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A semi-interpenetrating network of polyacrylamide and recombinant basement membrane allows pluripotent cell culture in a soft, ligand-rich microenvironment

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ABSTRACT

The physical properties of the extracellular matrix play an essential role in guiding stem cell differentiation and tissue morphogenesis both *in vivo* and *in vitro*. Existing work to investigate the role of matrix mechanics in directing stem cell proliferation, self-renewal, and differentiation has been limited by the poor attachment and survival of human pluripotent cells cultured on soft matrices (Young's modulus E $\lesssim$ 1000 Pa). To address this limitation we developed a protocol for generating semi-interpenetrating networks of polyacrylamide and recombinant basement membrane. Using these materials, we found that human embryonic stem cells (hESCs) remained proliferative and pluripotent even when grown in small colonies and on surfaces ranging in stiffness from 150 to 12000 Pa, spanning the range of tissue stiffnesses likely to be encountered in the embryo. Considerable recent attention has focused on the role of the transcriptional coactivator and Hippo effector YAP in regulating differentiation and cell proliferation both in the early embryo and *in vitro*. We found that while YAP localized to the nucleus on substrates of E $\gtrsim$ 1000 Pa, its localization was heterogeneous on substrates of moduli $\lesssim$ 450 Pa, with predominantly nuclear localization at the colony periphery and mixed cytoplasmic and nuclear localization for cells in the colony interior, a pattern reminiscent of YAP subcellular localization in the inner cell mass (ICM) of the early embryo. In addition, hESC colony dynamics were highly responsive to substrate stiffness, with cells assembling into monolayers, multilayer structures, and transient, hollow rosettes in response to decreasing substrate stiffnesses in the range of 12000 to 150 Pa. We suggest that soft, ligand-rich substrates such as are described here provide a promising means of recapitulating aspects of early mammalian development that are otherwise inaccessible, and more broadly may be useful in the derivation of complex tissues from pluripotent cells in an *in vitro* setting.

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1. Introduction

The ability of pluripotent stem cells to differentiate into any adult cell lineage presents a significant opportunity for scientific study and regenerative medicine. Accordingly, the mechanisms controlling self-renewal and differentiation of these cells both *in vivo* and *in vitro* are of great interest. For differentiated cell types, extracellular substrate stiffness and stress relaxation properties influence cell spreading, proliferation, migration, and matrix invasion [1,2]. In particular, for mesenchymal stem cells matrix stiffness can influence differentiation, biasing cells on a physiologically soft substrate (E $\lesssim$ 1000 Pa) toward adipogenesis and those on a physiologically stiff substrate (E $\gtrsim$ 25000 Pa) toward osteogenesis [3]. Substrate stiffness is also found to play a role in enhancing the proliferation and self-renewal of muscle satellite cells [4,5] and in neural stem cell differentiation [6]. More broadly, additional substrate properties, including topography and extracellular matrix protein spacing, have also been found to influence cellular morphology, organization, and fate specification in a variety of self-renewing populations including mesenchymal stem cells [7,8], pluripotent cells [9,10], and epidermal tissues [11].

Current evidence likewise suggests that matrix material properties can strongly influence pluripotent stem cell proliferation,

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self-renewal, and organoid formation. Substrate stiffness has been shown to influence the morphology and dynamics of colonies of pluripotent cells [12,13], and the differentiation of pluripotent cells into mesodermal lineages [14]. Recent work demonstrates that colonies of mouse and human pluripotent cells encapsulated in Matrigel, a soft protein hydrogel consisting mainly of basement membrane components, spontaneously form hollow spheres reminiscent of the proamniotic cavity [15–18]. However, the underlying mechanisms by which matrix material properties influence pluripotent stem cell differentiation and tissue morphogenesis, either *in vitro* or *in vivo*, remain poorly understood.

The Hippo pathway and its downstream transcriptional effector YAP (Yes-associated protein 1) has emerged as an important mechanically responsive signaling pathway involved in tissue development and homeostasis [19]. Mechanistically, a variety of studies support an emerging view of YAP as a mechanical signal integrator that regulates cell proliferation in response to cell-cell contact, substrate rigidity, and cell geometry [20]. YAP is likewise thought to play a key role in early embryogenesis. In particular, previous work indicates that YAP plays an essential role in trophectoderm specification [21]. Additionally, stiffness-dependent changes in YAP activity have been associated with differentiation of human embryonic stem cells (hESCs) into neuronal lineages *in vitro* [22].

The above considerations motivated us to generate simple, robust, and tunable materials that would replicate the physical and biochemical environment present in the early embryo. In contrast to the stiff environment provided by typical *in vitro* culture conditions, pluripotent cells in the early embryo experience soft, ligand-rich environments. Literature precedent suggests that conventional soft biomaterials, for example common preparations of ECM-functionalized polyacrylamide (PA) hydrogels, are poorly suited for hESC culture. In particular, initial studies found that long term human pluripotent cell attachment and survival on soft 2D microenvironments was poor [23]. Related studies overcame attachment limitations by seeding pre-formed embryoid bodies onto hydrogels early in the process of differentiation [22]. More recent work indicated that the limited attachment of pluripotent stem cells to soft (Young's modulus E = 400 Pa) hydrogels could be improved with the incorporation of multifunctional methacrylates, likely due to an increase in the extracellular matrix (ECM) ligand density on the gel surface [13,14].

