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Topographically and Chemically Modified Surfaces for Expansion or Differentiation of Embryonic Stem Cells

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1. Introduction

Cells in the body respond to signals emanating from the interaction with neighbouring cells, from the surrounding extracellular matrix, and from soluble signalling molecules. These signals are perceived by the cell as topographical, mechanical, and chemical cues (Martínez et al., 2009). One example of a defined physical topography embedded in the extracellular matrix (ECM) is provided by the collagen structure. Collagen molecules are about 300 nm long and 1.5 nm wide and can form fibrils up to tens of microns in length and with a diameter of 260-410 nm (Bettinger et al., 2009). Furthermore, the spatial organisation and density of ECM is characteristic of individual tissue types (Martínez et al., 2009), and these natural structures serve to guide interacting cells in terms of cell morphology, migration, and function. Among mechanical cues, tissue elasticity has been found to vary from 0.1-0.3 kPa for embryonic stem (ES) cells and endoderm through increasing values for various differentiated cells to more than 30 kPa for demineralised bone (Reilly & Engler 2010). Such mechanical cues are also recognised in early development with cell-cortex tension being involved in germ-layer sorting (Krieg et al., 2008), indicating that mechanical signals have implications for the decision of stem cell fate. Chemical cues from the surroundings are provided from biochemical mixtures of soluble chemokines, cytokines, and growth factors, as well as insoluble receptor ligands and ECM molecules. Stem cells reside in three-dimensional tissue-specific stem-cell niches were the cells are exposed to a controlled microenvironment including both chemical, mechanical, and topographical cues from the surrounding matrix and cells (Reilly & Engler 2010).

For the development of cell-based therapies, where growth and differentiation of cells must be controlled in the laboratory or in the body, it is therefore a challenge to develop biomaterials that exploit these biological principles of guiding cells through specific interactions with their environment. Human embryonic stem (hES) cells are potentially valuable in cell-based therapies since they are able to differentiate into cells of all three germ layers as well as to self-renew and being expanded without loss of pluripotency. However, one prerequisite for such clinical use is that expansion and differentiation protocols must fulfil defined quality standards including xenofree culture conditions (Unger et al., 2008), maintenance of pluripotency during expansion, and uniform differentiation into a specific cell type.

Reproducible and defined ES cell-culturing systems must ensure cell expansion without compromising the quality of the cells as monitored by maintained chromosomal integrity and expression of pluripotency markers. For murine embryonic stem (mES) cells the ultimate quality control is the ability to enter the mouse germ line after blastocyst injection and give rise to viable offspring (Longo et al., 1997). For hES cells the strictest control of maintenance of an undifferentiated state is the ability to differentiate into three germ layers during teratoma formation after injection into immunodeficient mice (Thomson et al., 1998).

Murine and human ES cells share common characteristics such as high alkaline phosphatase (ALP) levels, a high nucleus to cytoplasm ratio, as well as the expression of a set of ES-cell-characteristic transcription factors including Nanog, Oct4, and Sox2. They exhibit distinct growth properties with mES cells growing in attached three-dimensionally rounded colonies and hES cells growing in flat colonies (Koestenbauer et al., 2006). While mES cells can be re-seeded as single cells after trypsination, the maintenance of hES cells requires a laborious step of manually cutting colonies for re-seeding, which is labour-intensive and comprises the means for clonal expansion. Differences with respect to the specific signals needed to maintain pluripotency of ES cells are reflected by the different soluble additives incorporated in the specialised culturing protocols developed for mES or hES cells. Additives to the culture media include biological signalling molecules like LIF (leukemia inhibitory factor) (Williams et al., 1988), BMP4 (bone morphogenetic protein 4) (Ying et al., 2003), or artificial inhibitors targeting specific cellular enzymes (Ying et al., 2008) in mES cell culture, and bFGF (basic fibroblast growth factor) and activin A (Akopian et al., 2010) in hES cell culture.

Both hES (Thomson et al., 1998) and mES (Evans & Kaufman 1981) cells were originally derived on mouse feeder cells, (i.e. a layer of fibroblasts) in serum containing culture medium. The layer of feeder cells contributes soluble molecules, ECM proteins (Horák & Fléchon 1998), as well as topographical and mechanical cues. However, the development of defined, xenofree culture conditions is a requirement for the use of hES cells in human cell therapy. Control and reproducibility of hES cell protocols have been improved by applying a gelatinous protein mixture secreted by a mouse sarcoma cell line manufactured under the product names Matrigel or Geltrex (Akopian et al., 2010) as culture surface. This ECM protein mixture is currently used as the standard reference surface in experiments concerning feeder-cell-free culture of hES cells, but during routine expansion feeder cells are still the preferred substrate in many laboratories in spite of the laborious preparation demands (Brafman et al., 2009; Braam et al., 2008; Mei et al., 2010; Nagaoka et al., 2010). Furthermore, neither Matrigel nor Geltrex are xenofree or chemically defined products and consequently incompatible with human cell therapy. With Matrigel and Geltrex being the generally applied feeder cell free standard surface there is a perspective for new smart biomaterials in trying to mimic the environmental demand of the ES cells, thereby contributing to better defined, xenofree, and reproducible expansion protocols.

Similar to the advantages of means for controlled expansion of undifferentiated ES cells there is also a great need for stringent protocols for controlled differentiation of ES cells for scientific and potential therapeutic purposes. Differentiation of mouse and human ES cells takes place in cell aggregates called embroid bodies or on two-dimensional surfaces. Both strategies result in a mixture of cell types even though populations are enriched for the desired cell type by means of soluble supplements (Keller 2005). A major hurdle towards the clinical use of hES cells is that the ability of pluripotent cells to induce teratomas must be lost from the batch of cells eventually used for therapy (Fong et al., 2010). It is therefore

desirable that protocols for hES cell differentiation eliminate any pluripotent cells with teratogenic properties. To identify culture conditions that lead to efficient and controlled differentiation of ES cells much effort has been put into the development of specific media supplements. However, it is now also recognised that biomaterials influence differentiation of ES cells not only through ECM-ligand presentation to integrin receptors but that also signals inherent in the synthetic biomaterials influence stem-cell state (Bakeine et al., 2009; Evans et al., 2009; Mahlstedt et al., 2010; Villa-Diaz et al., 2010). Consequently cues contributed by biomaterials are a promising and relatively unexplored tool which could increase the control of differentiation into desired cell types and help eliminate potentially teratogenic pluripotent cells from batches intended for therapeutic purposes.

