

# MULTIPLEX MICROFLUIDIC PERFUSION IDENTIFIES SHEAR STRESS MECHANOSENSING MEDIATORS IN MOUSE EMBRYONIC STEM CELLS

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## ABSTRACT

Shear stress is a ubiquitous but obscure environmental cue in perfusion stem cell cultures, especially under self-renewing (pluripotency maintenance) conditions. We used a multiplex microfluidic array, which overcomes limitations in shear application throughput and precision faced by macro-perfusion systems, to comprehensively study shear effects on self-renewing mouse embryonic stem cells. When compared to static cultures with or without a saturated soluble environment, shear stress specifically upregulates the epiblast maker, *Fgf5*. Further targeting of related molecular elements revealed that heparan sulfate proteoglycans are involved in shear mechanosensing. This study demonstrates microfluidics' utility in parsing flow-dependent factors within the stem cell niche.

**KEYWORDS:** microfluidic, shear stress, embryonic stem cells

## INTRODUCTION

Recent breakthroughs in stem cell technologies have brought stem cell therapies closer to reality. It is now conceivable to generate patient-specific pluripotent stem cells<sup>1</sup> and expand them in bioreactors<sup>2</sup> for clinical applications. Perfusion culture during stem cell expansion subjects cells to flow-induced shear stress, and it is unclear how these shears alter cell fate, particularly under self-renewing conditions (*i.e.*, conditions that maintain pluripotency). Stem cell behavior in perfused self-renewing environments has typically been studied in macroscale bioreactors,<sup>3</sup> which have complex flow profiles and a limited ability to test multiple conditions, making it difficult to correlate shear stress to stem cell phenotypes directly and comprehensively. In comparison, microfluidic perfusion systems have more defined flow characteristics, consume minute amount of reagents, and are amenable to multiplexing (Figure 1A). Here, we present a microfluidic perfusion system that allows us to precisely apply shears (due to more defined flow) at high resolution (due to increased throughput), and identify, for the first time, the effects of shear on self-renewing mouse embryonic stem cells (mESCs).

## EXPERIMENTAL

### Cell culture

Mouse embryonic stem cells (mESCs) were routinely maintained in stem cell (SC) medium, consisting of DMEM basal medium supplemented with 15% fetal calf serum, 4 mM L-glutamine, 1 mM non-essential amino acid, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 100  $\mu$ M  $\beta$ -mercaptoethanol and 10 ng/ml Leukemia Inhibitory Factor (LIF). mESC-conditioned medium (CM) was prepared by conditioning SC medium with  $5 \times 10^3$  mESCs/cm<sup>2</sup> for 2 days, dialyzing with a 3 kDa filter, and reconstituting the concentrate with fresh SC medium.

### Perfusion device design and fabrication

The 1x6 logarithmic microfluidic array is a two-layered polydimethylsiloxane (PDMS) device, which consists of a 250  $\mu$ m-thick fluidic layer that is controlled by microvalves actuated via a second pneumatic layer (Figure 1B). The fluidic layer includes six 10 mm (length) x 1.25 mm (width) x 250  $\mu$ m (height) cell culture chambers with 100  $\mu$ m (width) x 100  $\mu$ m (height) flow-setting resistor channels at the outlet of the chambers. The lengths of the flow-setting resistor channels in each culture chamber were designed based on fluidic resistance calculation, such that the flow rates in each culture chamber vary by a factor of four. The pneumatic layer consists of 250  $\mu$ m high pneumatic lines connecting two sets of 150  $\mu$ m-high pneumatically actuated displacement chambers, which control fluid

