is Pore Size Dependent

Glasses is Pore Size Dependent

2. Results

2.1. Physical Properties of Nanoporous Glass Membranes with Different Pore Sizes

To probe the influence of nanoporous glass on living cell behaviour and to derive pore-size dependencies, we prepared membranes of different mean pore sizes. For a systematic investigation on the influence of nanoporous glass membranes’ pore sizes on living cells, we optimized the leaching and cooling process. We were able to fabricate reproducible, stable membranes of $16 \times 16 \times 0.25$ mm in batches of up to 90 membranes per leaching step. The membranes could be readily autoclaved prior to cell culture. With increasing demixing temperature, the membranes become more opaque, indicating an increase in the pore size (Figure 1a). This macroscopic observation is confirmed in SEM measurements of the nanoporous glass surfaces, displaying a significant increase in the nanopore sizes with increasing demixing temperatures. The overall pore structure is conserved (Figure 1b). The pore size analysis of the nanoporous glass samples was performed on binarized and segmented SEM micrographs. We were able to robustly prepare nanoporous glass membranes with mean pore sizes between 17 and 124 nm and a mean thickness of only $250 \mu$m (Figure 1c). As visible in the SEM micrographs, the overall pore structure is highly anisotropic, which is where the large deviations in the measurement of mean pore sizes originate from. The UV-vis transmission spectra clearly display a broadening of the absorbed wavelength range with increasing mean pore sizes (Figure 1d). The characterization results display the expected trend: The nanoporous glass samples show an increasing pore size with higher demixing temperature. For 80% SiO$_2$ samples, two demixing temperatures were applied, yielding nanoporous glass membranes with mean pore sizes of 10 and 20 nm (Figure S1, Supporting Information).

2.2. L929 Mouse Fibroblast Proliferation on Nanoporous Glasses is Pore Size Dependent

To elucidate cell behavior on nanoporous glass membranes, we analyzed the proliferation of L929 cells under standard cell culture conditions. For this purpose, cells were seeded onto nanoporous glass membranes with four different mean pore sizes (17, 45, 81, and 124 nm) and two flat reference surfaces (FLAT/TCPS). Microscopy and cell counting was performed for days 1, 2, 3, 4, 6, 7, and 10 after cell seeding (Figure 2a). On TCPS, proliferation started immediately, with a daily doubling rate of $\sim 0.6$. On the following days, cells on TCPS displayed the expected exponential proliferation behavior known from standard cell culture. Cell counting on TCPS had to be stopped at day 7 to ensure reliable cell counting results, which was not possible on the fully overgrown substrates. Cell proliferation on all of the glass samples, including FLAT, exhibited an initial proliferation delay during the first days of culture. While cells on the FLAT reference started to slightly proliferate after two days of culture, there was a delay in proliferation or a slight decrease in cell number on most nanoporous glass samples until day 4, indicated by negative cell doubling rates displaying a small degree of apoptosis. After this initial delay, cells started to proliferate steadily on the nanoporous glass samples with a clearly positive trend toward mean pore sizes above 80 nm, almost reaching the cell doubling rates of the reference samples (Figure 2b).

2.3. Differential mRNA Expression on Different Pore Sizes—Elucidation of a Cutoff Pore Size for Attractive Interaction

mRNA expression of L929 cells seeded on nanoporous glass membranes with mean pore sizes of 17, 26, 45, 81, and 124 nm and the two reference samples was analyzed by qRT-PCR after 48 h of culture, i.e., during the initial resting phase when the cells settle on the surface and explore their surroundings. Cells seeded on nanoporous glass membranes with mean pore sizes of 81 and 124 nm show an expression profile that is very similar to the FLAT reference that already started proliferating at this timepoint (Figure 2c). Even though the relative cell count is still slightly decreasing during the first days of culture, the induction of proliferation for cells on the 81 and 124 nm samples is
significantly increased compared to smaller pore sizes. This is visible in the increased expression of the proliferation-specific proteins (MKI67, MCM2). These findings indicate a more attractive initial cell-substrate interaction compared to smaller pore samples, even though overall proliferation has not started yet after 48 h. In addition, genes that regulate other standard cell functions such as adhesion (FAK, ITGB1), matrix production (COL1A1, FN1) and contraction (ACTA2) were analyzed. The expression of ACTA2 on nanoporous glass membranes with larger nanopores is slightly reduced in comparison to the FLAT samples. Below 80 nm pore size, a drastic change in expression can be observed as there is a clear upregulation of FAK, while the other essential genes are dramatically downregulated, indicating reduced mean cell vitality.

2.4. Primary hMSCs Proliferate Readily on Nanoporous Glasses with Different Pore Sizes

Primary hMSCs were seeded onto nanoporous glasses of three different mean pore sizes (17, 46, and 81 nm) and two flat reference samples (FLAT glass/TCPS). Samples were fixed and stained with phalloidin at different timepoints. All samples exhibited good cell adhesion and proliferation, proven by increasing cell density and confluent cell sheets visible by eye. During the first days of culture on the nanoporous glass membranes, cell clustering was observed (Figure 3a). No more clusters were present after day 3, indicated thorough cell spreading.