The contemporary production of smart biomaterials takes advantage of several manufacturing techniques (Reviewed in (Schmidt & Healy 2009; von der Mark et al., 2010)). Development is accelerated by the screening of large biomaterial arrays, including combinations of patterning of proteins and signalling molecules (Brafman et al., 2009; Flaim et al., 2005), topography (Lovmand et al., 2009; Markert et al., 2009), modified self-assembling monolayers (Derda et al., 2007), or polymers (Anderson et al., 2004; Mei et al., 2010). In this chapter we review how stem cells respond to mechanical, chemical, and topographical cues and how such cues in synthetic two-dimensional biomaterials determine the cellular response. We refer to the vast amount of data generated by combinatorial screening approaches using two-dimensional polymer libraries derived by organic synthesis, and illustrate through our own work that the equally important input from surface topography can also be addressed by the systematic screening of libraries. To rationally design biomaterials with a predictable effect on stem cells we need to integrate optimised surface topography with optimal mechanical properties and surface chemistry. We expect that such synthetic designs based upon integrated random screening approaches will provide superior materials for use in stringent protocols that control stem-cell fate as well as novel insight into how environmental cues affect the state and signalling processes of ES cells.

2. Mechanical cues

In this section, mechanical cues affecting the programming of ES cells towards specific lineage differentiation or self-renewal are discussed. To illustrate fundamental principles we also refer to studies using multipotent stem cells.

The main mechanical link between the ECM and the intracellular actin skeleton is made up of the integrin family of membrane-spanning proteins. Integrins bind to RGD sequences in ECM proteins like for instance laminin, fibronectin, and vitronectin (Geiger et al., 2001). A key cellular response to binding of extracellular ligands to integrins is the recruitment of multiple structural proteins and signalling molecules into focal adhesions (Geiger et al., 2001). Such binding of extracellular ligands to integrins has been found to influence the differentiation state of ES cells (Burdon et al., 1999; Li et al., 2007; Na et al., 2010). E.g. a distinct type of laminin, which is present in the ES cell niche in vivo (Cooper & MacQueen 1983), supported the propagation of undifferentiated ES cells through activation of a specific integrin while other laminin subtypes did not have the same effect (Rodin et al., 2010). In mES cells focal adhesion assembly following integrin ligation was found to lead to activation of the kinases FAK, Rho, and ERK1/2 (Park et al., 2010), where ERK1/2 activation is known to link focal adhesion formation to the control of ES cell differentiation state (Burdon et al., 1999).

Externally applied force through integrin ECM interactions promotes the local growth of focal adhesions through an assembly process dependent upon the Rho kinase (Riveline et al., 2001). However, not only the size of focal adhesions but also their molecular composition changes in response to applied tensional stress (Sawada & Sheetz 2002). When integrins pull on the ECM, more tension will result from stiffer than from softer extracellular substrates, and it has been hypothesised that this might in turn lead to various conformational changes in mechanosensory proteins that connect the cytoskeleton and the ECM (Reilly & Engler 2010). Such changes may cause a change in binding-site exposure and focal-adhesion composition, thereby potentially transforming mechanical signals into a downstream intracellular response.
By using growth supports that mimicked different tissue-specific elasticities, multipotent stem cells were found to be stimulated towards the expression of markers characteristic of tissues with the elasticity in question (Engler et al., 2006). In the same study, cells grown on a stiffer matrix were stiffer and more tense than cells grown on a more elastic matrix. In the case of ES cells, such an effect of matrix stiffness on cellular differentiation is less clarified than in the case of multipotent stem cells, but mES cell morphology has been found to respond to changes in synthetic matrix stiffness, and mesendoderm marker expression to be upregulated on stiffer substrates compared to softer ones (Blin et al., 2010; Evans et al., 2009). In addition, cell softness regulated the spreading of mES cells in response to mechanical stress imposed via integrin ligands in a process dependent on myosin II, F-actin, and cdc42, but not Rac (Chowdhury et al., 2010). Myosin II activity was also critical for the previously mentioned elasticity-directed lineage-specific differentiation of multipotent stem cells (Engler et al., 2006) indicating that cytoskeletal tension is involved in the regulation of stem cell differentiation state in response to mechanical cues. This response is mediated by modulation of focal adhesion length and myosin II activity (Engler et al., 2006). The finding that disruption of cytoskeletal structures manipulated cell morphology and specifically directed the cells towards an adipogenic fate (Feng et al., 2010) also supports an involvement of cytoskeletal tension in regulation of hES cell differentiation state. Besides externally imposed mechanical cues like matrix stiffness also cyclic biaxial strain affects the differentiation state of hES cells (Saha et al., 2006). Although diverse, these studies establish that mechanical cues affect the differentiation state of ES cell by means of an interplay between cell shape, focal adhesion organisation, cell stiffness, myosin II activity, and cytoskeleton rearrangement.

3. Chemical cues

3.1 Immobilisation of signalling ligands

In vitro, many signals are imposed on cells through addition of soluble factors to the culture medium. This approach, however, is poorly compatible with transplantation based therapies. Hence, the immobilization of naturally soluble macromolecules to affect intracellular signalling poses an attractive alternative. Biomaterials with immobile growth factors can be used to modulate cell behaviour including attachment, proliferation, alignment, and migration (Mieszawska & Kaplan 2010), but have also recently been used to control the ES cell differentiation state (Alberti et al., 2008; Brafman et al., 2009; Chiang et al., 2010; Nagaoka et al., 2008).
Immobilised LIF activated the same intracellular pathways as soluble LIF and independently supported formation and growth of pluripotent mES cells (Alberti et al., 2008; Makino et al., 2004). The intensity of activation of intracellular signalling pathways by immobilised LIF was dose-dependent illustrating the possibility of fine tuning the cellular response to immobilised

signalling molecules (Alberti et al., 2008). Furthermore, lower amounts of immobilised LIF was necessary to maintain the pluripotent phenotype compared to soluble LIF (Nagaoka et al., 2008). The efficient cytokine induced signalling observed with immobilised LIF might be related to the fact that the soluble form induced LIF receptor internalisation whereas the immobilised form did not. This suggests that the high efficiency of immobilised LIF is due to prolonged signalling owing to the lack of receptor internalisation.

