Exploring combined influences of material topography, stiffness and chemistry on cell behavior at biointerfaces
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CHAPTER SEVEN

GENERAL DISCUSSION
The biomaterial interface (biointerface) has emerged as a key determinant for directing cellular response and tissue function in the development of tissue engineering and regenerative medicine (TERM) [1–5]. Increasing evidence suggests that biointerfaces within the biological microenvironment not only serve as a structural support for cells but also offer biochemical and biophysical cues to regulate cell fate and tissue development observed both in vitro and in vivo [6,7]. However, understanding biointerface complexity in regulating cellular responses is still in its infancy due to a lack of highly efficient experimental platforms. In this thesis, advanced material interfaces, i.e., multi-parameter interfaces and gradient platforms, have been developed to explore and elicit their interactions with (stem) cells for improving and accelerating the development of high-performance biomaterials. The main finding is that cell-material interactions are so complex that still with efficient analysis approaches developed here, huge technological challenges are ahead to really understand and in future times even predict cellular behavior as a consequence of material properties.

In Chapter 2&3, multi-parameter interfaces (i.e., stiffness and topography, topography and chemistry) were developed using imprinting lithography and nanoskiving, respectively, which are expected to elucidate cellular responses toward combined surface properties [8].

Cells are selected from different tissues with an intrinsically different stiffness, i.e., osteoblast-like cells from the stiff bone, fibroblasts from the moderately stiff skin, as well as epithelial cells and mesenchymal stem cells from the soft lens and bone marrow, respectively. We found that cells (i.e., osteoblast-like cells, fibroblasts, and mesenchymal stem cells) on aligned nanotopographies (wrinkles and nanowires) were more oriented than on the smooth counterparts. On the same wrinkles, epithelial cells were much less sensitive and did not align along the wrinkle direction as compared to other cell types, mainly because the wrinkle size (wavelength: 168 nm and amplitude: 937 nm) is too small and not enough to induce epithelial cell orientation as observed from our other experiments (data not shown). Additionally, various mechanical properties on aligned nanotopographies significantly affected the orientation of osteoblast-like cells, probably because osteoblasts are well-known to be very sensitive to material mechanical cues [9,10].

Osteoblast-like cells on the hydrogel wrinkle surface were more oriented after 2 days, but equal after 5 days compared to that on the PDMS wrinkle surface. The elongation of osteoblast-like cells had the same trend with cell alignment after 2 and 5 d of incubation. However, the orientation and elongation of fibroblasts and epithelial cells on wrinkle surfaces were not influenced by different material stiffness. In general, cells are more elongated on directional topographical surfaces as compared to those on the planar surfaces. However, it has to be noted that this depends on the cell-type and the range of feature sizes as larger sized topographies are needed to influence epithelial cells. We observed a positive correlation between cell orientation and elongation.

In addition to the effect of cell morphology, subcellular structures, e.g., focal adhesions, can be significantly influenced by surface topography and stiffness. On flat surfaces, the focal adhesion area/cell of osteoblast-like cells on the PDMS displayed more than that on the hydrogel; In contrast, the focal adhesion area/cell of epithelial cells on the PDMS showed less than that on the hydrogel. These results are in line with our initial hypothesis that osteoblast-like cells would respond more to the hard substrates and epithelial cells would respond more to the soft substrates. However, the trend of focal adhesions on soft and hard wrinkle surfaces were opposite to the results on flat surfaces and did not match with our initial hypothesis.
Importantly, these cell responses are closely related to cell function and differentiation as well as even tissue development [9]. Further, we measured the metabolic activity and functional protein expression on the molecular level. For cell metabolic activity tested by the XTT assay, after 2 and 5 days, we found that the metabolic activity of osteoblast-like cells on flat PDMS surfaces was less than that on hydrogel flat surfaces; In contrast, the metabolic activity of osteoblast-like cells on wrinkle PDMS surfaces was higher than that on wrinkle hydrogel surfaces. The metabolic activity of fibroblasts on soft wrinkles was higher compared to on hard wrinkles. The level of metabolic activity of epithelial cells on PDMS wrinkled surface was the highest compared to on other surfaces. Importantly, both ALP expression and collagen I production of osteoblast-like cells were more on the hard materials after 5 d, which is consistent with their natural microenvironment. The soft wrinkle surfaces caused fibrosis generation, because of the overexpression of collagen I and α-SMA for fibroblasts and epithelial cells, respectively. Therefore, material topography and stiffness have a significant effect on cell behaviors and functionalization.