2.5. Chondrogenic Differentiation is Induced on Glasses with 17 nm Mean Pore Size—Distinct Differences in Cytoskeletal Ordering

mRNA expression analysis was performed to identify any differentiation inducing effects of the nanoporous glass samples on primary hMSCs. For this purpose, mRNA was extracted at different timepoints, converted to cDNA and then analyzed by qPCR. For the sample with 17 nm pore size, we found distinct upregulations for some chondrogenic factors. In comparison to the controls, a clear increase in the cartilage-specific genes SOX9, COL2A1, and COL10 can be observed compared to FLAT and TCPS (Figure 3b,c). Phalloidin stainings of the
Figure 2. Analysis of the interaction of L929 mouse fibroblasts with nanoporous glass samples of different pore sizes (17, 26, 46, 81, and 124 nm), compared to flat references (FLAT glass and TCPS). a) Exemplary fluorescence microscopy image series of one (out of 10 total) sites of analysis at different timepoints from day 1 to 10. Images show L929 GFP cells (green) on different substrates at different timepoints. b) Mean cell doubling rates for each timepoint at days 2, 3, 4, 7, and 10; brackets to connect the bar graphs with the corresponding image columns from (a). Cell doubling rates were calculated via an exponential function with mean cell counts at day 1 as reference points. c) Relative mRNA expression after 48 h culture on the different pore size samples and FLAT glass, normalized to TCPS expression.
Figure 3. Analysis of the interaction of primary human mesenchymal stem cells (hMSCs) with nanoporous glass samples of different pore sizes (17, 46, and 81 nm) and flat references (FLAT glass and TCPS). a) Microscopy images of hMSC at different timepoints with actin cytoskeleton stained by phalloidin and DAPI stained nuclei. b) Heatmap of mRNA expression of chondrogenic biomarkers at different timepoints (7–12, 21, and 28 d). c) Relative mRNA expression of chondrogenic markers at different timepoints (7–12, 21, and 28 d), normalized to the expression at the first timepoint on TCPS for each marker.
hMSC actin cytoskeleton at different timepoints display that actin fiber alignment is much more pronounced on the 2D reference surfaces, while the actin cytoskeleton is rather disordered on the nanoporous glass samples (Figure S2, Supporting Information).

2.6. Resisting Gravity: Short-Term Adhesion and Proliferation Studies of SK-MEL-28 Reveal Increased Adhesion and Cell Spreading on Nanoporous Glass

For the investigation of general cell adhesion and viability on nanoporous glass membranes in upside down culture, we used nanoporous glass membranes with mean pore sizes of 10 and 20 nm with 80% SiO₂. 10 µL droplets of a cell suspension containing ≈500 SK-MEL-28 melanoma cells were pipetted onto the nanoporous glass membranes and FLAT reference glasses. After 3 h of incubation to allow for slight adhesion, the samples were turned upside-down and cells were incubated under the influence of gravity, putting cell adhesion to the test. Microscopy and cell counting was performed for days 1, 2, 3, 4 and 9 after seeding (Figure 4a). Strikingly, the initial adhesion of SK-MEL-28 cells is significantly increased on nanoporous glass, with 490 ± 18 cells (10 nm) and 459 ± 27 cells (20 nm) versus 259 ± 32 cells on FLAT after 3 h adhesion time (Figure 4b). After the typical resting phase upon seeding on uncoated or non-TCPS surfaces, the...
cells on nanoporous glass membranes proliferated very strongly even under the influence of gravity, growing to a mean relative cell count of 11.3 ± 0.2 on day 9 after seeding. In contrast, the number of cells on smooth glass surfaces steadily decreased to a mean relative cell count of 0.17 ± 0.05 (Figure 4c). Additional morphological investigations carried out with binarized cell outlines from SEM micrographs (Figure 4d) revealed a significantly higher circularity and solidity for the cells on FLAT surfaces. This indicates a rather passive spreading process with a more circular morphology and less protrusions. Cells on nanoporous samples had more protrusions and an increased area, indicating an active spreading process with strong focal adhesions, clamping the cell body to the topographic surface under the influence of gravity in upside down culture.

### 2.7. Giving in to Gravity: Deadhesion of MDA-MB-231 Breast Cancer Cells upon Paclitaxel Treatment

To further study the effect of differential cellular adhesion to nanoporous glass compared to the FLAT reference, the effect of a cytoskeleton-altering chemotherapeutic agent on MDA-MB-231 breast cancer cells was investigated. Cells were seeded onto nanoporous glass membranes with different mean pore sizes (17, 26, 46, 81, and 124 nm) and onto FLAT samples and were incubated for 24 h in order to achieve homogeneous cell colonisation of all substrates. The samples were then cultured upside down (as previously described) for 48 h and divided in two groups: “Control” and “Paclitaxel.” The “Control” samples were cultured in normal culture medium, the “Paclitaxel” group was cultured with 500 \( \times 10^{-9} \) m Paclitaxel (PACLITAXEL). Paclitaxel blocks the dynamics of microtubules essential for maintaining focal adhesions. 24 h into the experiment, all cells on “Control” samples are proliferating, except on the 17 nm sample. After 48 h of culture, proliferation has continued on the FLAT and 124 nm sample, while cell numbers only slightly increased on the other samples. In the “Paclitaxel” group, no distinct differences between samples can be observed after 24 h. After 48 h the relative cell count on all nanoporous samples decreases by ≈35–55%. The influence on the FLAT substrate is significantly lower, with a drop of ≈5% during the first 48 h (Figure 5).