With respect to hES cells, formation of undifferentiated colonies was supported by immobilising bFGF in combination with ECM molecules (Brafman et al., 2009). The same approach was used to induce hES cell differentiation by co-immobilisation of BMP4 and retinoic acid (Brafman et al., 2009). These experiments proved the applicability of immobilising growth factors for differentiation purposes. Taking this a level further, a cutting-edge setup showed that 100 μm wide lanes of VEGF (vascular endothelial growth factor) and collagen IV stamped onto an otherwise non-adhesive surface directed site-specific differentiation of mES cell derived angiogenic progenitor cells into endothelial cells (Chiang et al., 2010). Taken together, the immobilisation of signalling molecules appears to be a versatile approach offering numerous possibilities of direction of site-specific differentiation in a dose-dependent manner.

Following a different line of thinking, non-adhesive surfaces with immobilised E-cadherin were used to mimic ES cell-cell interactions (Nagaoka et al., 2006; Nagaoka et al., 2010). Under these conditions, mES cells exhibited scattered growth of discrete cells with pseudopodial protrusions but still they maintained their germline competence (Nagaoka et al., 2006). Also, the morphology of hES cell colonies was affected by the non-adhesive surfaces with immobilised E-cadherin (Nagaoka et al., 2010). Interestingly, the cells proliferated at the same rate as cells cultured on Matrigel even though no integrin binding was involved in the initial attachment to the E-cadherin surfaces.

Immobilised signalling molecules thus provide an extremely powerful tool for mimicking the ES cell niche and thereby modulating ES cell differentiation and colony morphology in a site specific manner. This opens new perspectives for transplantation based therapies.

3.2 Modified organic polymers

Another promising approach for the development of chemically defined biomaterial surfaces for ES-cell culture involves the synthesis of organic polymers and takes advantage of screening of large combinatorial arrays. Acrylate polymers synthesised from different combinations of two monomers have been applied in high through-put screening of hES cells (Anderson et al., 2004; Mei et al., 2010). In such screenings the initial arrays identify hits based upon a small number of events whereas subsequent secondary arrays with a high number of replicates are used to decrease experimental error, thereby achieving an efficient and thorough analysis. Acrylate polymers possess both mechanical, chemical, and topographical cues (Mei et al., 2010). By means of different arrays, specific polymers were found to support proliferation and differentiation into cytokeratin-positive cells (Anderson et al., 2004) and some were found to support clonal propagation of undifferentiated hES cells (Mei et al., 2010). Especially the hydrophilicity and the chemical composition of the surface appeared to be important for the biological performance (Mei et al., 2010).

Extensive analysis of surface chemistry by secondary ion-mass spectrometry revealed that biological performance could in fact be predicted from the surface chemical composition with small hydrocarbon ions, oxygenated hydrocarbon ions derived from esters, as well as

ions from cyclic structures being associated with favoured formation of undifferentiated colonies (Mei et al., 2010).

Polymers built from monomers with a high acrylate content supported the clonal expansion of undifferentiated ES cells (Mei et al., 2010). Further coating of di- or triacrylate polymers of moderate surface wettability with recombinant vitronectin supported clonal expansion in chemically defined media, thus providing a protocol for the clonal expansion of hES cells under fully defined conditions (Mei et al., 2010). Tissue-culture plastic coated with recombinant vitronectin was previously shown to support hES-cell expansion (Braam et al., 2008) but under these conditions clonal propagation led to differentiation (Mei et al., 2010), thereby emphasising the importance of the physicochemical characteristics of the coated material. This result might partly be related to the fact that surface chemistry alters the exposure of integrin binding sites of ECM proteins (Keselowsky et al., 2005). The importance of ECM conformation is emphasised by the finding that cyclic RGD strongly sustained hES cell expansion as compared to a linear RGD sequence (Kolhar et al., 2010). Melkoumian et al. applied an acrylate surface with covalently bound short (~ 15 aa long) peptides of vitronectin and bone sialoprotein including the RGD motif and found that this surface supported long-term self-renewal of hES cells (Melkoumian et al., 2010). This points to a fruitful avenue for enhancing the biological function of optimised polymer surfaces through covalent coupling of chemically synthesised minimal peptides recognised by specific cellular receptors.

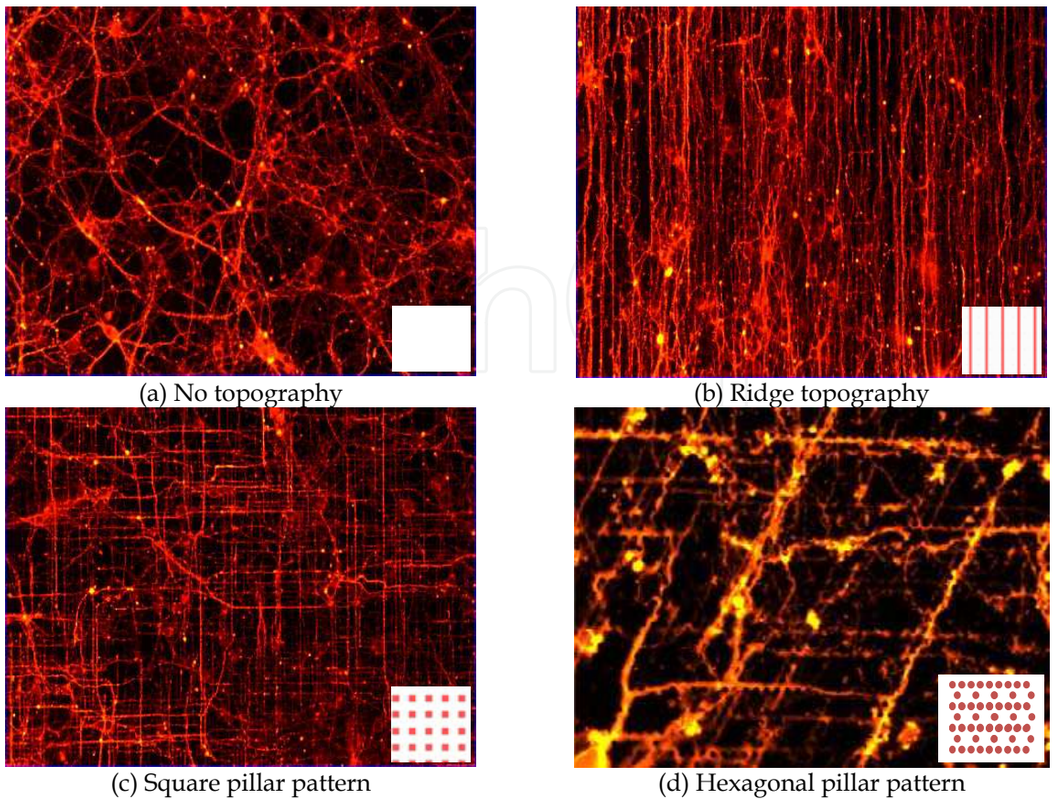
Through analysis of the combinatorial arrays of acrylate polymers, surfaces of moderate hydrophilicity (i.e. a water contact angle ~ 70°) were found to robustly support the expansion of hES cells (Mei et al., 2010). However, another study reported that plasma etching of tissue-culture plast, which was found to reduce the water contact angle from the value 66° of non-etched plast to less than 10°, supported hES-cell expansion (Mahlstedt et al., 2010), indicating that hydrophilicity is not the sole determining parameter. This is also confirmed by studies including poly(α-hydroxy esters) (Harrison et al., 2004b), where the surface that best facilitated mES cell colonisation had a water-contact angle around 70° while other surfaces of the same hydrophilicity performed less well. Interestingly, surface hydrophilicity also affects embroid body formation (Valamehr et al., 2008). Embroid body size as well as the expression of differentiation markers could be tuned by varying surface hydrophilicity. Hence, hydrophilic surfaces resulted in smaller aggregates and hydrophobic surfaces in intermediate, regularly sized aggregates, which were found to exhibit sustained expression of germ-layer markers.

