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Nanoscale Topography and Chemistry Affect Embryonic Stem Cell Self-Renewal and Early Differentiation

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Abstract

Adherent cells respond to a wide range of substrate cues, including chemistry, topography, hydrophobicity, and surface energy. The cell-substrate interface is therefore an important design parameter in regenerative medicine and tissue engineering applications, where substrate cues are used to influence cell behavior. Thin films comprising 4.5 nm (average diameter) gold nanoparticles coated with a mixture of two alkanethiols can confer hemispherical topography and specific chemistry to bulk substrates. The behavior of murine embryonic stem cells (ESCs) on the thin films can then be compared with their behavior on self-assembled monolayers of the same alkanethiols on vapor-deposited gold, which lack the topographical features. Cells cultured both with and without differentiation inhibitors are characterized by immunofluorescence for Oct4 and qPCR for Fgf5, Foxa2, Nanog, Pou5f1, and Sox2. Nanoscale chemistry and topography are found to influence stem cell differentiation, particularly the early differentiation markers, Fgf5 and Foxa2. Nanoscale topography also affects Oct4 localization, whereas the chemical composition of the substrate does not have an effect. It is demonstrated for the first time that ESCs can sense topographical features established by 4.5 nm particles, and these findings suggest that nanoscale chemistry and topography can act synergistically to influence stem cell differentiation. This study furthers the understanding of the effects of these substrate properties, improving our ability to design materials to control stem cell fate.

1 Introduction

The effects of substrate cues on cell behavior is of considerable interest to the fields of regenerative medicine and tissue engineering, where the cell-substrate interface has been repeatedly shown to affect cell behavior, and is therefore an important design parameter.¹ In recent years, the design of the cell-substrate interface in tissue engineering scaffolds has moved away from a trial and error approach, instead adopting rational design informed by systematic studies of cell response to a number of substrate properties.² This study

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establishes the response of murine embryonic stem cells (ESCs) to substrate nanoscale topography and chemical composition.

Many bulk and interfacial substrate properties are known to affect cell behavior. Chemical groups on a substrate can influence protein adsorption and subsequently modulate cell behavior such as adhesion and differentiation in cell types including pre-osteoblasts and mesenchymal stem cells (MSCs).^{3–5} A combinatorial approach to the investigation of substrate chemistry demonstrated that polymers composed of hydrophobic acrylate monomers could promote colony formation of human ESCs, which was attributed to the moderate wettability of the resulting substrates.⁶ In that study, the cell–substrate interaction was shown to be mediated through vitronectin binding integrins, though there are many ways in which a chemical cell–substrate interaction can occur.⁷ Beyond chemistry, substrate elasticity is another widely studied feature that has been demonstrated to affect cell behavior, with recent interest in guiding stem cell differentiation.^{8–10} Elasticity has also been related to self-renewal, with soft matrices preventing differentiation by mitigating cell spreading.¹¹ These two parameters can be combined and it was recently shown that surface chemistry and elasticity can work synergistically to influence MSC osteogenesis.¹²

In addition to these substrate parameters, materials with topographical features or patterns designed to control cell shape are known to influence cell behavior, independent of biochemical cues. An early study demonstrated that endothelial cells can be switched from growth to apoptosis simply by using micropatterned substrates containing progressively sparser protein islands to restrict cell extension.¹³ Nanometer scale features aimed at guiding cell behavior are of particular interest because of the many biological processes and interactions that occur at that scale. For example, collagen in the extracellular matrix (ECM) supports cells and contains nanoscaled banding, and the diameter of cell surface receptors is on the order of nanometers.¹⁴ One recent study used a combinatorial approach, previously reserved for chemistries, to present many different topographical cues to MSCs and found effects on proliferation and osteogenic differentiation.¹⁵ Many other processes known to be affected by surface nanotopography are key in cell survival and phenotype, including adhesion,^{16, 17} orientation,¹⁸ cytoskeletal organization,¹⁹ self-renewal,²⁰ and differentiation.^{21, 22} These effects have been attributed to a number of signaling pathways, many of which involve integrin clustering as the effectors.^{23, 24}