### 3. Discussion

For a systematic study of the pore size of nanoporous glass on cell behavior, glass membranes of different mean pore sizes were produced and characterized in order to test their applicability as a novel nanostructured topographic cell culture substrate. A membrane thickness of 250 μm was found suitable for robust handling, while thinner samples were more likely to break during the preparation process and in the lab. In comparison to thicker samples, 250 μm samples provided a sufficient optical transparency for basic microscopy tasks even for the macroscopically opaque samples. This opaqueness is caused by the typical coarsening and phase separation occurring during spinodal decomposition of such glass systems.[12] Opacity increased with the demixing temperatures, pointing toward increasing nanopore sizes. The UV-vis measurements confirmed this assumption with decreased transmission for increasing demixing temperatures – the pores in the system act as a scatterer. This is in good accordance to the Mie-scattering theory, where the in-line transmission decreases at a defined wavelength, if the size of the scatterer increases. The cutoff wavelength for transmission increases with increasing size of the scatterer, i.e., with increasing mean pore sizes.[13,14]

We then investigated the interaction of living cells with nanoporous glass membranes by analyzing the proliferation of L929 mouse fibroblast cells, the standard cell line used for biocompatibility testing of novel materials. We used samples with four different mean pore sizes (17, 45, 81, and 124 nm) under standard cell culture conditions, and FLAT glass samples and TCPS as controls. As the “gold standard” commercial product for cell behavior studies, TCPS surfaces are optimized for immediate protein adsorption by surface treatment, facilitating initial cell adhesion and proliferation.[15] The TCPS samples exhibited the expected exponential cell proliferation known from conventional cell culture. On the FLAT glass reference, cell proliferation starts after the second day. Compared to TCPS, proliferation is significantly reduced on FLAT, due to the fact that protein adsorption is significantly decreased on non-treated glass.[16] While proteins from the culture medium can still form a uniform, but less dense layer on the FLAT samples, the protein layer adsorbed at the adhesion surface of the nanoporous
samples is interrupted by the pores. Thus, surface protein density at the nanoscale is radically reduced, inhibiting cell spreading and cell proliferation during the first days of culture. After this initial delay, the proliferation rate constantly increases to near-exponential rates for cells on mean pore sizes >80 nm until confluence. As fibroblasts are matrix-producing cells that can modify their environment,[31] this effect can be ascribed to ongoing matrix secretion by the cells, remodeling the adhesion surface bit by bit—in our study, depending on the pore size of the substrate. In general, larger nanopore sizes (>80 nm) show a positive influence on cell proliferation, as displayed in the larger increase of the relative cell numbers on nanoporous glass membranes with pore sizes of 124 and 81 nm. This clear trend points toward a systematic mechanism for L929 fibroblast adhesion and proliferation on nanoporous glass, indicating a proliferation mechanism and –rate that is closer to the in vivo situation than on flat substrates.[5] It is widely known that cells can perceive the nanoroughness of a surface and that mechanical and topographical properties influence the adhesion and migration behavior of cells. Cells probe surface features with mechanosensitive elements consisting of actin, myosin, paxillin or filopodia.[18,19] In fact, cell proliferation can be strongly influenced by the (nano)structure of a cell’s direct vicinity and the substrate topography.[20,21] Cell proliferation in a lot of cell types is diminished on surfaces exhibiting nanotopographical stimuli.[19,22–24] For example, corneal epithelial cells cultured on nanogrooves mimicking the diameter of collagen fibrils in the corneal stroma exhibited reduced proliferation.[19] Altering the nanoscale roughness by just a few nanometers can have a distinct influence on cell proliferation, highlighting living cells’ sensitivity to topographic cues that are one million times smaller than their diameter.[25,26] Milner et al. demonstrated the influence of nanochannel architecture by investigating the proliferation of human fibroblasts on PLLA substrates with randomized nanoscale bumps or nanoislands: The cultures maintained significantly lower densities and lower rates of proliferation on patterns with features >200 nm. Continued reduction of feature sizes eliminated the discrepancy between nanotextured and flat substrates with respect to cell proliferation.[27] A cutoff size for cell proliferation was also found for human HepG2 cells seeded on nanoporous aluminum surfaces with pore sizes of 40 and 270 nm: On the larger pores, cell adhesion and proliferation was much better than on the smaller pores.[28] Two studies on surfaces modified with gold nanodot patterns of different nanospacings showed results on the integrin clustering at the surface: There is a distinct turnover of adhesion strengths for fibroblasts in the range between 50 and 70 nm for these surfaces.[29] With spacings <70 nm, the adhesion is highly increased, even for very short adhesion times <10 min.[30] These results point to a cell-type specific influence of nanoscale topographies on cellular adhesion and proliferation behavior. In agreement to that, our study showed that there is a cutoff size for the proliferation of L929 fibroblast cells on nanoporous glass that could also be fostered for cell cultures that need diminished proliferation rates, e.g., to better reflect in vivo proliferation for very short adhesion times <10 min.[30] These results point to a cell-type specific influence of nanoscale topographies on cellular adhesion and proliferation behavior. In agreement to that, our study showed that there is a cutoff size for the proliferation of L929 fibroblast cells on nanoporous glass that could also be fostered for cell cultures that need diminished proliferation rates, e.g., to better reflect in vivo proliferation rates in long-term experiments.[3,31]