Studies of individual polymers or other chemically defined biomaterials are informative in their specific context (Harrison et al., 2004a; Horák et al., 2004; Kroupová et al., 2006; Li et al., 2006). However, the pioneering polymer-array study (Anderson et al., 2004) showed that differences among polymers with respect to their effect on proliferation of hES cells in some cases depend upon soluble media supplements. This re-enforces the value of systematic high through-put screening for biomaterial effects in various cellular protocols to avoid overlooking superior effects from specific combinations.

4. Topographical cues

4.1 The basis of topographical effects on cells

In their natural environments cells are exposed to both nano and microscale topographical cues provided by other cells and the surrounding ECM. For years it has been known that designed topographical cues influence important cellular processes such as adhesion, morphology, migration, proliferation (Curtis & Wilkinson 1997), and differentiation



(a) No topography (b) Ridge topography

(c) Square pillar pattern (d) Hexagonal pillar pattern

Fig. 1. Topographical alignment of neurites of fetal rat cortex cells on poly-D-lysine-coated structured surfaces. The topographical patterns are indicated by the inserts (not to scale) in the lower right corner of each panel. (a), (b), (c): Stained for Tau1 expression in axons. (d): Stained for β-tubulin expression in neurites. The images were kindly provided by Trine Elkjær Crovato.

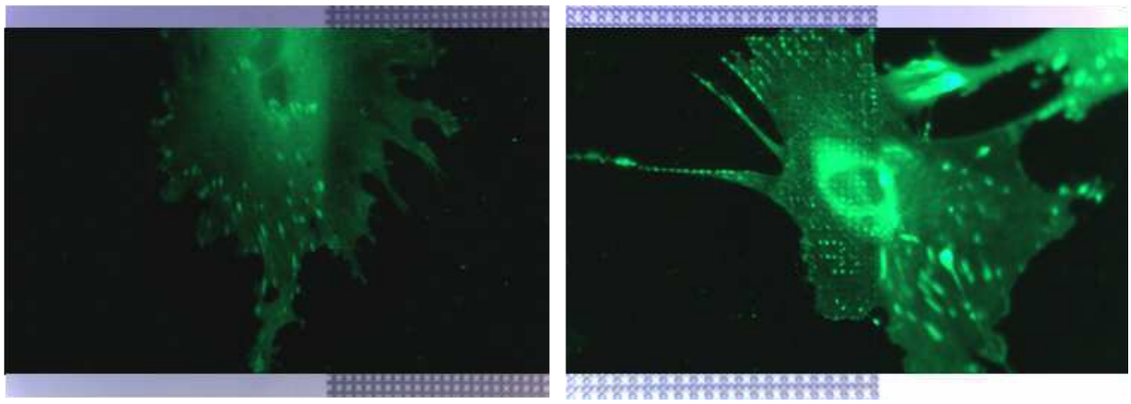
(Dalby et al., 2007), and it has become clear that also ES cells are affected by topographical signals (Gerecht et al., 2007). The most commonly designed topographies applied in biomaterials are usually groove/ridge patterns, defined or random pillar patterns, pit patterns, or surface roughness (Bettinger et al., 2009). Figure 1 shows an illustrative example of the guidance of cell morphology by topography, namely the alignment of neurites according to topographical patterns. A variety of cell types have been applied to numerous nano and microscale variations of these different topographies and this scattered approach has brought about a lot of non-systematic information, which makes it difficult to draw general conclusions. There are, however, consistent ideas of how topography imposes an effect on mammalian cells. In this regard a distinction between micro and nanoscale topographies must be made.

Biomaterials with designed microscale topographies are aimed at controlling cell shape and position. The generally appreciated idea of how microscale topography induces a cellular response involves irregular tension imposed on the cell in the encounter with the geometric features. This cytoskeletal stress leads to a change in nucleus shape and possibly concomitant alterations in gene expression (Martínez et al., 2009).

Nanoscale topography, where feature sizes lie in the range of the dimensions of single proteins, modulate integrin binding and focal adhesion formation in that integrins require

less than 73 nm separation between the ligands presented to them in order to cluster and facilitate cell spreading and attachment (Arnold et al., 2004). In addition, topography in the range 0-250 nm affects adsorption, conformation, and therefore biological functionality of ECM proteins (Martínez et al., 2009).