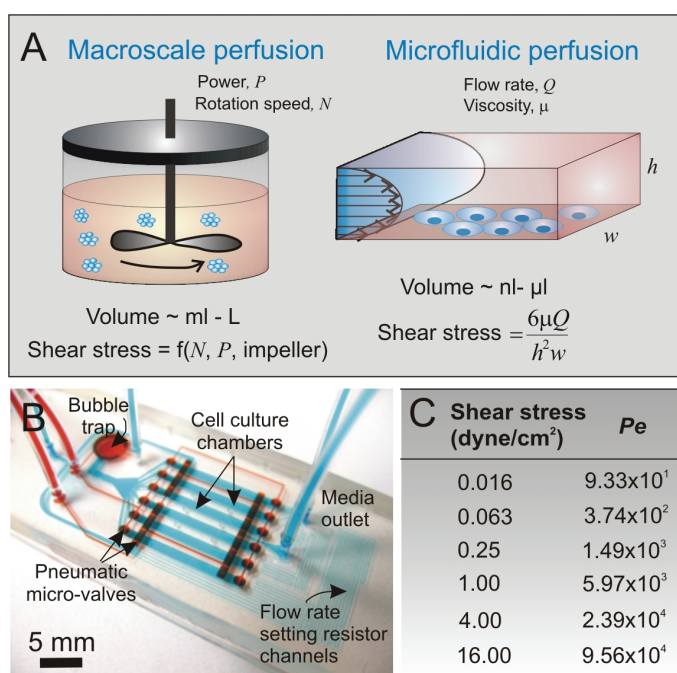


Figure 1: Multiplex logarithmic microfluidic perfusion array for probing shear stress effects on stem cells. (A) Microfluidic perfusion systems exhibit more defined shear stress profiles and consume less reagents than macro-scale perfusion systems *e.g.*, bioreactors, which we capitalized in this work. (B) A 1x6 multiplex logarithmic microfluidic array for simultaneous application of shear stress varying over a range of 1000x. Shear stress in each culture chamber is controlled by the flow rate-setting resistor channels at the chamber outlet. (C) Range of shear stress with corresponding Peclet number ( $Pe$ ) for a typical soluble factor ( $MW \sim 20$  kDa) secreted by mouse embryonic stem cells (mESCs) investigated in this study.

paths during cell seeding and medium perfusion. The 2 layers were first fabricated by PDMS replica molding with plastic molds, obtained from stereo lithographic rapid prototyping, before being plasma bonded together.

### Perfusion culture and assessment of mESCs

The device and connectors were autoclaved, assembled and coated with 0.1% gelatin.  $1 \times 10^6$  cells/ml of mESCs were seeded and allowed to attach overnight. Perfusion was initiated by circulating 25 ml of SC medium or CM continuously with a peristaltic pump. Open dish cultures were used as static controls, where cells were seeded at an areal density equivalent to that of perfusion culture ( $0.85 \times 10^4$  cells/cm<sup>2</sup>). Functions of heparan sulfate proteoglycans (HSPGs) were disrupted with 15 mU/ml Heparinase III, which digests heparan sulfate chains from the core glycoproteins. mESC phenotypes were assessed after 72 hours of culture by quantifying proliferation and transcriptional expression. Proliferation was determined by quantifying the fold increase in cell attachment area at a time point over the initial cell attachment area after seeding by image analysis. Transcriptional expression was measured by quantitative real-time PCR (qRT-PCR).

## RESULTS AND DISCUSSION

Current shear-applying microfluidics typically focus on mimicking physiologically relevant shear experienced by endothelial cells, and thus operate over a narrow range of shears.<sup>4</sup> Because we want to comprehensively investigate shear stress effects that stem cells may be subjected to in a bioreactor, we designed a 1x6 multiplex logarithmic microfluidic array (Figure 1B) to screen across a wide range of flow rates, corresponding to 1000× variation in applied shear stress while in a mass transport convective regime (Peclet number >1) (Figure 1C).