The immediate influence of nanoporous glass on L929 fibroblast cell functions was further investigated in real time PCR experiments. The mRNA expression patterns of L929 cells after 48 h of culture on all samples further elucidate the underlying mechanism of the initial cell reaction to the different substrates. After two days on the respective culture surfaces, two different main types of expression profiles on the nanoporous samples could be distinguished: The mRNA profiles on the three small (17 nm/26 nm/46 nm) and on the two larger nanopore samples (81 nm/124 nm) each show a similar pattern. This indicates a transition of cell behavior for adhesion on smaller versus larger nanopores. On smaller nanopores, the expression of most proteins that play crucial roles in healthy cells was strongly down-regulated after 48 h of culture. This is in good accordance with the trends observed in the L929 proliferation experiments. Additionally, the resemblance of the expression patterns of the larger nanopore samples and the FLAT sample is striking, proving the general cell reaction to the material glass. Taking a closer look at the mRNA expression, the decreased expression of K167 and MCM2 after 48 h concurrently underlines the diminished proliferation rates on nanoporous glass during the first days of exploring and remodeling the adhesion surface by matrix production. A more and more natural protein layer on the surface, produced by the cells, could then facilitate cell adhesion and proliferation on the nanoporous samples in the subsequent days. The production of ECM molecules like collagen and fibronectin by L929 cells is described in studies on collagen-PLLA hybrid nanopore structures where a similar trend toward larger nanopores was found.[31] In our experiments, the expression of ACTA2 is reduced on larger pores, indicating less tension inside the cell caused by decreased cellular contraction. An overall reduced cytoskeletal elongation and contraction can have a decisive effect on other cell functions such as intracellular transport,[33] which can then take place in a more physiological way inside cells that are not artificially contracted by stretching across a surface lacking topographic stimuli. Below 80 nm pore size, another drastic change in expression can be observed: A peak for FAK (Focal Adhesion Kinase) is clearly visible, which, together with a lower proliferation for small pores, indicates that a large portion of cells on those samples has a tendency toward anoikis—programmed cell death due to poor cell-substrate adhesion.[34,35] Thus, we assume that the up-regulation of FAK is due to the fact that the majority of cells on the smaller nanopores are fighting against anoikis: This could be caused by a lack of properly sized “adhesion-islands” inbetween the pores—a high percentage of initiated adhesions might be retracted due to a lack of available surface area, which should be further examined by high-resolution microscopy in future studies. On samples with larger pores, they are already producing ECM molecules and start to proliferate. After 4 days of delay, sufficient surface remodeling has occurred on the smaller porous samples and proliferation slowly begins.

Furthermore, the nanoporous glass membranes were analyzed for their interaction with primary hMSCs. This specific cell type is a very promising candidate for translation research in tissue engineering due to its differentiation potential. The cell clustering observed during the first days of culture on nanoporous glasses indicates a diminished initial cell adhesion on the uncoated, rough surfaces, where cell-cell contacts are preferred. After three days of culture, no clustering is observed,
cells have fully spread onto the surface. An overall good proliferation could be observed on all surfaces, indicating the suitability of nanoporous glass for the robust expansion of mesenchymal stem cells. This fits well with the findings of a study on the interaction of osteogenic cells with nanoporous titanium surfaces with mean pore diameters of ≈20 nm, where an overall increased adhesion and upregulations of adhesive genes were found.[36] In our mRNA expression experiments on hMSCs, a trend toward chondrogenic differentiation could be observed for nanoporous glass membranes during the first two weeks of culture, most pronounced for samples with a mean pore size of 17 nm. This manifested in the increased expression of COL10, COL2A1, and SOX9, which are all key players in chondrogenic differentiation.[37] Especially COL2A1 and SOX9 are characteristic for immature chondrocytes,[38] potentially indicating early chondrogenic differentiation states after 7–12 days of culture. The rather disordered actin organization on nanoporous samples could also point toward a trend for chondrogenic differentiation. A material-intrinsic differentiation effect, without external stimulation, is a very promising result and has the potential to controllably modulate stem cell fate.[19] The overexpression of COL10 might point to a bias toward hypertrophic differentiation, an effect that could be fostered for the buildup of models for osteochondral ossification on nanoporous membranes.[40] These first results for hMSC interaction with nanoporous glass membranes presented in our study indicate that cell differentiation could be induced by these surfaces without any further media supplements. Furthermore, standard differentiation experiments could be sped up by surfaces that facilitate proliferation.[41]

In the short-term cell adhesion and proliferation experiments with SK-MEL-28 melanoma cells, the quick adhesion to the nanoporous glass surface proves the nanoporous glass membranes’ function as a nanotopographical, three-dimensional environment for cell adhesion. In contrast to the FLAT glass surfaces, cells on nanoporous glass resist the gravitational force after only 3 h of adhesion time. The initial cell adhesion on the FLAT samples is decreased by 47%, indicating diminished initial adhesion. In the first four days of upside down culture, the constant relative cell number on the nanoporous glass surfaces is an indicator for the fact that cells on the nanoporous surfaces perceive the gravitational force and that their proliferative behavior is influenced by their struggle for adhesion. However, cell numbers on the nanoporous surfaces did not decrease during the first days of upside down culture while there was a progressing decrease on FLAT samples. While a diminished, but still measurable proliferation could have been attributed to lower cell density caused by the 47% lower initial cell count, the absence of proliferation displays that the cells are losing their struggle for adhesion and detach from the surface. The subsequent constant proliferation on the nanoporous glass membranes in contrast to the continuous decay of the relative cell number on the smooth glass surfaces strengthens the hypothesis of positive cell-material interaction: The nanoporous glass surface supports cell adhesion by its inherent nanoscale surface topography. Thus, the cells perceive the nanoporous surfaces as a 3D environment, enabling increased initial cell adhesion by nano-sized topographic stimuli that facilitate the quick manifestation of focal adhesions at the surface after only 3 h of adhesion. This leads to the conclusion that for strongly adherent cells like SK-MEL-28, a nanoporous glass surface—presenting topographic stimuli for filopodia stabilization—is much closer to their natural environment in the human body than smooth 2D surfaces. This result is strengthened by the morphological findings, displaying increased protrusive activity and an increased area on nanoporous glass membranes. This points to an active adhesion mechanism with strong focal adhesions that can resist the gravitational force and keep the cells in place. Furthermore, cells also undergo extensive proliferation on the nanoporous glass membranes. Thus, they manage to partly deadhere, divide and spread on the surface again during the process of mitosis, proving that cells not only adhere but perform their normal function in 3D topographic environments.