Whenever the effect of topography relies on focal adhesions it is relevant that both nano and microscale topographies contribute a spatial organisation of focal adhesion formation. Also, focal adhesion formation is modulated by the size of integrin ligand-presenting protein patches presented to the cell on planar surfaces (Malmström et al., 2010). Hence, 200 nm patches only supported formation of small and few focal complexes while 500 and 1000 nm patches facilitated increasingly more and large complexes with matching effects on cell attachment and spreading. Increasing pillar diameter from one to two micrometers has been observed to facilitate focal adhesion formation in embryonic fibroblasts (Figure 2). In fact, individual focal adhesions can adopt sizes up to the extent of several μm^2 (Geiger et al., 2001).



(a) Pillar diameter 1 μm (b) Pillar diameter 2 μm

Fig. 2. Embryonic fibroblasts on topographies with pillar distances of 1 μm and pillar diameters of 1 μm (a) and 2 μm (b) compared to planar surfaces. Cells were stained with anti-vinculin antibody. Notice the border between the topographical pattern and the planar surface. The images were kindly provided by Annette Füchtbauer.

Therefore, when designing topographical biomaterials it should be taken into consideration at which level the different topographical magnitudes affect the cells depending on which cue one intends to impose on the cells.

4.2 ES cell response to topographical surfaces

4.2.1 Morphology

Actin polymerisation is essential for cell morphology and attachment and is expected to be involved in the cellular response to topography by a mechanism involving filopodia formation during contact guidance (Bettinger et al., 2009). In this connection signalling through the Rho, Rac, and Cdc42 pathway, which is involved in the formation and organisation of the actin cytoskeleton, is a possibility. Clarification of this, however, awaits further investigation.

One of the most intensively studied topographical designs is line gratings to which ES cells morphologically respond in ways similar to somatic cells (Curtis & Wilkinson 1997). Hence, line gratings directed elongation of hES cells in an orientation parallel with the topographical features under culture conditions which conventionally favour self-renewal (Gerecht et al.,

2007) or neuronal differentiation (Lee et al., 2010). Under other conditions morphological aspects like cell spreading and protrusion formation of hES cell derived osteoprogenitor cells were changed in response to topography (Smith et al., 2010). Taken together, this implies that topography is able to cause versatile morphological responses of ES cells.
This morphological response was reflected in alteration of the organisation of the cytoskeletal components α-actin, tubulin, and vimentin (Gerecht et al., 2007), thus emphasising the effect of topography on the ES cell cytoskeleton.

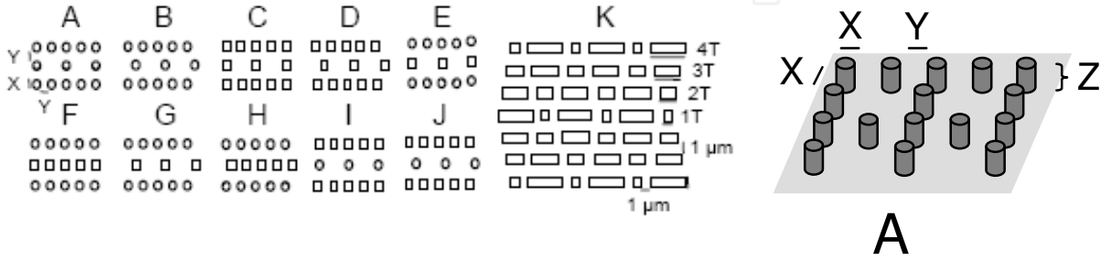
4.2.2 Attachment and proliferation

Biomaterial topography also affects cell attachment and proliferation. When the topography of the biologically derived ECM material Matrigel was changed owing to adsorption onto different surfaces this affected both attachment and proliferation of hES cells (Kohen et al., 2010). The importance of surface topography for attachment was further emphasised by the finding that mES cells were not able to adhere to a planar polyamide surface in contrast to a fibrillar surface of the same material (Nur-E-Kamal et al., 2006). Proliferation of hES cells, mES-cell-derived cardiomyocytes, and ES-cell-derived neural precursors was reduced in response to defined topographical patterns as different as fibronectin-coated 600 nm groove/ridges (Gerecht et al., 2007), fibronectin coated pillars (diameter 5 μm) of 15 μm but not of 5 μm height (Biehl et al., 2009), and nano-roughness (Bakeine et al., 2009), respectively. More specifically, proliferation and/or attachment of mES cell derived neural precursors inversely correlated with increasing gold surface roughness in the range 0-30 nm (Bakeine et al., 2009). The reduction in proliferation caused by nano grooves/ridges was abolished by addition of actin skeleton-disrupting agents (Gerecht et al., 2007) and the inhibitory effect of microscale topography was reversed by blocking the actions of Rho or myosin light-chain kinase (Biehl et al., 2009), indicating that the actin skeleton organisation and tension play an important part in mediating the topographical cue that affects proliferation of ES cells. The fact that this breadth of topographical features produced from different materials inhibits cellular proliferation of ES cells in spite of differentiation state and species of origin suggests that topography in general inhibits proliferation of ES cells compared to planar surfaces. However, a single study showed that mES-cell proliferation was increased by the three-dimensionality of a nanofibrillar polyamide surface (Nur-E-Kamal et al., 2006). The increase in proliferation on this topographic surface correlated with an increase in PI3K (phosphoinositide 3-kinase) and Rac activity while Rho and Cdc42 activities were practically unchanged. PI3K activity has been linked to regulation of both proliferation and self-renewal of ES cells in other studies (Welham et al., 2007).

4.2.3 Self-renewal and differentiation

In recent years it has become clear that topography is also involved in the regulation of differentiation. The first convincing report in this field came from Dalby et al., who showed that mesenchymal stem cells were induced to differentiation into bone-mineralising cells in response to nothing but topography (Dalby et al., 2007). Lately also a few reports on the influence of defined topography on the differentiation of ES cells or ES-cell-derived progenitor cells have been published (Markert et al., 2009; Bakeine et al., 2009; McFarlin et al., 2009; Lee et al., 2010).
The few available studies concerning ES cells and topography have been carried out with different biomaterials and feature-size ranges which makes comparison difficult. In our

laboratory we chose a systematic, array-based approach called BioSurface Structure Array (BSSA) (Lovmand et al., 2009), in order to achieve a more general insight into the connection between topography and ES-cell phenotype (Markert et al., 2009). We designed 10 defined topographical pattern series based on circular and/or square protrusions (Figure 3). Also a single pattern series with interrupted groove/ridge features inspired by shark-skin configuration was included. Iteration series of the patterns were generated by varying the horizontal pattern dimensions illustrated by X and Y in figure 3. Thus, protrusion-based patterns with varying feature (X=1, 2, 4, or 6 μm) and gap sizes (Y = 1, 2, 4, or 6 μm) were generated giving rise to 16x10 topographies with highly different protruding areas.