To address the limited attachment of pluripotent stem cells to soft materials, we sought to increase the concentration of extracellular matrix protein presented at the substrate surface. Because covalent conjugation of protein to a PA hydrogel is limited by the density of available crosslinking sites at the gel surface, we considered whether an alternative method for protein incorporation might yield soft, E $\lesssim$ 1000 Pa hydrogels with sufficiently high ligand densities for robust pluripotent cell attachment. Interpenetrating networks (IPNs) present a promising alternative strategy in that they allow separate control of biochemical and physical substrate properties, and have been used successfully with both collagen and Matrigel in alginate for 3D cell culture [24–26]. Interpenetrating materials are generally formed by the sequential or simultaneous polymerization of intermixed precursor solutions, resulting in polymer networks that are physically interwoven upon polymerization. We therefore hypothesized that an IPN of Matrigel and PA could provide the surface protein densities required for pluripotent cell attachment while allowing independent control of the hydrogel stiffness via the PA network.

In this study we describe a semi-interpenetrating matrix of Matrigel and PA and demonstrate its utility for robust culture of hESCs across a wide range of substrate stiffnesses. Consistent with earlier reports, hESCs maintained pluripotency factor expression on semi-IPNs with Young's moduli $\gtrsim$ 450 Pa [14]. Building on prior work, we observed that hESC pluripotency, survival, and proliferation are maintained on extremely soft, E ~ 150 Pa substrates, even in small colonies of 6–8 cells. Substrate stiffness had a pronounced influence on hESC colony morphology. Clusters of hESCs cultured on E ~ 12000 Pa and 450 Pa surfaces formed monolayer and multilayer colonies, respectively. Additionally, on composite substrates with Young's moduli $\lesssim$ 450 Pa, cells exhibited heterogeneous YAP subcellular localization and a decreased cell proliferation rate in colony interiors, indicative of a role for substrate stiffness and colony geometry on regulating proliferation. Remarkably, colonies cultured on E ~ 150 Pa substrates exhibited complex morphological rearrangements that included the formation of hollow, polarized rosettes. This process of polarization in pluripotent cells is reminiscent of the formation of the proamniotic cavity, a behavior that has been previously reported for hESCs suspended in 3D Matrigel [16,17]. Our findings thus demonstrate that hESCs can replicate the range of structures formed by pluripotent cells in the epiblast (flat sheet), inner cell mass (multicellular mass), and proamnion (hollow sphere) in response to changes in substrate rigidity. We suggest that the ease of use, preparation from commonly available reagents, and tunability make the Matrigel/PA composites described here a potentially useful class of materials for a wide variety of cell biological and tissue engineering applications.

2. Materials and methods

2.1. Preparation of composite substrate

Acrylamide (40% w/v, Fisher BioReagents), bis-acrylamide (2% w/v, Fisher BioReagents), and 10X phosphate buffered saline (PBS, pH 7.4, no Mg/Ca, Quality Biological #119-069-491) stock solutions were diluted in deionized MilliQ-filtered water to yield a polyacrylamide (PA) precursor 2x stock solution of acrylamide and bis-acrylamide in 1X PBS. For this study the 2X PA stock solutions were prepared as follows: (1) E ~ 150 Pa gel: 6% acrylamide, 0.08% bis-acrylamide, (2) E ~ 450 Pa gel: 6% acrylamide, 0.12% bis-acrylamide, and (3) E ~ 12000 Pa gel: 15% acrylamide, 0.2% bis-acrylamide. The PA precursor stock was then passed through a 0.2 μm syringe filter. If protected from light and stored at 4 °C the PA precursor solution is stable for several months. Growth Factor Reduced Matrigel (BD #354230) was provided by the manufacturer at approximately 10 mg/mL and was stored in flash-frozen aliquots at −80 °C. Prior to composite gel preparation, aldehyde-functionalized glass of desired substrate geometry was prepared as previously published [27] with slight modifications. In particular, to facilitate the activation of small coverslips, four changes were introduced: (1) fresh coverslips were laid in a single layer on a Petri dish and cleaned and dried by brief exposure to open flame rather than the soap and ethanol washes, (2) solutions were mixed briefly by gently swirling of the Petri dish without continuous stirring, (3) the concentration of 3-aminopropyltrimethoxysilane (APTES) was increased from 2% to 4% (v/v) in 5 mL of isopropanol, and (4) the concentration of glutaraldehyde was increased from 1% to 2.5% (v/v) in 10 mL of water. After activation, coverslips can be stored at room temperature, covered and protected from light, for up to two months.

If necessary, Matrigel was diluted in cold Dulbecco's Modified Eagle medium (DMEM; Gibco #11885-084) to 2x the final desired concentration before the preparation of composite solutions. For composites used in cell culture, which contained a final Matrigel concentration of 5 mg/mL, dilution was not necessary as stocks were provided at ~10 mg/mL. Matrigel-containing solutions must be prepared, thawed, and kept on ice at all times. Matrigel stock and acrylamide/bis-acrylamide solutions were mixed on ice 1:1 to form

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