This is also confirmed in other studies, e.g., it was shown that osteoblast-like SaOS-2 cells adhere much better to titanium surfaces with 0.5 and 2.0 μm pores than on flat reference surfaces after an equal short incubation time of 1–2 h.[42] In a study on nanoporous anodic aluminium oxide with a similar anisotropic surface structure, Poinern et al. found an overall good cell adhesion and slightly increased proliferation for the Vera cell line.[43] In our experiment, the nanoporous membranes facilitate initial cell adhesion without additional functionalization/coating, indicating a 3D scaffold effect and an altered adhesion mechanism compared to flat surfaces.

The effect of differential adhesion on nanoporous and flat glass was further examined in a functional drug assay, utilizing one of the most common cell lines for breast cancer and the prominent anti-cancer drug paclitaxel. The aim of this assay was to find a quick test for having a distinct effect of a drug on a specific cell function. An assay that gives quick outputs for drug effects on patient cells could speed up drug screening in personalized medicine, e.g., to find a suitable drug for a specific patient and cancer type. We proved this in an adhesion assay with MDA-MB-231 breast cancer cells that were treated with paclitaxel in upside down culture. Paclitaxel is an active agent that inhibits the disassembly of microtubules. These play a crucial role in diverse vital processes like the chromosome separation during mitosis, adhesion and focal adhesion disassembly.[44] Microtubule-inhibiting agents are particularly effective in fast dividing cells like cancer cells and are often used for the treatment of breast cancer. Paclitaxel can lead to a dwelling during mitosis due to a mitotic spindle stabilization, followed by apoptosis. Alternatively, it can induce a mitotic block that leads to multipolar cell division and subsequent apoptosis of the daughter cells with reduced chromosome number. Cells on nanoporous glass and FLAT surfaces were pre-incubated for 24 h under standard cell culture conditions (standard procedures, upright cell culture) and then cultured upside down. At this timepoint, all samples exhibited normal, comparable cell densities with spread cells, indicating a good starting point for the assay. For nanoporous surfaces, we observed a pore size independent drop in the relative cell numbers 48 h after 500 × 10⁻⁹ M paclitaxel was added to the medium, while cell numbers on the FLAT surface were almost constant. This indicates an altered cellular adhesion mechanism on the porous glass substrates that seems to be highly dependent on microtubule dynamics. While proliferation is highly diminished or even stopped on all samples, a
high number of cells detaches from nanoporous samples while cells on FLAT samples remain adherent. In a recently published study, we found that the microtubule-diminishing drug Benomyl has a similar effect on the adhesion of amoeboid cells of *Dictyostelium discoideum* to nanostructured SiO2 fibers: Upon drug treatment, almost all of the cells deadhere from the nanostructured fibers, adhesion to flat glass is persisting. These findings indicate a suitability of these nanoporous substrates for the quick detection of active substances that interfere with cytoskeletal and adhesion processes. This or similar effects found in future studies could be fostered for clinical application, e.g., in personalized cancer treatments to see if a specific patient’s cancer cells respond differently to a set of potential drugs or drug combinations.

4. Conclusions
We have shown that nanoporous glass membranes are a bioactive material that provides an adhesion surface for various cell types from pathologic and from healthy human tissue. Our pioneering study targeted the identification of potential effects of nanoporous glass surfaces on different murine and human cell types, for both cell lines and primary cells. We were able to identify cell-type specific effects of certain nanoporous glass membranes within our specific experimental setups. Future studies should aim at confirming these findings for other cell types, identifying differences in cell behavior and broadening the knowledge on cell behavior in different experimental setups.

We found differential adhesion characteristics and modulation of gene expression in L929 fibroblasts, just by changing the pore size, which points toward a direct influence of the pore size on nanoscale cellular processes: Glass with a mean pore size above 80 nm can serve as a macroscopically flat, but intrinsically nanostructured topographic surface for cell culture applications even without additional bio-functionalization. The change in gene expression for L929 and hMSCs as a function of pore size implies a cell-specific cutoff pore size for a positive cell adhesion effect. In future studies, the bioactivity of the nanoporous glass samples could be further tailored toward a positive or negative interaction with specific cell types by varying the pore sizes or introduction of surface functionalization or coating. Potential effects on differentiation, as observed in the trend toward chondrogenic lineage for hMSCs on nanoporous glass membranes with 17 nm mean pore size, might enable various future applications for nanoporous glass membranes in hMSC research and tissue engineering. Additionally, a strongly increased adhesion and proliferation of SK-MEL-28 cells on nanoporous glass compared to flat glass references in upside down culture was identified. This underlines that nanoporous glass membranes are suitable for use in 3D cell culture vessels or bioreactors — with cell adhesion to all surfaces in 3D. The material can provide cell adhesion while still allowing constant perfusion for nutrient flow and cell-cell signalling. Finally, paclitaxel treatment of MDA-MB-231 breast cancer cells shows more rapid effects on nanoporous glass membranes in upside down culture compared to FLAT glass references, with strongly diminished cell adhesion and proliferation after only 48 h. Drug assays and the development of personalized treatments could be sped up by fostering this differential cell adhesion effect on nanoporous glass membranes. Furthermore, complex functional assays, including microfluidics and other stimuli, could benefit from a more physiological cell behaviour and proliferation rate on nanoporous glass surfaces.