In addition to the mechanical properties of the material, chemical properties play an important role in cell response. While in the previously mentioned study directional topography was combined with mechanical properties, directionality itself can also be a multi-parameter properties, i.e. offering a cell the same mechanical/chemical topographical cue but in different directions simultaneously. It was found that the organization or orientation of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) on glass substrates with directional Au nanowire arrays was greatly enhanced by introducing stem cell-material affinity differences. Interestingly, when the angle of the Au nanowires on the glass was increased from 0° to 90°, hBM-MSC arrangement exhibited a transition from a unidirectional distribution induced by a vector response to a bimodal polarization pattern. The degree of cell vector response decreased with increasing nanowire angles from 0 to 90° where the 90° angle between wires resulted in a loss of vector response and displayed a bimodal directionality. These findings illustrate that even one parameter or one type of physical cue can still be used to direct cellular behavior and that cells are susceptible even for these kinds of delicate influences. Therefore, the importance of multi-parameter contributions was investigated by using newly developed complex interfaces, which plays a critical role in TERM. In brief, two parameters combined are different than the two individually joint and perhaps in some cases two are better than one.

It is well-demonstrated that biomaterial surfaces with one-/two-parameter provide pivotal signals to regulate (stem) cell behavior and not always in a predictive manner [11–18]. Therefore, it is logical to identify optimal surface characteristics for controlling cellular response and accelerating the design of biomedical devices applied in TERM. In Chapter 4-6, masked plasma oxidation treatment was used to prepare PDMS gradient surfaces with single or double features for identifying optimal cell response in a high-throughput screening (HTS) manner [16,17]. PDMS was selected because of its cost-efficiency, nontoxic, inert, easy to process properties, FDA approved and its ubiquity as an implantable biomaterial and tissue engineering scaffold [18,19]. The approaches developed here are simple, cost-efficient, and highly reproducible, do not require significant chemical or technical background or complicated devices or clean room facilities, and do not have the residual toxicity of initiators. Importantly, as compared to the cell results from discrete substrates, our work in Chapter 4 have exemplified single gradient surfaces as an accurate
and efficient high-throughput screening tool to assess (stem) cell behavior and function and identify the optimal material parameter for guiding critical cell response. As is well-known, tissue repair and regeneration require scaffolds with proper material and performance. For instance, inorganic biomaterials (e.g., metals, metal oxides, bioceramics, bioglass, etc.) are often used for orthopedic, orthodontic, and dental applications, because of their excellent mechanical properties. Natural polymers (such as collagen, gelatin, hyaluronic acid and so on) are generally selected for soft tissue development due to their excellent cell attachment and biological interaction. Generally, every biomaterial has particular physicochemical features, including chemistry, wettability, stiffness, charge and so on. The optimal cell response towards topography, identified from PDMS-based gradient surfaces, is not necessarily the same when other biomaterials are used. Therefore, it is logical to translate the gradient structure to other biomaterials, which provide insights in combined effects of materials chemistry and topography. We developed new strategies (i.e., metal deposition or imprinting lithography) to translate PDMS-based wrinkle gradients to other biomaterials, such as inorganic biomaterials (e.g., Ti/TiO\textsubscript{2}, Cr/CrO\textsubscript{3}, and Al\textsubscript{2}O\textsubscript{3}) and nature and synthetic polymers (e.g., collagen, poly (lactic acid), poly(lactic-co-glycolic acid)) of which the latter ones have been developed by other PhD-students within the group. Particularly, for inorganic biomaterials, it is a huge technological challenge to cost-efficiently prepare topographical gradients due to their inherent material properties. For the first time, biomimetic topographical gradients of various clinically relevant inorganic biomaterial interfaces were developed via a combination of masked plasma oxidation and metal deposition-oxidation methods. The gradient platform system developed in Chapter 5 enables condensation of experiment quantities vastly that would generally need thousands of discrete inorganic biomaterial topographies to an individual substrate. In addition, cell behavior on our gradient surfaces was observed by automated fluorescence microscopy (TissueFAXS) in a high-throughput manner, which enabled the observation of the surfaces as a whole. The gradient platforms containing both wrinkles and materials chemistry can generate synergistic effects on the response of hBM-MSCs, indicating that we need to apply a screening to assess optimum conditions for both current and new biomaterials. Compared with the unidirectional single gradients developed in Chapter 4&5, the enhanced HTS platform designed in Chapter 6 is the orthogonal double gradient. In this case, surface stiffness and wettability vary independently and continuously in perpendicular directions within a single sample. Importantly, a true HTS platform system was developed using the novel orthogonal double gradient surface, automated imaging technology (TissueFAXS), and efficient analysis software (TissueQuest), which can elicit the relationship between biomaterial properties and biological performance and accelerate multiscale design and optimization of biomaterial properties. As compared to the orthogonal double gradient represented by a plane in the schematic diagram (Figure 1), a uniform sample with fixed material properties represents one single point; A unidirectional gradient surface displays a line parallel to the border; The unidirectional double gradient shows a vector line made by two parameters (reported in previous PhD-work by P.T. Kühn). Significantly, an orthogonal double gradient would output exponentially more information than the uniform sample and unidirectional gradients. Importantly, if only the unidirectional gradients would be employed, interesting cell responses would have remained unidentified as demonstrated in Chapter 6. In addition to the orthogonal stiffness and wettability gradient, it is possible to create orthogonal double gradients with
different combinations (e.g., wettability and topography, stiffness and topography) using the shielded plasma strategy developed in Chapter 4&6. All these gradient platforms would uncover the effects of all three parameters towards cell responses and are currently being explored. Expectantly, the big data obtained from HTS, named “materiobiology[7] genome”, could in the future be utilized as input for computer models to simulate and generate the accurate prediction of cell response as a function of biomaterial parameters.