ESCs are an attractive cell type for regenerative medicine applications. Similar to other adherent cells, they also respond to substrate cues, and it is especially important for their application in regenerative medicine to be able control their differentiation. There have been a few recent studies on the effects of nanoscale topography on ESCs. Mouse ESCs in contact with electrospun fibrous scaffolds differentiated into neural lineages and had enhanced neurite outgrowth,²⁵ while cells cultured on polydimethylsiloxane substrates with nanoscale topography maintained self-renewal.²⁶ Human ESCs seeded on nanoscale grooved substrates underwent neuronal differentiation even in the absence of differentiation-inducing agents,²⁷ and human ESCs on glass substrates with nanoscale roughness underwent spontaneous differentiation.²⁸ On polycarbonate substrates with a near square arrangement of nanopits, human ESCs were predisposed towards mesodermal differentiation.²⁹

Despite recent work in the field, there remains little known about topographical effects in the <10 nm region on any cell type,³⁰ particularly about ESC response.³¹ Furthermore, it is difficult to isolate the effects of only substrate chemistry or topography when many systems employed have these variables changing concurrently. In this study, we used a template-free nanoparticle system, which enabled us to specify the substrate chemical composition and topography in a single step with a simple synthesis and no specialized equipment.³² We fabricated thin films composed of 4.5 nm (average diameter) gold nanoparticles coated in alkanethiols. The alkanethiolate coatings conferred a specific chemical composition to the nanoparticles and the subsequent thin films, allowing us to tailor substrate hydrophobicity.³³ We also made a series of alkanethiolate self-assembled monolayers on vapor-deposited gold, in order to create substrates of the same nominal bulk chemistry as the nanoparticle thin films, but without the nanoparticle topography. These tailorable surfaces of varying chemical composition and topography enabled the exploration of their effects on feeder-free murine ESCs. For the first time, it was shown that ESCs can sense topographical features under 5 nm, and the effect of changing the chemical composition of the substrate was also established.

2 Results and Discussion

One of the major aims of tissue engineering is to design materials that can control cell behavior and ultimately drive the cells to create a therapeutically relevant tissue. While there are many studies demonstrating the potential for using substrate cues to generate this control, there remains a dearth of information on the effects of nanometer scale cues and, in particular, their effect on ESCs. In this study, materials science and biology were combined to fabricate two classes of materials to allow us to study the effects of nanoscale chemical composition and topography on murine ESCs. Gold nanoparticles with stoichiometrically defined shell chemical composition were synthesized and covalently bound to glass slides to create closely packed nanoparticle films (“nanoparticle thin films”) of defined topography and tailorable chemistry. The effect of nanoscale topography alone was isolated by fabricating self-assembled monolayers on vapor-deposited gold (“flat SAMs”) composed of the same nominal alkanethiol composition as in the nanoparticle thin films, thereby holding bulk chemistry constant. Similarly, the effects of nanoscale chemistry alone were studied by preparing both the nanoparticle thin films and the flat SAMs with varying ratios of hydrophobic and hydrophilic ligands, while holding substrate topography constant. In order to characterize cell behavior, the expression of important stem cell markers was observed at the transcript and protein level.

2.1 Substrate Fabrication

In order to form domains of varying hydrophobicity on vapor-deposited gold, the substrates were incubated for 1 week in ethanolic alkanethiol (6-mercapto-1-hexanol (MH) and 1-octanethiol (OT)) solutions of 5:1, 2:1, 1:1, 1:2, and 1:5 molar ratios (Figure 1). To create the same nominal bulk SAM chemical composition with topographical features of approximately 4.5 nm, monodisperse nanoparticles were synthesized with SAM coatings in the same molar ratios of MH and OT, and a layer-by-layer assembly technique was used to build up thin films of closely packed particles. It has previously been reported that this

technique creates substrates with complete coverage, no pin-hole defects, and statistically similar roughness.³³ A considerable advantage of using SAMs on both gold nanoparticles and vapor-deposited gold is that they do not reorganize or denature and their chemical composition can be easily tailored.³⁴