We conclude that nanoporous glass membranes present nanoscale cues that act as bioactive triggers to influence cellular cascades for the regulation of cell functions. This proves the suitability of the substrates as a functional cell culture surface. Our findings underline the versatile application potential of nanoporous glass membranes for cell culture. Deepening the understanding of our findings can lead to new approaches for existing needs in both science and the biotechnical and biomedical industry.

5. Experimental Section

**Membrane Preparation:** We produced nanoporous glass samples with two different material compositions and multiple mean pore sizes (Table 1). Smelting of initial components was performed at 1500 °C for 2 h. The smelted samples were slowly cooled down (10 K h⁻¹) to room temperature and cut into pieces of 16 × 16 mm before they were grinded down to a thickness of 250 μm. To achieve nanoporous glasses of different pore sizes, a second heat treatment step was performed at temperatures between 500 and 730 °C for 8 h (Table 1). After slowly cooling down, the samples were leached out for 2 h in distilled water at a temperature of 90 °C. After the leaching process, the hot water was exchanged in parts with 25 °C warm water. When the water in the beaker reached 60 °C, the samples were taken out of the beaker and washed in warm distilled water before they were placed in the oven over night to dry at 60 °C. This process was necessary to prevent cracks in the glass samples. All samples were stored at room temperature. The nomenclature for this work is defined in Table 1. Borosilicate microscopy cover glass slips were used as flat glass reference (FLAT) (Menzel-Gläser, Thermo Fischer Scientific) to compare the results with a flat surface of the same material class. Tissue culture polystyrene (TCPS) was utilized as the “gold standard” control for standard cell behavior in 2D cell culture.

**Membrane Characterization:** Membranes with 70% SiO2 were characterized in detail by UV–vis spectroscopy and SEM measurements, followed by pore size calculation. 80% SiO2 samples were characterized by SEM (Figure S1, Supporting Information).

**UV–vis:** Transmission spectra of nanoporous glass membranes with 70% SiO2 were recorded with a Cary 50 UV–vis spectrophotometer (Varian Inc.). The wavelength range was from 180 to 900 nm.
SEM and Pore Size Analysis: We acquired SEM micrographs of all samples. Measurements were performed with a Supra 25 Scanning Electron Microscope (Carl Zeiss Microscopy) at different magnifications and voltages. For pore size analysis, images were binarized with Fiji (ImageJ, National Institutes of Health, USA) by manual thresholding. Then, the plugin „DiameterJ“ was used to determine the mean pore diameters. „DiameterJ“ includes „OrientationJ,“ which consists of several scripts for analyzing fiber orientation and the pores inside scaffolds, e.g., the mean pore diameter used as a sample classifier in this study.

Cell Culture: Lentivirally transduced L929 mouse fibroblasts expressing a free Green Fluorescent Protein (L929-GFP) in the cytoplasm were used for the experiments. Cell culture was performed at standard conditions. For proliferation and SEM studies, cells were kept in “Minimum Essential Medium (MEM) EBS with stable Glutamine without Phenolred” (Amimed), 10% FCS (Bio&Shell) and 1% PenStrep (Sigma-Aldrich). For mRNA expression experiments, cells were cultured in „Dulbecco’s Modified Eagle Medium (DMEM) with GlutaMAX“ (Gibco), 10% FCS and 1% PenStrep. Medium exchange was performed at standard rates for cell culture (every 2–3 days).

Proliferation Experiments: Prior to the experiments, nanoporous glass membranes with four different mean pore sizes (17, 45, 81, and 124 nm) and FLAT glass reference samples were glued to glass sample holders for upside-down culture and autoclaved. The glasses and TCPS reference surfaces were pre-incubated with phosphate buffered saline (PBS, Sigma-Aldrich) for 15 min and culture medium for 30 min. Cells were seeded on the glass samples at a density of \( \approx 15.500 \) cells cm\(^{-2}\). For the TCPS control, cells were seeded directly into multi-well plates at a density of \( \approx 4.000 \) cells cm\(^{-2}\). The difference in initial seeding density was chosen to be correct for the height of the sample holder / sample compound (\( \geq 50\% \) of total medium level) that decreases effective seeding density on the glass substrates. For microscopy, the glass samples were reversed in the well. Images were acquired at days 1–4, 7, 10, and 14. For each time point, bright field and the fluorescent images of the same ten positions were taken. The images were processed and aligned with Corel Photo-Paint X5 (Corel DRAW) prior to cell counting. Cell counting was performed manually with the “Cell Counter” plugin of Fiji. Relative cell counts compared to day 1 were calculated. The cell doubling time \( T_d \) was calculated for each time interval. For this, the following exponential function was applied

\[
N(t) = N_0 e^{kT_d} \quad T_d = \frac{\ln 2}{k}
\]

where \( N_0 \) is the cell count at day 1, \( k \) is the growth rate, \( t \) the time, and \( T_d \) is the doubling time. For further analysis, the mean daily doubling rate \( k \) per cell for each day was calculated as the inverse of the doubling times with the general assumption of exponential growth.