(a) 2-D projection of BSSA topographical pattern design (b) 3-D view of topography A

Fig. 3. Topography pattern designs of BSSA. (a) Two-dimensional projections of pattern A-K. Series A-J are each produced with 16 different combinations of feature size X and gap between features Y: (X,Y) =(1,1), (1,2), (1,4), (1,6); (2,1), (2,2), (2,4), (2,6); (4,1), (4,2), (4,4), (4,6); (6,1), (6,2), (6,4), (6,6). Iteration of the K series specified by T = 1,2,3,4,5,6,7, or 8 defines K patterns of varying length, but with constant gap and feature broadness (1 μm). (b) Three dimensional presentation of topography A where Z is the protrusion height; A(X,Y,Z). X, Y, Z and T are in μm.

Interrupted groove/ridge topographies were produced in different lengths by letting T adopt the values 1 through 8 μm resulting in 8 topographies (Figure 3). This total of 169 different topographies including one non-structured surface was produced as a tantalum BSSA for cell culturing. Furthermore, the topographical BSSA was produced in three different heights (Z = 0.6, 1.6, or 2.4 μm).

The surface of the BSSA was coated with a layer of tantalum (i.e. tantalum oxide) which has previously been successfully applied for cell culture and is a promising material for stem cell-based implantation procedures (Stiehler et al., 2008). To our knowledge it is, however, unprecedented to seed ES cells on this material.

When seeding two feeder-dependent mES cell lines on the topographical tantalum BSSA without feeder cells, we found that colony number and morphology varied among the different topographies. The examples shown in figure 4 are representative of the differences observed. It appears that colony number increased with structure height for both cell lines. Also both cell lines exhibited larger/more spread colony morphology on the F(X,Y)=F(4,1) topography than on the F(X,Y)=F(1,2) where the colonies formed were mainly compact and well defined as expected for undifferentiatied mES cells. On the K structures, here exemplified by K(T)=K(7), the colonies were elongated and tended to spread out. Even though the two cell lines behaved differently on the non-structured control surface, they exhibited similar responses to the topographies, thus emphasising the strength of the topographical cues.

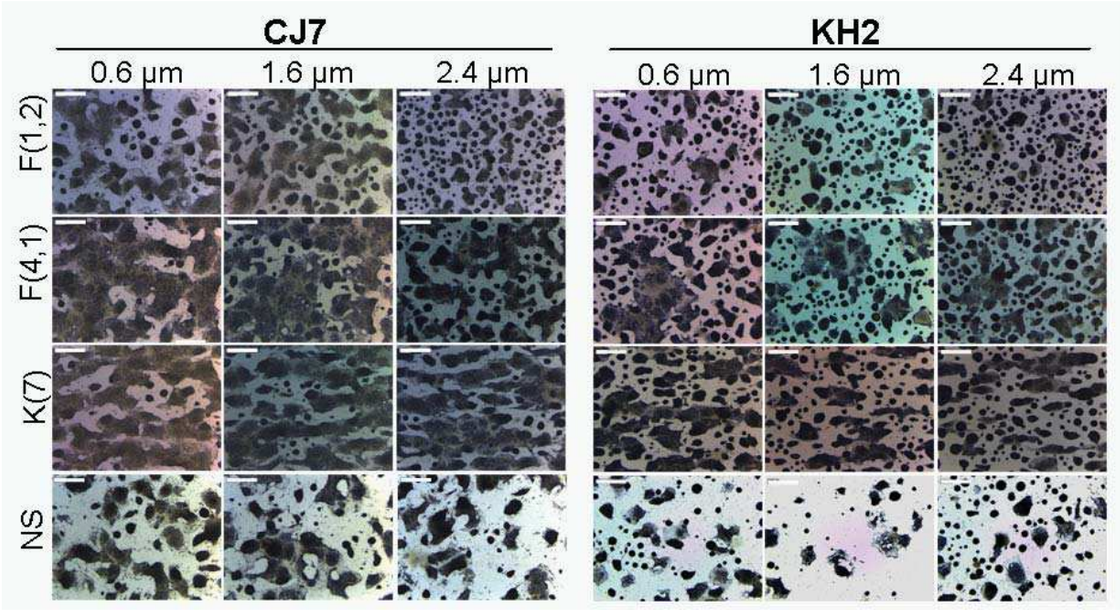
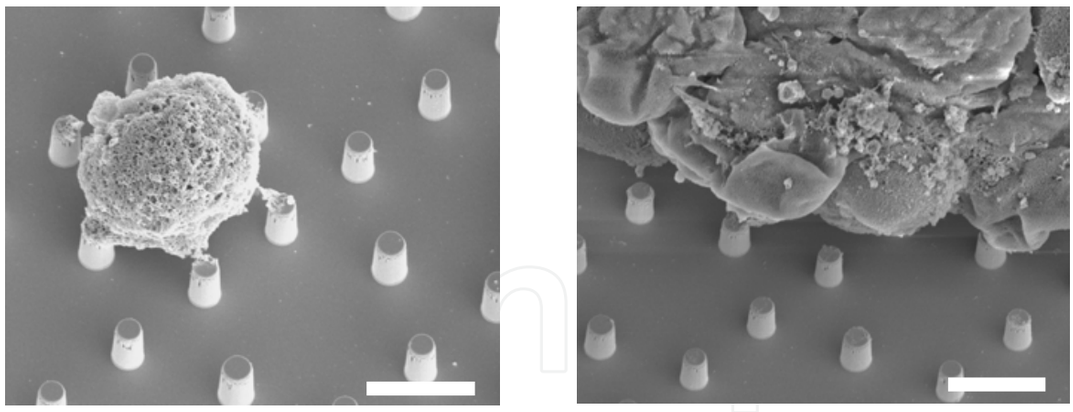


Fig. 4. Mouse ES cell colonies on selected tantalum BSSA topographies. CJ7 and KH2 mES cells were seeded at a density of $2.6x10^4$ cells/cm^2 in conventional medium containing LIF. Colonies were fixed after three days and stained for alkaline phosphatase activity. Colony formation on the selected topographies F(1,2), F(4,1), and K(7) is shown at three different vertical feature heights (Z=0.6,1.6, or 2.4 µm). A non-structured (NS) control surface is also included. Scalebar: 500 µm.