A minimum of three batches of each nanoparticle with defined SAM chemistry (5:1, 2:1, 1:1, 1:2, and 1:5 molar ratios of MH:OT) were synthesized. Based on their UV-vis spectrum absorbance and analysis of their size distribution, one batch of each type was used to fabricate the nanoparticle thin films used in further experiments. Figure 2a–c demonstrates a representative batch (2:1 MH:OT) of particles. Particle diameter was measured from transmission electron micrographs (TEM; Figure 2a) of the particles dried from solution, which was then plotted as a frequency distribution (Figure 2b) and used to calculate particle polydispersity. The UV-vis spectrum (Figure 2c) shows a surface plasmon peak of approximately 510 nm, characteristic of gold nanoparticles this size. Figure 2d lists the mean particle diameter, polydispersity index (PDI), and plasmon peak of each batch of nanoparticles used for thin film fabrication. Importantly, the variations of particle diameter between nanoparticles of different chemistries (ranging from 5:1 to 1:5 MH:OT) were not statistically significant ($p > 0.05$), as plotted in Figure 2e.

2.2 Quantitative PCR of Stem Cell Markers

In order to evaluate how nanoscale chemistry and topography affect the genes crucial in stem cell maintenance, we used quantitative polymerase chain reaction (qPCR) to probe for mRNA levels of Pou5f1 (Oct4), Nanog, and Sox2, markers of ESC pluripotency and self-renewal. Cells were seeded on gelatin- and gold-coated glass as reference substrates, nanoparticle thin films of varying chemistry, and self-assembled monolayers (flat SAMs) of the same nominal bulk chemical compositions as the nanoparticle thin films. Six days after seeding and having been cultured in the absence of leukemia inhibitory factor (LIF), a differentiation inhibiting cytokine, cells on all substrates had diminished expression of Pou5f1, Nanog, and Sox2 compared with cells cultured in the presence of LIF (data not shown for the latter). Not surprisingly, in the absence of LIF, the highest expression of these genes, which are central to stem cell maintenance, was observed in cells cultured on gelatin-coated glass, a standard substrate used for ESC expansion in vitro (Figure 3). For all three stem cell markers, nanostructure had a statistically significant effect on transcript expression ($p < 0.05$, comparing the group of flat SAMs to nanoparticle thin films), but despite the statistical significance, the changes were very small. For Pou5f1 (Figure 3a) and Sox2 (Figure 3c), expression was unaffected by changing the substrate chemistry. For Nanog (Figure 3b), there were some differences that could be attributed to chemistry and the interactive p-value was < 0.05 , implying some combined effects of substrate chemistry and nanotopography.

2.3 Immunofluorescence of Oct4

To better understand how Oct4, a critical ESC transcription factor, was affected by the nanoparticle substrates, immunofluorescence was performed with an antibody against Oct4 and cells were further stained with wheat germ agglutinin (WGA), a lectin that labels the cell membrane to help visualize the cells, and DAPI to stain the nuclei. There were few

differences noted in colony shape or size, or Oct4 staining across the nanoparticle thin films or flat SAMs of varying chemistry, providing more evidence that the substrate chemistries in this study did not play a significant role in affecting Oct4. However, we observed a marked difference in the Oct4 staining when comparing nanoparticle thin films to flat SAMs of the same nominal bulk chemistry. Figure 4 shows a representative comparison between 1:1 MH:OT nanoparticle thin films and 1:1 MH:OT flat SAMs. After 6 d in culture either with or without LIF supplementation, Oct4 staining was only present diffuse in the cytoplasm in cells cultured on nanoparticle thin films (Figure 4b,e). In contrast, the protein was located in the nuclei of cells cultured on the flat SAMs (Figure 4c,f), similar to cells cultured on gelatin-coated glass (Figure 4a,d). This result demonstrates that while the population-wide transcript expression of Pou5f1 was only slightly affected by nanoscale topography, Oct4 protein expression clearly indicates that cells were undergoing differentiation. Similar cytoplasmic Oct4 expression has been observed in cells undergoing spontaneous differentiation in the absence of LIF.³⁵ It is interesting that in our system, it appears the flat SAMs are mitigating this downregulation of nuclear Oct4.