qRT-PCR Analysis: The expression of different adhesion-related and physiologically relevant genes of L929-GFP cells in reaction to nanoporous glass membranes was analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Nanoporous glass membranes with mean pore sizes of 17, 26, 45, 81, and 124 nm were analyzed, as well as flat reference samples (FLAT, TCPS). Pieces of the glass samples were placed in petri dishes, while for TCPS, the bottom of the petri dishes was used as culture substrate. All samples were pre-incubated with cell culture medium for 15 min prior to the experiments. L929-GFP cells were seeded at a density of \( \approx 10.000 \) cells cm\(^{-2}\). The glass samples were moved to new petri dishes the day after seeding to exclude the influence of the cells at the bottom of the original petri dishes on qRT-PCR results. After 48 h of culture, the cells were harvested and lysed directly in the petri dishes with the RNase MicroKit (Qiagen). Equivalent amounts of all samples were reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Biorad). qRT-PCR was performed in a CFX96 Real-Time PCR system (BioRad) using the SsoFast EvaGreen Supermix (Bio-Rad). The primers for the experiments are listed in Table 2. ACTB was used as the housekeeping gene for normalization.

Cell Culture: Human mesenchymal stem cells from two patients (male, 35 and female, 62) were isolated under the approval of the Local Ethics Committee of the University of Würzburg (approval number 182/10) and informed consent of the patients. Cells were cultured at standard conditions. The cell culture medium was “DMEM/F12” (Gibco) with 10% FCS (Bio&Shell) and 1% PenStrep (Aldrich). Medium exchange was performed at standard rates for cell culture (every 2–3 days).

Phalloidin Staining: Small pieces of nanoporous glass samples with 17, 46, and 81 nm mean pore size and FLAT glass were autoclaved, distributed to 48-well plates and pre-incubated with PBS for 20 min and cell culture medium for 1 h. Additionally, TCPS petri dishes were cut into small pieces, sterilized and pre-incubated in the same way in 48-well dishes. Cells were seeded at a density of \( \approx 70.000 \) cells cm\(^{-2}\) on nanoporous glass samples and \( \approx 25.000 \) cells cm\(^{-2}\) on references (to prohibit confluence). Cells were cultured at standard conditions until fixation on days 1, 2, 5, 7, 9, 14, 21, and 28 after seeding. For fixation and Phalloidin / DAPI staining, the samples were washed three times with PBS, then fixed for 20 min in 4% Histofix (Carl Roth) and washed three times with PBS again. After that, the cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) and washed three times with PBS again. Then, blocking was performed by incubating with 1% BSA (Sigma-Aldrich) in PBS for 20 min before staining in Alexa Fluor 555 Phalloidin (Invitrogen) (diluted 1:40 with PBS). Samples were then mounted with Fluoromount with DAPI (ThermoFisher) and left to dry overnight. Imaging was performed with a Keyence fluorescence microscope.

qRT-PCR Experiments: Nanoporous glass samples with mean pore sizes of 17, 46, and 81 nm, FLAT reference glasses and TCPS were used for hMSC experiments. We took the decision to limit the number of different nanoporous glass samples to three due to limitations in the availability of primary human cells, in order to ensure sufficient RNA material for all samples at all timepoints. These three mean pore sizes were the logical choice, being not too far apart and still representing three distinctly different regimes of mean pore sizes. Samples were

| Name | Forward primer | Reverse primer | PCR product length |
|------|----------------|----------------|--------------------|
| ACTB | ACTGTCGACTCCCTGGTCCA | TCATCCATGCGAACACTGGTG | 88 |
| MKI67 | CAGACCTAACTTTGGCTGAC | CCGTTGATGTCGACCGAGTG | 148 |
| MCM2 | ACATCCAGCCCTGATATCCGG | ACATCCGCCTTGAATCCGG | 133 |
| FAK | ACCAGAGAATCTTCTCCGCCC | AGCCACAGATACCCACTCTTG | 110 |
| ITGB1 | CGAGCCCTGGAAAGAATGCAA | CCAATTTGCGCTCTTGGC | 152 |
| COL1A1 | GACAGCTGAAACAGGTCGG | AACACTCTTCGCTCTTGCC | 153 |
| FN1 | CGACTCTGACTGCGTTTACC | ACCGTCTAAGGGCTAAAACCA | 166 |
| ACTA2 | GTCAGTGGTGCCTCTGCTA | ACTGGGACGACATGGGAAAGA | 101 |

Table 2. Primers for L929 qRT-PCR experiments.
placed into 12-well plates and cells were seeded with a density of \( \approx 85.000 \text{cells cm}^{-2} \) (nanoporous glass samples) and \( \approx 30.000 \text{cells cm}^{-2} \) (references). The glass samples were moved to new well plates the day after seeding to exclude the influence of the cells at the bottom of the original wells on qRT-PCR results. At timepoints 7, 12, 21, and 28 d of culture, cells were harvested and lysed directly in the wells. For subsequent analysis, samples from day 7 and day 12 were pooled due to limitations in cells available for initial seeding. All further steps were performed as described in 2.3.1. The primers for the experiments are listed in Table 3. GAPDH was used as the housekeeping gene for normalization.