To substantiate the topographically induced variations in colony number and colony spreading the findings were quantified using automated estimation procedures. It became obvious that the heights of the topographies were of major importance for formation of undifferentiated colonies and colony spreading while the shape of the geometric features (circle, square) in the A-J topography patterns did not contribute consistently to colony variations. More specifically, we found that increasing feature height, Z, from 0.6 µm to 2.4 µm heavily increased the number of undifferentiated colonies formed on the tantalum topographies while decreasing feature height resulted in colony spreading and colony areas of reduced alkaline phosphatase activity, which suggested that differentiation had commenced in spite of the presence of LIF. Others have reported that increasing surface roughness of acrylate polymers in the root mean-square range 0-110 nm had no major impact on the formation of undifferentiated hES-cell colonies (Mei et al., 2010). Our findings, however, indicate that an even higher number of undifferentiated colonies might be achieved by further increasing the height of our defined micro topographies. Moreover, we found that the lateral dimensions of the A-J topographies were decisive for the morphology and the number of colonies formed, in the sense that the largest numbers of undifferentiated mES-cell colonies were generated on topographies with feature width, X, 1 µm and a distance between adjacent features, Y, of 2 or 4 µm. At the same time colony spreading was most heavily induced on the topographical patterns with the smallest distances between features, Y=1. Combining the latter observation with unpublished results from our lab showing that mES cells lie on top of topographical features (Figure 5) suggests that cells spread more easily onto adjacent protrusions when the distance between them is reduced.



(a) Single mES cell on pillar topography

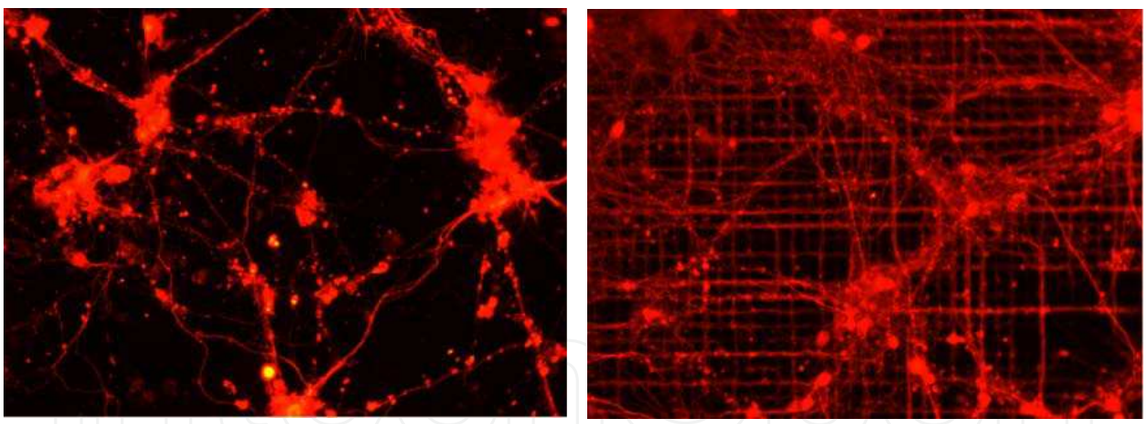
(b) Colony of mES cells on pillar topography

Fig. 5. Scanning-electron microscopy of CJ7 cells on B(X,Y,Z)= B(1,4,2.4) topography. (a) Single mES cell. (b) mES cell colony. We thank Jenny Malmström for kindly sharing her scanning electron microscopy expertise. Scalebar 5 μm.

The elongation of the mES-cell colonies induced by the interrupted groove/ridge K topography is in line with the elongation induced in individual cells in response to line gratings (Gerecht et al., 2007; Lee et al., 2010). The observed colony elongation was accompanied by increased colony spreading and loss of alkaline-staining intensity in the presence of LIF (Markert et al., 2009) - both characteristics of mES-cell differentiation. This makes topography a plausible cue for aiding the elimination of pluripotent cells which is critical for cell based therapies.

Colony morphology is an important pluripotency marker for mES cells. Therefore, we investigated the strength of the topographical cues on mES-cell-colony morphology in the absence of LIF. Surprisingly, it turned out that the variations in colony spreading induced by the iterated A-J topographies were also valid when LIF was not added to the culture (Markert et al., 2009). To further explore the sustained impact of the topographical cues we serially passaged mES cells 6 times on the F(X,Y,Z) = F(1,2,2.4) topography in conventional mES-cell-culture medium containing LIF. Under these conditions we found that the F(1,2,2.4) topography sustained the germ line competence of mES cells throughout 6 passages. It is an intriguing question to which extent the topographical cues provided by the various topographical patterns in the BSSA affect the differentiation state of mES cells. Equally exciting would it be to find out in more detail how the signals imposed by the defined topographies are translated into an intracellular response. However, our observations that topography has a positive effect on ES-cell self-renewal has been reported by others as well (McFarlin et al., 2009). The expression of the pluripotency marker Nanog in mES cells was found to be upregulated in response to a topographic polyamide surface (Nur-E-Kamal et al., 2006) and when hES cells were cultured on gelatin-coated polyurethane groove/ridge patterns of 300 nm feature height and a number of pitches in the range of 400-4000 nm, these were also found to affect the mES-cell differentiation state (McFarlin et al., 2009). It is, however, unclear exactly from which pitch ranges the published data have been obtained. Still, these authors found that topography reinforced the soluble cues in the sense that topography inhibited spontaneous differentiation under conditions which promoted self-renewal and stimulated differentiation under culture conditions which promoted