2.4 Quantitative PCR of Early Differentiation Markers

Following our finding that Pou5f1 and Sox2 expression were affected by nanoscale topography and Nanog was affected by both nanoscale topography and chemistry, two markers of early differentiation events were examined with qPCR to further investigate the role of these substrate properties on ESC differentiation. Foxa2, a marker of early endoderm commitment, was highly affected after 6 d in culture without LIF (Figure 5a). It was upregulated on all nanoparticle thin films and downregulated on all flat SAMs. Interestingly, there were differences noted between the nanoparticle thin films and the flat SAMs of the same nominal bulk chemistry ($p < 0.05$) but there were also differences noted between the surfaces of similar topography but varying chemistry ($p < 0.05$), suggesting both nanoscale topography and chemistry play a role in endoderm specification. We recently found that substrates with 400 nm topographical features mitigate endoderm specification.³⁶ However, there is growing evidence that features of different sizes can have opposite effects on cell fate, which might explain how substrates with 4.5 nm features resulted in an upregulation of Foxa2, a key endoderm marker.²⁴

Fgf5, a marker of differentiation into primitive ectoderm, which then contributes to all germ layers, is an indicator of early differentiation events and is therefore useful for determining how substrates affect stem cell differentiation. Fgf5 was highly upregulated on all substrates after 6 d when LIF was removed from the culture medium (Figure 5b). To obtain some information about the temporal pattern of Fgf5 expression, qPCR was performed two days earlier, at day 4. A nearly identical trend for both time points was observed (data not shown) but at the earlier time point, a lower degree of upregulation was measured. This suggests the cells are losing their stem cell phenotype and as time progresses, more are differentiating. Fgf5 was significantly higher on all nanoparticle thin films compared with a flat SAM of the same bulk chemistry ($p < 0.05$), indicating that it was affected by topography. However, expression was also affected by changing the bulk chemical composition, and this was particularly apparent on the nanoparticle thin films. Interestingly, Fgf5 expression does not correspond to the changes in bulk hydrophobicity as the nanoparticle chemistry changes

from 5:1 to 1:5 MH:OT, perhaps indicating a combined role for chemistry and topography in influencing this key marker. Hydrophobicity has previously been reported to improve ESC differentiation; however, in that study, cells went through an embryoid body (EB) intermediary and hydrophobicity was employed to limit the EB size.³⁷ A number of studies have suggested that substrate properties may act by affecting morphology, which subsequently directs differentiation^{11, 38} but in our study, the ESCs were typically spherical and did not exhibit morphological changes related to either chemistry or topography.

3 Conclusion

A better understanding of how stem cells respond to substrate cues is crucial for their use in regenerative medicine and is also an interesting scientific question. This study demonstrates for the first time that ESCs are able to sense topographical features provided by gold nanoparticles 4.5 nm in diameter, and these cues caused an upregulated of endoderm and primitive ectoderm markers. The cells responded to substrate chemistry in non-linear manner, suggesting a possible synergistic role of these substrate cues for influencing cell behavior.

4 Experimental Section

Nanoparticle Synthesis

Monolayer-protected metal nanoparticles were synthesized using a previously published modified Stucky method.^{33, 39} All of the reagents were purchased and used as received from Sigma-Aldrich (UK). Chloro(triphenylphosphine)gold(I) (1×10^{-3} M; 99%) was dissolved in dichloromethane (80 mL) heated to 55 °C in a water bath. After 10 min, alkanethiols (6-mercapto-1-hexanol (MH; 97%) and 1-octanethiol (OT; 98%), 1×10^{-3} M total) were added in either 5:1, 2:1, 1:1, 1:2, or 1:5 molar ratios and stirred for 10 min, after which borane tert-butylamine complex (10×10^{-3} M, 97%) was added and the flask was capped and stirred for 1 h at 55 °C. After cooling to ambient temperature, diethyl ether (Et₂O; 80 mL) was added to precipitate the particles overnight at ambient temperature. The supernatant was removed and the particles were collected, washed in Et₂O, dissolved in a small volume of ethanol, reprecipitated in Et₂O, and washed again. The particles were dried in air and transferred to a glass vial for storage.