### Cell Culture: SK-MEL-28 with lentivirally transfected GFP

For subsequent analysis, samples from day 7 and day 12 were pooled due to limitations in cells available for initial seeding. All further steps were performed as described in 2.3.1. The primers for the experiments are listed in Table 3. GAPDH was used as the housekeeping gene for normalization.

**Cell Culture:** SK-MEL-28 with lentivirally transfected GFP were cultured under standard conditions in “Dulbecco’s Modified Eagle Medium (DMEM)” with GlutaMAX” (Life Technologies), with 10% fetal calf serum (FCS) (Bio&Shell) and 1% Penicillin Strepomycin (PenStrep) (Sigma-Aldrich). The cells were kindly gifted by Sebastian Kress (Fraunhofer ISC, Würzburg). Before fluorescence microscopy experiments, the medium was switched to “Minimum Essential Medium (MEM) EBS with stable Glutamine without Phenolred” (Amimed), 10% FCS and 1% PenStrep. Medium exchange was performed at standard rates for cell culture (every 2–3 days).

**Adhesion Experiments:** Prior to the experiments, the glass samples were autoclaved. Cells were trypsinized, counted and suspended to \( \approx 50.000 \text{cells mL}^{-1} \). 10 \( \mu \)L drops of this suspension containing \( \approx 500 \text{cells} \) were applied onto nanoporous glass samples with a mean pore size of 10 and 20 \( \mu \text{m} \) and FLAT reference glass samples. After 4 h incubation in upright culture to allow the cells to adhere to the surfaces under the drops, the samples were put upside-down into “\( \mu \)-Dishes 30 mm” (IbiDi GmbH) containing cell culture medium and spacers to prevent the sample surface from touching the bottom of the dish. After incubation, samples were incubated overnight. Image acquisition (brightfield and fluorescence) was performed on days 1, 2, 3, and 4 after seeding. A stage top incubator (Tokai Hit) was used to ensure optimal conditions for living cells during imaging. After imaging at day 4, the samples were stored in the incubator until day 9. Then, the samples were embedded in a solution of Mowiol (Sigma) and DAPI (D 8417, Sigma) andcovered with a coverslip. They were imaged in a fluorescence microscope (Keyence) with a DAPI excitation filter. Automatic cell counting of all images was performed with the “Spot Detection” feature of NIS-Elements Software (Nikon). The mean error of the automatic detection of cells was determined by manual cell counting of three samples on different days with the “Cell Counter” plugin in FIJI. Relative cell counts normalized to day 1 cell counts were calculated as ratio of cell count at current day to cell count at day 1 (i.e., a relative cell count of 1.5 at day x equals to 50% increase in cell numbers relative to day 1). Furthermore, the mean value for the different samples were determined.

**SEM Preparation and Morphological Analysis:** For SEM visualization of SK-MEL-28 cells on nanoporous glasses and FLAT reference samples, 50 \( \mu \text{L} \) droplets containing \( \approx 625 \text{cells} \) were placed onto all samples and incubated for 4 h. Then, full culture medium was added to the dishes and samples were incubated for 12 h. After that, the samples were rinsed three times with warm and once with ice cold PBS (Sigma-Aldrich) for one minute. Subsequently, they were placed on ice and treated with 6% glutaraldehyde (Sigma-Aldrich) in PBS (Sigma-Aldrich) for 30 min. Then the samples were stored in PBS (Sigma-Aldrich) on ice for five minutes three times, medium was exchanged between steps. Afterward, PBS was exchanged with distilled water at room temperature. Then water was replaced by acetone in five concentration steps: 15 min 30% acetone, 20 min 50% acetone, 30 min 75% acetone, 45 min 90% acetone, and 5 times for 30 min 100% acetone. Finally, acetone was exchanged with CO2 by critical point drying in a pressure chamber at 7 °C by repeated sucking of acetone from the chamber and replacement by CO2. This was repeated 15 times before heating the chamber up to 40 °C over the critical point of CO2 (31 °C, 73.8 bar) to reach a final pressure of about 80 bar. Then the CO2 was slowly released from the chamber. After that, the samples were sputtered with platinum and analyzed in a scanning electron microscope (Supra 25, Zeiss). For morphological analysis, SEM micrographs of the cells on the different culture surfaces were used. Prior to analysis, the image magnification was normalized, single cells were cropped by hand in a detailed process and images were binarized. Cell morphology was then analyzed using the “Analyze Particle” tool in FIJI.

**Cell Culture:** MDA-MB-231 cells were cultured at standard conditions. The cell culture medium was “RPMI 1640 modified with GlutaMAX” (Gibco) with 10% FCS (Bio&Shell). Medium exchange was performed at standard rates for cell culture (every 2–3 days).

**Paclitaxel Treatment Experiments:** Nanoporous glass samples with mean pore sizes of 17, 26, 46, 81 and 124 \( \mu \text{m} \) and FLAT reference glasses were analyzed using the “Analyze Particle” tool in FIJI.

### Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
Part of this research is part of the US Patent Application #20190345443 “Method for the Culturing and Differentiation of Cells” and the EU Patent Application #19170636.5 “Intrinsisch nanostrukturierte 2.5D-Zellkulturen für Zellkultur”, both filed by the authors Martin Emmert, Ferdinand Somorowsky and Doris Heinrich.

Data Availability Statement
Research data are not shared.

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