differentiation. While it has been amply demonstrated that topography per se has an influence, indirect effects owing to altered access of the topographically elevated ES-cell colonies to the growth medium may also play a role in this case.
Topographical biomaterials have also been applied for directed differentiation. Neuronal differentiation of neuronal precursors generated from mES cells was increased by a gold surface roughness of 21 nm compared to a planar surface or a surface with 30 nm roughness (Bakeine et al., 2009). These authors combined the optimal (21 nm) nanoscale roughness with microscale groove/ridge topographies and found that neurons differentiating on this surface extended their neurites in parallel with the gratings. Moreover, unpublished results from our laboratory show that mES-cell-derived neuronal cells also can align in a characteristic pattern on pillar-structured surfaces (Figure 6). Also, neurons derived from hES cells showed extensions of neurites in parallel with the topographical features (Lee et al., 2010). Topography alone was able to stimulate the differentiation of hES cells into neurons when cells were cultured in media free from traditional neuronal inducers on ridge/groove (350 nm/350 nm) topography with ridge height 500 nm (Lee et al., 2010).
Altogether, these results emphasize the importance of considering topographical cues when designing biomaterials for both self-renewal and differentiation of ES cells. Our work highlights the important effects of defined micro-topography on ES-cell-colony formation and morphology. Since cell shape (Feng et al., 2010) and colony morphology (Lee et al., 2009) are linked to ES-cell differentiation, topography offers an extra level of possibilities for directing ES-cell fate through modulation of cell and colony morphology. Furthermore, the BSSA approach has been applied to other cell types as well, thereby facilitating immediate comparison between cell types over the same topographical patterns and ranges produced in the same material (Kolind et al., 2010; Lovmand et al., 2009).



(a) Planar surface (b) Surface with pillar topography

Fig. 6. Neuronal cells derived from mES cells on (a) planar surface and (b) pillar-structured surface F(X,Y,Z)=F(6,4,2.4) from the BSSA library. The cells were stained for β-tubulin expression in neurites. The pictures were kindly provided by Trine Elkjær Crovato.

5. Conclusion

In this chapter we have highlighted the controlled self-renewal or directed differentiation of ES cells through an integrated input from chemical, topographical, and mechanical cues.
In general contemporary methodologies for self-renewal of ES cells a layer of feeder cells grown on standard tissue-culture plastic and the feeder-cell generated ECM provide

topographical, and mechanical signals, whereas the chemical signals include specfic media supplements as well as products of the feeder-layer. Among the developments towards easy and stringent protocols for expansion or differentiation of ES cells under xeno-free and fully defined conditions, we emphasise development of novel biomaterials in which cell fate is partly or fully controlled by signals embedded in the surface of these materials.

The coupling of natural ligands such as specific peptides or recombinant proteins to surfaces have already led to sophisticated biomaterials with specific influence on cell behaviour. Immobilisation of soluble signalling molecules such as differentiation blockers or growth factors may enhance control and alleviate the need for specific soluble media additives. In another development integrin-ligands such as ECM proteins or artificial RGD-containing peptides may control not only cell-adhesion and morphology, but also the state of differentiation.

Array-based screening of surfaces generated by combinatorial chemistry of non-biological polymers have provided immense information on differences in cellular response to variations in surface chemistry. Remarkably, in such arrays a statistical analysis of the relationship between surface chemistry and biological read-out allowed the prediction of the cellular response to a given surface chemistry, even though the underlying mechanisms are far from understood (Mei et al., 2010; Yang et al., 2010). In a more recent development, polymer-based screenings have also been applied to screening of the impact of mechanical properties of the growth substrate on cellular behaviour. In fact, in such polymer-based screenings it may not be possible to fully discriminate between a chemical or a mechanical effect. Array-based screenings of the response of ES cells to chemical and mechanical cues currently contribute vast amounts of information that may aid the design of specialised biomaterials and provide new insight into regulatory mechanisms in ES cells.

It is well established that surface topography plays a role for the behaviour of ES cells, with nanosized features affecting interaction at the single protein level and microsized features affecting overall cell attachment and geometry. In this area, array-based screenings for the impact of topography have defined optimal structures for self-renewal or differentiation, but only on a given type of material and within a given window of analysis. However, we expect the overall potential of optimising topography for ES cell cultivation to be far from exhausted.

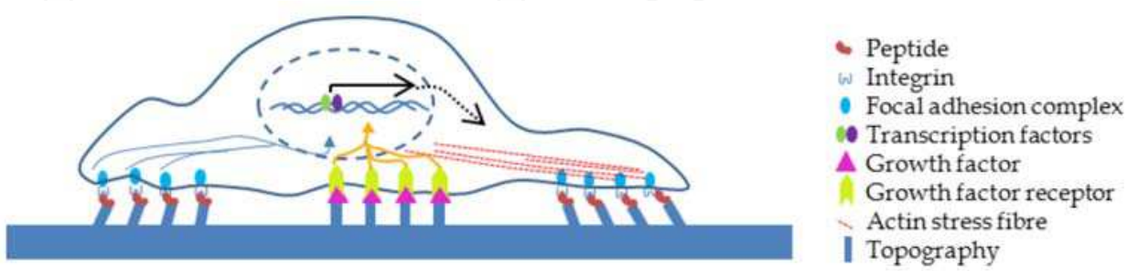


Fig. 7. Cellular reponse to topographical, chemical, and mechanical cues provided by synthetic biomaterials. The responding cell integrates signals from various external cues through complex regulatory circuits to determine the morphology and mechanical properties of a cell as well as its gene expression programme and state of differentiation.

Future specialized biomaterials may mimic the natural stem-cell niche by combining optimised surface topography with mechanical features, signals from specialised ECM-

components and other chemical cues as illustrated in figure 7. Such materials may be fine-tuned to allow improved signalling in a concentration-dependent manner and optimisation of geometric patterning for site-specific attachment and differentiation to allow extended applications in regenerative medicine. The signals from mechanical, chemical, and topographic cues act in concert to determine cell fate through an influence on cell shape and cytoskeleton organisation as well as gene expression and differentiation state. Sophisticated biomaterials with cues that control stem cell fate will allow defined means for investigation of the integrated regulatory mechanisms that determine stem cell fate in response to signals in the environment.

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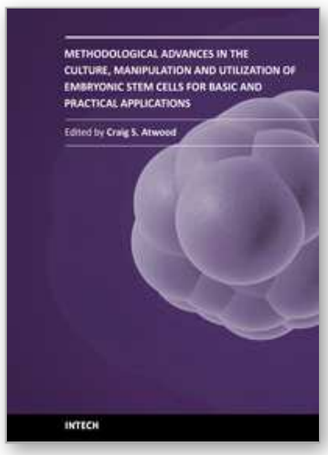

Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes methodological advances in the culture and manipulation of embryonic stem cells that will serve to bring this promise to practice.

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