Nanoparticle Thin Film Surface Fabrication

Glass chamber slides (Nunc, Germany) were oxygen plasma treated under 0.2 mbar and 50 W for 1 min (Plasma Prep 5, GaLa Instrumente, Germany). They were vapor-silanized in a light vacuum desiccator with a dish of 3-mercaptopropyl(trimethoxysilane) (>97%; Sigma-Aldrich, UK) for 2 h. The slides were washed profusely with ethanol, dried, and incubated in a methanolic nanoparticle solution (0.25 mg mL^{-1}) overnight at ambient temperature. Slides were washed profusely in ethanol, dried, and incubated for 1 h in ethanolic 1,9-nonanedithiol (5×10^{-3} M, 95%; Sigma-Aldrich). The alternate incubation of dithiol followed by nanoparticles was repeated to obtain seven layers of particles.

“Flat SAMs” on Au-Coated glass

Self-assembled monolayers (SAMs) of the same alkanethiols used in nanoparticle synthesis were generated on gold substrates prepared by electron beam physical vapor deposition (EB-PVD) in an Auto 306 Coating Unit (Edwards, UK). Cleaned microscope slides were ozone plasma treated before being placed in the evaporator where they were coated with a 5 nm layer of chromium followed by 80 nm of gold. A copper mask was used to limit the areas of the metal evaporated to 1 cm². The substrates were transferred directly from the evaporator into ethanolic solutions (10×10^{-6} M) of mixed alkanethiols in the same ratios as for the nanoparticle synthesis and incubated for 1 week. They were rinsed profusely in ethanol and stored in air until use. Gold substrates without any self-assembled monolayers were used as a reference material.

Transmission Electron Microscopy

Characterization of particle size distribution was performed with transmission electron microscopy (TEM). 5 μ L of an ethanolic nanoparticle solution (0.125 mg mL^{-1}) was pipetted on a 300 mesh carbon-coated copper TEM grid (Agar Scientific, UK), wicked away, and allowed to dry in air for at least 30 min. Microscopy was performed on a TEM (JEOL 200CX, Japan) at an accelerating voltage of 200 kV and 60 000–80 000 $\times$ magnification. At least 300 particles were measured from micrographs using ImageJ software (available at <http://rsbweb.nih.gov/ij/>) and the size distribution was plotted as percent composition versus diameter. Polydispersity index (PDI) was calculated as M_w/M_n , where M_w is the weight average diameter, and M_n is the number average diameter.

Cell Culture

ES-E14TG2a (ATCC CRL-1821 from LGC Standards, UK) were subcultured on 0.1% (w/v) bovine gelatin- (Sigma–Aldrich, UK) coated polystyrene dishes (Nunc, Germany) in ESC medium (DMEM supplemented with 10% (v/v) FBS, L-glutamine (2×10^{-3} M), non-essential amino acids (100×10^{-6} M) (all from Invitrogen, UK), β -mercaptoethanol (100×10^{-6} M, Sigma–Aldrich) and LIF (1000 U mL^{-1} ; leukemia inhibitory factor, Millipore, UK). On day 0, cells were seeded at $7500 \text{ cells cm}^{-2}$, and starting on day 1, they were fed daily either with or without supplemented LIF until they were collected for analysis.

RNA Extraction

Cells were lysed in 100 μ L Buffer RLT (Qiagen, Germany) supplemented 1% (v/v) β -mercaptoethanol and stored at -20°C for up to 2 weeks. An additional 250 μ L was added and lysates were thawed on ice. Lysates were homogenized using a QiaShredder column and RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA quantity and quality were assessed on a NanoDrop 2000c (Thermo Scientific, USA).

cDNA Synthesis and Quantitative Real-Time PCR (qPCR)

100 ng RNA was reverse transcribed using SuperScript III VILO (Invitrogen) in a 20 μ L reaction according to the manufacturer's instructions. Real-time PCR was performed on a Rotorgene 6000 (Corbett, Qiagen) with primers for Fgf5, Foxa2, Nanog, Pou5f1, and Sox2,

with Gapdh (Quantitect, Qiagen) as an internal control using EXPRESS GreenER (Invitrogen) according to the manufacturer's instructions. Thermocycling was performed as follows: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 60 s at 62 °C, then a melt curve from 60–95° to ensure a single amplicon. Relative gene expression was quantified using the 2^{-CT} method with day 0 ESCs on gelatin as the reference sample, and was plotted as 2^{-CT-1} for values >1 (corresponding to upregulation, such that the baseline expression is 0) and $-1/(2^{-CT})$ for values <1 (corresponding to downregulation, such that downregulation is presented on a negative scale).

