Intrinsic Fibronectin Matrix Properties Regulate Differentiation of Embryonic Stem Cells Better than Canonical Growth Factors

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Introduction: The embryonic stem cell (ESC) niche employs a wide variety of external cues that are presented with precise spatial and temporal patterning to direct appropriate cellular arrangements and patterns. Complex sets of soluble growth factors, intended to recapitulate the early developmental regulation of a specific lineage, have long been used to differentiate ESCs into specific lineages. However, some growth factors, such as Activin A used in endoderm differentiation, have also been linked to upregulation of fibronectin (FN), which assembles into a fibrillar structure. Though FN matrix is known to regulate development, it is not clear if its presence and resulting intrinsic properties can also regulate earlier fate choices as with ESCs. To address this question, we employed two different differentiation conditions where ESCs were grown as multicellular embryoid bodies (EBs), where all 3 germ layer lineages are present, and on FN materials with specifically engineering properties.

Methods: *Cell culture* of murine ESCs employed conditions previously described [1]. Cells were induced to differentiate into EBs upon removal of an adherent growth substrate. ESCs were also grown with or without the presence of endoderm growth factors and plated on FN matrices to investigate their ability to induce an endoderm phenotype. To make FN matrix for ESCs, NIH3T3 cells were cultured at confluence for 5 days to assemble a matrix and then removed via hypotonic detergent solubilization prior to ESC plating. Matrices were selectively crosslinked with organics. FN-coated surfaces were prepared using 10 ug/ml stock solution. *Immunofluorescent staining* employed R457 rabbit anti-FN and anti-GATA-4 rabbit polyclonal antiserum. Samples were labeled with a DAPI stain and examined by a CARV II confocal microscope. *Quantitative PCR* was performed using select self-renewal, mesoderm, ectoderm, and endoderm genes as well as microarrays. *Western blots* used the anti-GATA4 rabbit polyclonal antibody, anti-α_5 integrin rabbit polyclonal and anti-GAPDH monoclonal antibody.

Results: To first establish a correlative link between FN expression and ESC fate, stem cells were cultured as EBs. As matrix is first secreted within EBs, FN is upregulated 4-fold and preferentially localized at the edge of EBs at the same time when GATA4, an endoderm marker, is also preferentially expressed at the edge of the EB. Nanog, an undifferentiated marker, is inversely correlated to this expression as the EBs mature, indicating both a temporal and spatial correlation. Since complex soluble signals could influence matrix production and endoderm expression in EBs, we further cultured ESCs as cell monolayers grown on FN materials in chemically defined environments.

ESCs were plated onto FN-coated surfaces and fibrillar FN matrix. At day 7 in culture, ESCs cultured on the fibrillar FN matrix showed more GATA4 positive nuclei compared to the FN-coated substrate (Figure 1). Microarrays and quantitative PCR also showed that ESCs cultured on the fibrillar FN matrix relative to FN-coated surfaces expressed higher levels of 21 endoderm genes and downregulation of 60 markers for undifferentiated cells and mesoderm and ectoderm lineages. Due to matrix remodeling by day 14, ESCs on both the FN-coated coverslips and fibrillar FN matrix have 15- and 25-fold upregulated and specific expression of endoderm markers, respectively. Also by day 14, 10-fold stiffer, crosslinked matrix (4.5 kPa versus uncrosslinked 0.4 kPa matrx) had the largest increase in endoderm expression at a 61-fold increase, which is also significantly different from the other lineages. Specifically, endoderm genes, such as Cdh5, Cxcr4, GATA4/6, Sox17, and FoxA2[2], were upregulated on this crosslinked matrix as much as 600-fold compared to less than 2-fold upregulation at day 7, again suggesting temporal regulation of ESCs by fibronectin materials. On the other hand, treatment of these conditions with endoderm growth factors did not significantly enhance endoderm expression, i.e. 15-fold upregulation with growth factors versus 25-fold upregulation without growth factors.

As for the mechanism(s) of intrinsic matrix property sensing, 2-fold more α5 integrin was in the bound state for differentiated cells on fibrillar FN matrix compared to undifferentiated ESCs. Moreover, when interfering RNA was used to block expression of α5 integrin, GATA4 expression was also inhibited. Downstream of integrins, inhibition of Src kinases and ROCK prevented ESCs from sensing matrix assembly and stiffness, respectively.

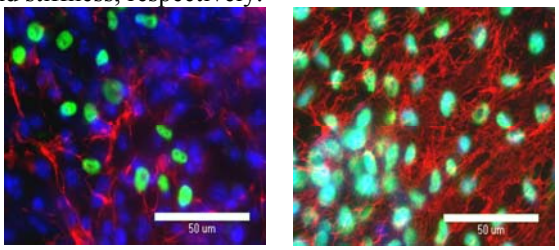

Figure 1. Immunofluorescent images of GATA4-stained (green), FN (red) or DAPI (blue) in ESCs cultured on a FN-coated surface (left) and fibrillar FN matrix.

Conclusions: The results of present studies confirmed that matrix assembly and mechanics, when displayed at the right time and place, induce endoderm expression in ESCs.

References:

1. Robertson et al. Nature. 1986; 323: 445-448.
2. D'Amour et al. Nature Biotech. 2005; 23: 1534-41.