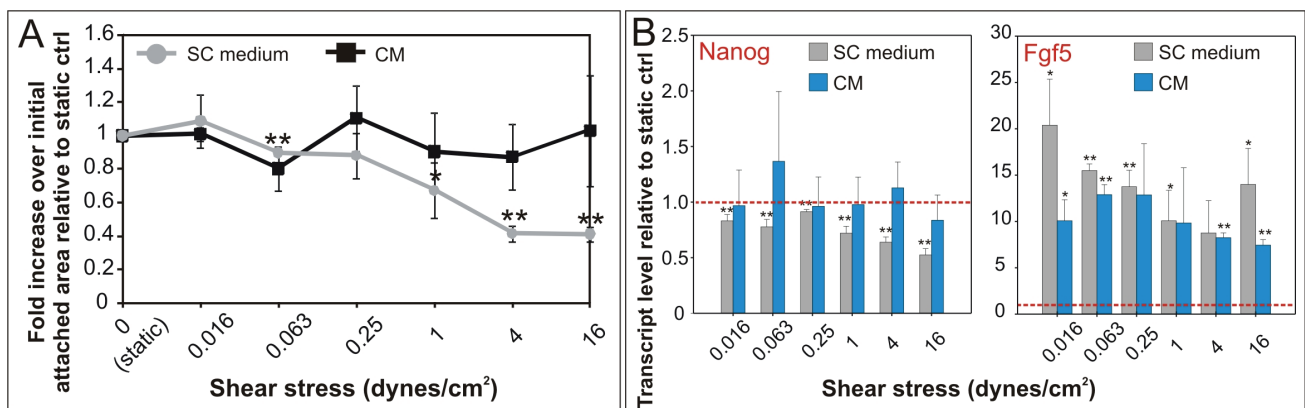
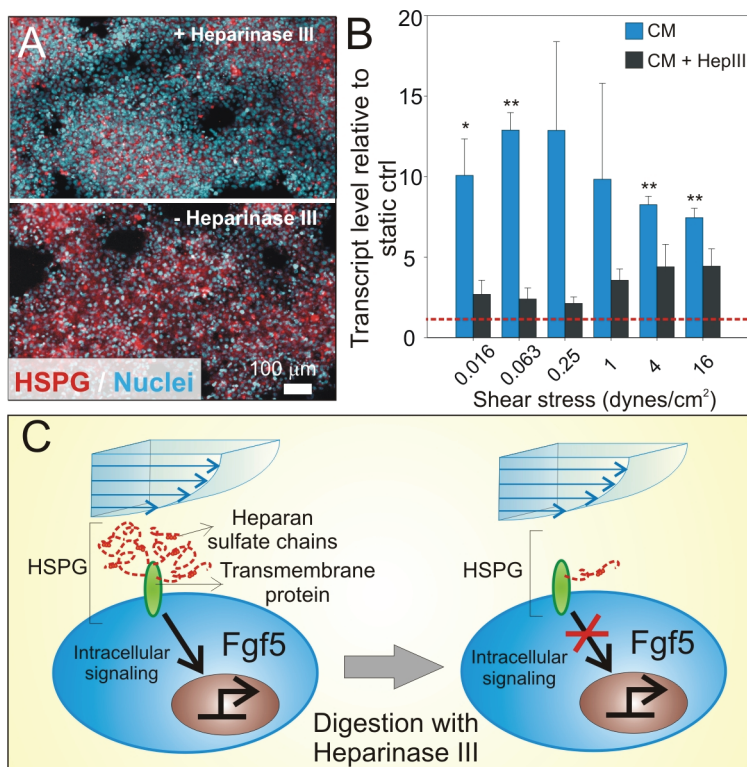


Figure 2. Perfusion culture of mESCs with either normal stem cell (SC) medium or mESC conditioned medium (CM) to saturate the soluble environment. (A) Proliferation of mESCs after 72 hours of perfusion at different shear stresses. (B) Comparison of Nanog and Fgf5 expression after 72 hours of perfusion at different flow rates CM can rescue proliferation and Nanog expression to levels observed in static control (red line) but did not alter perfusion-mediated Fgf5 upregulation. Data are average  $\pm$  s.e.m of 3 experiments. Asterisks indicate statistically significant difference between perfusion and static cultures (Student's t-test). \*  $p < 0.1$ , \*\*  $p < 0.05$ .

We assessed mESCs' proliferation and mRNA expression of molecular markers indicative of various stem cell states, ranging from maintenance of pluripotency (self-renewal), to exit from self-renewal (epiblast) and finally lineage commitment (differentiation). The multiplex nature of our system allows for both pairwise comparison with static (*i.e.*, open dish) controls and correlation across shear magnitudes, which enabled us to identify proliferation (Figure 2A), Nanog and Fgf5 (Figure 2B) as flow-dependent phenotypes. Fluid flow can affect cell phenotype either via shear or by sweeping away soluble factors, and so to determine whether the observed flow-dependent phenotypic changes were indeed caused by shear stress, mESC conditioned medium (CM) was used to saturate the soluble environment during perfusion culture. This CM was able to restore proliferation (Figure 2A) and Nanog expression (Figure 2B) to static values, but did not alter Fgf5 expression (Figure 2B), suggesting that Fgf5 was modulated by shear stress while proliferation and Nanog were mediated by soluble factors.