Immunofluorescence and Confocal Microscopy

All reagents were purchased from Sigma-Aldrich (UK) unless specified otherwise. To detect proteins of interest, cells were fixed in fresh 3.7% (w/v) paraformaldehyde for 15 min, washed in PBS, permeabilized for 10 min in 0.25% (v/v) Triton X-100 in PBS, washed in PBS, and blocked in 5% (v/v) goat serum in PBS/T (PBS with 0.1% (v/v) Tween-20) for 20 min. Samples were incubated in rabbit anti-Oct4 (1/1000; Abcam, UK) and WGA-Alexa633 (wheat germ agglutinin, 1/1000; Invitrogen, UK) in 2.5% (v/v) goat serum in PBS/T for 2 h at ambient temperature in the dark. They were washed in PBS/T and incubated in Alexa Fluor-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1/2000; Molecular Probes, Invitrogen) in 2.5% (v/v) goat serum in PBS/T for 1 h at ambient temperature in the dark then washed in PBS/T. To counterstain the nuclei, samples were incubated for 1 min in 300×10^{-9} M DAPI (4',6-diamidino-2-phenylindole dilactate; Invitrogen). They were coverslipped using ProLong antifade gold reagent (Invitrogen), left to dry for 24 h, and stored at 4 °C prior to confocal microscopy. Imaging was performed on an inverted confocal microscope (SP5 MP/FLIM, Leica, Germany) with a 63X oil objective under standardized gain and offset. Line averages were used. For z-stacks, each stack was 0.84 μ m thick.

Statistical Analysis

Statistical analysis between samples was performed using Student's t-test and between groups was performed using ANOVA, with $p < 0.05$ considered significant.

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Figure 1.

Two immiscible alkanethiols (6-mercapto-1-hexanol (MH) and 1-octanethiol (OT)) were used in the fabrication of self-assembled monolayers ("flat SAMs") and thin films composed of gold nanoparticles with an alkanethiolate shell ("nanoparticle thin films"). The flat SAMs were self-assembled on a vapor-deposited gold-coated glass slide and formed alkanethiolate domains of >5 nm. The nanoparticle thin films were composed of gold nanoparticles synthesized by a modified Stucky method, dried, and solubilized in methanol. The 4.5 nm nanoparticles were covalently attached to a silanized glass slide by a layer-by-layer technique with alternating incubations of a dithiol crosslinker and the nanoparticle solution. The atomic force microscopy (AFM) image demonstrates the substrate after the thin film was built up (scale: 500 nm). The two types of substrates had different topography (domains of either >5 nm or ~4.5 nm for the flat SAMs and nanoparticle thin films, respectively) and tailorable bulk chemistry, governed by the stoichiometric ratios of MH and OT used in the synthesis.

Figure 2.

The size and polydispersity of 2:1 MH:OT nanoparticles are shown as a representative sample along with parameters of all batches of nanoparticles used. The ratio refers to the molar ratio of the alkanethiolate self-assembled monolayer composed of 6-mercapto-1-hexanol (MH) and 1-octanethiol (OT) surrounding the gold nanoparticle. A transmission electron micrograph, a), was taken to measure the particle size and distribution (scale: 20 nm), which is plotted in b). Absorbance in the UV-vis spectrum, c), was taken of particles dissolved in ethanol. These data are shown for all batches of particles used in subsequent thin film fabrication in d), and a box and whisker plot of nanoparticle size indicates similar size and polydispersity in e). The horizontal line inside the box indicates the median, the dark circle indicates the mean, the box spans the 25th-75th percentile, and the whiskers span the 5th-95th percentile ($N > 300$ particles, $p > 0.05$, indicating no statistically significant difference in particle diameter of the different types of nanoparticles).

