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# The effect of Young's modulus on the neuronal differentiation of mouse embryonic stem cells

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ABSTRACT

There is substantial evidence that cells produce a diverse response to changes in ECM stiffness depending on their identity. Our aim was to understand how stiffness impacts neuronal differentiation of embryonic stem cells (ESC's), and how this varies at three specific stages of the differentiation process. In this investigation, three effects of stiffness on cells were considered; attachment, expansion and phenotypic changes during differentiation. Stiffness was varied from 2 kPa to 18 kPa to finally 35 kPa. Attachment was found to decrease with increasing stiffness for both ESC's (with a 95% decrease on 35 kPa compared to 2 kPa) and neural precursors (with a 83% decrease on 35 kPa). The attachment of immature neurons was unaffected by stiffness. Expansion was independent of stiffness for all cell types, implying that the proliferation of cells during this differentiation process was independent of Young's modulus. Stiffness had no effect upon phenotypic changes during differentiation for mESC's and neural precursors. 2 kPa increased the proportion of cells that differentiated from immature into mature neurons. Taken together our findings imply that the impact of Young's modulus on attachment diminishes as neuronal cells become more mature. Conversely, the impact of Young's modulus on changes in phenotype increased as cells became more mature.


## 1. Introduction

Embryonic stem (ES) cells, derived from the early mammalian embryo are unique in their ability to both self renew indefinitely *in vitro* [1] as well as differentiate into cells from any of the 3 germ layers (endoderm, mesoderm and ectoderm) and thus form any tissue within the body [2]. As a result of these two properties, there has been much interest generated in potential therapeutic applications for embryonic stem cells, including treatments for Parkinson's disease [3–6], diabetes [7–9] and cardiovascular disease [10–14]. Despite promising early studies in animals, production of the large quantities of cells required for therapeutic use in humans will require a substantial improvement on the current cell processing technology [15,16].

One approach to improve the efficiency of stem cell processes has been to investigate the role of the mechanical microenvironment. This includes factors such as cyclic strain [17], shear from fluid flow [18] and extracellular matrix elasticity [19–21] which have all been shown to be key regulators of stem cell differentiation. Culturing cells on a matrix elasticity similar to that of the *in vivo* microenvironment has in particular shown to have a profound effect on the behaviour of somatic cells, including increased beating in cardiomyocytes [22], increased spreading of fibroblasts [23] and direction of differentiation in myoblasts [24].

A seminal study by Engler et al. in 2006 [19] showed that culturing mesenchymal stem cells on polyacrylamide gels with a Young's modulus associated with a specific tissue type (such as brain, muscle or cross-linked collagen in osteoids) promoted directed differentiation into that lineage. In an investigation into the effect of stiffness on ES cell differentiation, Evans et al. [25] showed that osteogenic gene expression increased when mESC's were differentiated on stiff PDMS materials, whose elasticity corresponded with that of bone. There has been little study as of yet as to how Young's modulus affects the formation of neuronal precursors and immature neurons from mESC's. There have however been many attempts at studying the effect of stiffness on primary cultures of mixed cortical neurons [26], mixed hippocampal neurons [27], embryonic rat spinal cord [28] or neural stem cells [29,30]. In the case of mixed cortical neurons [26], 80% of cells were positive for β-III tubulin on soft, 100 Pa polyacrylamide gels compared to 45% on stiffer, 9 kPa polyacrylamide gels. Neuronal adhesion did not vary between materials in this study. Jiang and colleagues [28] showed a similar finding using mixed embryonic rat spinal cord,

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where MAP2-positive neuronal adhesion did not vary with Young's modulus from 0.3 kPa to 27 kPa. In that study, both the adhesion and maturation of immature astrocytes increased with an increase in Young's modulus from 0.3 kPa to 27 kPa. The number of mixed hippocampal neurons (per $cm^2$) found on polyacrylamide gels at 24 h post-attachment also did not vary [27] with Young's modulus from 2 kPa to 18 kPa. In the case of neural stem cells, β-III tubulin gene expression peaked on soft materials (200 Pa in Banerjee et al.'s study on 3D hydrogels [29]; between 500 Pa and 1 kPa in Saha et al.'s study on 2D gels [30]).

In most of the aforementioned studies [26,29,30], soft materials in the range of 0.1 to 1 kPa, corresponding to the elasticity range of tissues found in central nervous system and brain [31], generally lead to higher expression of markers such as βIII tubulin and MAP2, which are early markers for maturation into cells with neuronal morphology [32].

Adhesion of primary neuronal and neural stem cell cultures was unaffected by Young's modulus in most cases [26–28,30]. In Flanagan et al.'s study, soft (50 Pa) polyacrylamide gels were found to increase neurite branching [33] in comparison to stiffer (550 Pa) gels. This is an important consideration when introducing reparative cells into diseased areas, where the mechanical microenvironment may be considerably different to that of healthy tissue, as well as *in vitro* conditions. For example, glial scarring, associated with spinal cord damage can significantly increase matrix modulus [26,27], thereby inhibiting neuronal outgrowth. Elasticity is an important consideration when designing biomaterials for tissue engineering purposes, where matrix stiffness is likely to affect the ability of engrafting neurons or precursors to attach, proliferate, differentiate and ultimately survive.