By pin-pointing Fgf5 as a shear-modulated mESC phenotype, we then targeted molecular regulators of Fgf5. Heparan sulfate proteoglycans (HSPGs) are a known shear stress mechanotransducer in endothelial cells<sup>5</sup>, and modulator of Fgf signaling in stem cells.<sup>6</sup> Hence, we hypothesized that HSPGs are involved in shear stress sensing in this study, causing the upregulation of Fgf5 in perfused mESCs. We test this hypothesis by disrupting HSPGs functions with Heparinase III treatment to digest away heparan sulfate chains from the core glycoproteins (Figure 3A). When compared to static controls similarly treated with Heparinase III, we observed that the upregulation of Fgf5 in perfused mESCs was attenuated when HSPGs function was disrupted (Figure 3B). This suggests that HSPGs are involved in transducing shear stress to direct mESCs to exit from a self-renewing program and transition into an epiblast-like state (Figure 3C).



*Figure 3. Disruption of heparan sulfate proteoglycans (HSPGs) in mESC attenuates shear-mediated Fgf5 upregulation. (A) Addition of Heparinase III (HepIII) decreases the amount of heparan sulfate chains (red) as shown by immunofluorescence staining. (B) Comparison of Fgf5 expression after 72 hours of perfusion with CM alone or CM + HepIII across different shear stresses. HepIII addition decreases Fgf5 expression to levels of static control, indicating that blocking HSPGs removes the ability to mESCs to upregulate Fgf5 in response to shear. Data are average  $\pm$  s.e.m of 3 experiments. Asterisks indicate statistically significant difference between perfusion and static cultures (Student's t-test). \*  $p < 0.1$ , \*\*  $p < 0.05$ . (C) Schematic illustrating how HSPGs may be involved in sensing shear stress acting on mESCs.*

## CONCLUSION

Our results are the first instance where multiplex microfluidic perfusion is used to probe shear stress effects in a graded, quantitative manner, and has identified HSPGs to be involved in shear mechanosensing in self-renewing mESCs.

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## REFERENCES

- [1] I.H. Park, R. Zhao, J.A. West, A. Yabuuchi, H. Huo, T.A. Ince, P.H. Lerou, M.W. Lensch, G.Q. Daley, Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*, 451, pp. 141-146 (2008).
- [2] D. Steiner, H. Khaner, M. Cohen, S. Even-Ram, Y. Gil, P. Itsykson, T. Turetsky, M. Idelson, E. Aizenman, R. Ram, Y. Berman-Zaken, B. Reubinoff, Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension. *Nature Biotechnology*, 28, pp. 361-364 (2010).
- [3] S.M. Dang, S. Gerecht-Nir, J. Chen, J. Itskovitz-Eldor, P.W. Zandstra, Controlled, Scalable Embryonic Stem Cell Differentiation Culture. *Stem Cells*, 22, pp. 275-282 (2004).
- [4] E.W.K. Young, C.A. Simmons, Macro- and microscale fluid flow systems for endothelial cell biology. *Lab Chip*, 10, pp. 143-160 (2010).
- [5] J.A. Florian, J.R. Kosky, K. Ainslie, Z. Pang, R.O. Dull, J.M. Tarbell, Heparan Sulfate Proteoglycan Is a Mechanosensor on Endothelial Cells. *Circ Res*, 93, pp. 136-142 (2003).
- [6] F. Lanner, K.L. Lee, M. Sohl, K. Holmborn, H. Yang, J. Wilbertz, L. Poellinger, J. Rossant, F. Farnebo, Heparan Sulfation Dependent FGF Signalling Maintains Embryonic Stem Cells Primed for Differentiation in a Heterogeneous State. *Stem Cells*, 28, pp. 191-200 (2009).

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