Figure 1. The representative illustration of the stiffness and wettability combinations obtained by a uniform sample, a unidirectional single gradient, a unidirectional double gradient and a orthogonal double gradient.

For exploring cell and biointerface interactions, in addition to cell behaviors studied in this thesis, HTS platform system based on gradient surfaces developed here could be utilized to determine the optimal material parameters for cell function and particularly stem cell differentiation. In other words, the platform could be utilized to train cells. This key information may output enhanced material-induced stem cell differentiation protocols, which would be a valuable tool to predict and control stem cell fate. For cell migration, many eukaryotic cell types can respond to gradients with physical and/or chemical features of the material substratum to study directed cell migration (e.g., topotaxis [25], chemotaxis [26], and durotaxis [27]), which could offer new insights into orchestrating directed cell migration during wound healing, immune responses, tissue repair and collective cancer cell invasion. The single and double gradient surfaces developed here provide an ideal platform to explore complex cellular directed migration as a consequence of multiple physical surface parameters.

Although the excellent HTS system has been established in this thesis, it still remains a huge challenge to collect and analyze the big data efficiently and accurately. So far, the HTS strategies have mainly focused on analyzing simple biological performances (e.g., cell adhesion, spreading, viability or metabolic activity), or fixing cells for subsequent analysis of functional markers using the fluorescent microscope. If the HTS platforms could combine with high-content characterization tools (e.g., microfluidics and assay) to detect
cell-material interactions, they will become more valuable and powerful. Microfluidics offer important advantages over conventional analysis systems, e.g., integration of sensors for direct readout, higher reliability and the possibility to enhance throughput of screening by utilizing parallelization, multiplexing and automation [28,29]. Microfluidics-based HTS platforms may result in more breakthrough discoveries not only in basic research but also related to clinical translation. In addition, grad-wellplates (gradient surfaces integrated into commercial well plate technology) could be developed, which consists of a standard well plate with bottoms made from PDMS gradients. The grad-wellplate offers several key merits compared to the prior art, including preventing cross-communication of cells and cross-contamination of soluble factors, incorporating high throughput assays such as ELISA, as well as allowing for robotic liquid handling and implementation of multi-well plate-based instrumentation. Importantly, the grad-wellplate enables straightforward studying of synergetic effects of drugs/biomolecular (gene, protein, enzyme) and material properties, to identify the optimal conditions. Due to the dynamic feature of natural ECM microenvironment, smart biomaterials are being designed and developed that can temporally and spatially change their properties in a controlled manner. Therefore, it is expected that combinatorial HTS platforms with real-time detection and analysis systems will be developed for deeper exploring of dynamic interactions between cells and biomaterials over time, which could significantly advance the field of biomaterials. For advanced TERM, it is necessary to have a comprehensive understanding for the combinatorial effects of biomaterial characteristics and biomolecule on cellular behaviors due to the complex nature of natural cellular microenvironment mention in the General Introduction. HTS platforms can be utilized to screen cell and biomaterial interactions to identify optimal conditions of biomaterials for specific target applications. Most importantly, the true success of HTS platforms will lie on their translation towards commercial uses and the clinic, e.g., drug discovery, toxicology, pharmaceutical science and cellular therapies.
References