The objective of this study was to investigate how Young's modulus can influence the formation of neurons from mESC's. In this study, we have taken a novel approach by looking at the effect of Young's modulus on differentiation on a stage-by-stage basis. Previous studies of mechanical effects on differentiation have only been characterised across the entire differentiation process from start to finish, not over individual intermediate stages. The first stage looked at formation of neural precursors from mESC's, the second at immature neurons from neural precursors and the third at more mature types from immature neurons. In order to direct mESC differentiation we used a well characterised adherent monolayer protocol [32,34–36], which avoids the need to create three dimensional aggregates. The material used for the study, GXG, consists of gelatin cross-linked with glutaraldehyde. GXG was chosen as a material as gelatin is also the coating used for Ying et al.'s [32] protocol, allowing for a direct comparison. It has previously been used to characterise mechanical effects on cells in a number of studies [37–39]. Changing the percentage of gelatin in either phosphate buffer saline (PBS) or water varies the Young's modulus of the gel. As tissue from the brain and central nervous system tends to be softer than tissue from other parts of the body, the hypothesis of this study was that softer materials favour the formation of neurons from mESC's over stiffer materials. Cellular attachment, expansion and phenotype changes were investigated for both mESC's and partially -differentiated mESC's at time points of 24 h, 48 h (D2), 72 h, 96 h (D4) and 144 h (D6) in neuronal differentiation medium. Any increase in cell numbers on the materials from 0 h to 24 h was considered as attachment. In addition, any increase in number from 24 h to 72 h was considered expansion. Differentiation was considered from 0 h to 144 h (D6). Finally, inhibitors for myosin-dependent cell contraction and microtubule formation (blebbistatin and nocodazole, respectively) were used in order to elucidate whether either of these cytoplasmic components had an effect on the ability of mESC's and partially-differentiated mESC's to attach to GXG.

## 2. Materials and methods

### 2.1. Cell culture

E14Tg2A and 46C mouse embryonic stem cells (Stem Cells Inc., Palo Alto, USA) were cultured on tissue culture flasks (NUNC, Waltham, USA) coated with 0.1% v/v gelatin (Sigma–Aldrich, St Louis, USA) in distilled water. Cells were cultured in the absence of feeder cells and passaged every 2 days. On the day of passaging, cells were washed with phosphate buffer saline (PBS) and trypsinised by incubating with trypsin for 3 min before being resuspended in fresh medium and plated onto fresh gelatin-coated tissue culture polystyrene (TCP) flasks.

MESC's were cultured in GMEM (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) (Sera Lab, Haywards Heath, UK), 1% Glutamax (Sigma–Aldrich, St Louis, USA), 1% non-essential amino acids (Sigma–Aldrich, St Louis, USA), 1% Antibiotic–Antimycotic (Invitrogen, Paisley, UK), 0.2% β-mercaptoethanol (Sigma–Aldrich, St Louis, USA), and 0.1% LIF (Millipore, Billerica, USA).

For differentiation experiments, cells were harvested by trypsinisation and resuspended in N2B27 medium. N2B27 medium consists of DMEM-F12 (Invitrogen, Paisley, UK) and N2 growth factor (PAA Laboratories, Pasching, Austria) mixed with Neurobasal Medium (Invitrogen, Paisley, UK) and B27 Neuromix growth factor (PAA Laboratories, Pasching, Austria) at a 1:1 ratio.

β-mercaptoethanol (Sigma–Aldrich, St Louis, USA) was added to a final concentration of 0.1 mM from a 0.1 M stock. 1% v/v FBS was added in order to allow mESC's to attach. Cells were then counted and seeded at a density of $1 \times 10^4$ cells/$cm^2$.

For studies using partially-differentiated cells, mESC's were seeded onto TCP in N2B27 medium for 4 or 6 days before being harvested by trypsinisation. Cells were then seeded onto GXG and TCP as with mouse ES cells. 4 days was chosen as the partial-differentiation time as this is the timeframe in which nestin-positive neural precursors begin to substantially appear in colonies when mouse ES cells are differentiated in N2B27 medium (Fig. 2A). 6 days was chosen as a second time-point as this was the when immature neurons begin to form in adherent monoculture [32] (also see Supplementary Fig. 3).

### 2.2. GXG synthesis

The GXG synthesis protocols were outlined in two earlier studies by Al-Rekabi and Pelling [38,39]. Briefly, stock solutions of gelatin (Sigma–Aldrich, St Louis, USA) were made up in either water or phosphate buffer saline (PBS). Concentrations of gelatin in the solution varied from 3% (corresponding to 2 kPa Young's modulus) in PBS to 4% (18 kPa) and 6% (35 kPa) in water.

The gelatin stock solution was then mixed with glutaraldehyde (Sigma–Aldrich, St Louis, USA) at a ratio of 5 μL glutaraldehyde per 1 mL of gelatin stock solution. The mixture was used to coat 6-well plates and left overnight.

The next day, 1 g/L sodium borohydride (Sigma–Aldrich, St Louis, USA) solution (in PBS) was added to the plates to wash out any residual unreacted glutaraldehyde. After 1 h, plates were washed overnight in PBS. The following day, growth medium (-LIF) was added and plates were allowed to equilibriate for 4–5 h prior to cell seeding.

### 2.3. Atomic force microscopy

An atomic force microscope (AFM) (Nanowizard I, JPK Instruments, Berlin, Germany) was used to measure GXG Young's modulus. For nanomechancal analysis the AFM was mounted on