Figure 3.

Quantitative PCR was used to determine the relative transcript levels of a) *Pou5f1* (Oct4), b) *Nanog*, and c) *Sox2* six days after seeding ESCs, cultured without LIF supplementation. The cells were grown on either self-assembled monolayers on gold substrates, “flat SAMs,” (gray bars) including gelatin-coated glass (“Gel.”) and gold-coated glass (“Au”) as reference materials, or thin films of densely packed monolayer-protected gold nanoparticles, “nanoparticle thin films” (black bars). The ratios refer to the molar ratio of 6-mercapto-1-hexanol (MH) and 1-octanethiol (OT) within the self-assembled monolayers. For *Pou5f1*, expression was downregulated in all samples relative to day 0 ($p < 0.05$) though to a slightly greater degree in the flat SAMs than on the nanoparticle thin films ($p < 0.05$). The chemical composition of the surface had no effect on *Pou5f1*. For *Nanog*, both the nanostructure and chemistry of the surface played a role in transcript expression (interactive $p < 0.05$). For *Sox2*, the substrate nanostructure was also found to affect transcript expression ($p < 0.05$). Bars represent mean $\pm$ standard deviation ($N = 3$ independent experiments, performed in technical triplicates), relative to *Gapdh* as an internal control and day 0 cells cultured on gelatin-coated glass as the baseline of 0. Statistically significant ($p < 0.05$) differences between flat SAMs and nanoparticle thin films of the same nominal chemistries are denoted with an asterisk (*).

Figure 4.

Immunofluorescence demonstrates different localization of Oct4, a protein crucial for stem cell identity in cells on nanoparticle thin films. ESCs were cultured on either self-assembled monolayers on gold substrates, “flat SAMs,” (including gelatin-coated glass and gold-coated glass as reference materials), or thin films of densely packed monolayer-protected gold nanoparticles, “nanoparticle thin films.” Both substrates were coated with a series of 6-mercapto-1-hexanol (MH) and 1-octanethiol (OT) self-assembled monolayers.

Immunofluorescence shows the nucleus (DAPI, blue), membrane (WGA (wheat germ agglutinin), red) and Oct4 (green) expression in ESCs seeded on gelatin, 1:1 MH:OT nanoparticle thin films (representative of all nanoparticle samples) and 1:1 MH:OT flat SAMs (representative of all flat SAM samples) either with LIF (a–c) or without LIF (d–f). The ratio refers to the molar ratio of MH:OT within the self-assembled monolayers. In both cases, the nuclear and membrane staining appear similar. Oct4, however, is located differently in cells on the nanoparticle substrates (b,e) where it is staining in the cytoplasm rather than the nucleus. Scale: 20 μm .

Figure 5.

Quantitative PCR was used to determine the relative transcript levels of a) *Foxa2* (endoderm specification) and b) *Fgf5* (primitive ectoderm, early differentiation) 6 d after seeding ESCs cultured without LIF supplementation. The cells were grown on either self-assembled monolayers on gold substrates, “flat SAMs,” (gray bars) including gelatin-coated glass (“Gel.”) and gold-coated glass (“Au”) as reference materials, or thin films of densely packed monolayer-protected gold nanoparticles, “nanoparticle thin films” (black bars). The ratios refer to the molar ratio of 6-mercapto-1-hexanol (MH) and 1-octanethiol (OT) within the self-assembled monolayers. For both genes, the nanotopography of the substrate was important ($p < 0.05$ for the differences between gray and black bars) but there was also an important influence of the substrate chemical composition ($p < 0.05$ for the differences among gray, and among black bars). Bars represent mean $\pm$ standard deviation ($N = 3$ independent experiments, performed in technical triplicates), relative to *Gapdh* as an internal control and day 0 cells cultured on gelatin-coated glass as the baseline of 0. Statistically significant ($p < 0.05$) differences between flat SAMs and nanoparticle thin films of the same nominal chemistries are denoted with an asterisk (*).