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Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm

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Abstract

Nodal, a member of the TGF-β family of signaling molecules, has been implicated in pluripotency in human embryonic stem cells (hESCs) [Vallier, L., Reynolds, D., Pedersen, R.A., 2004a. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol. 275, 403–421], a finding that seems paradoxical given Nodal's central role in mesoderm/endoderm specification during gastrulation. In this study, we sought to clarify the role of Nodal signaling during hESC differentiation by constitutive overexpression of the endogenous Nodal inhibitors Lefty2 (Lefty) and truncated Cerberus (Cerb-S) and by pharmacological interference using the Nodal receptor antagonist SB431542. Compared to wildtype (WT) controls, embryoid bodies (EBs) derived from either Lefty or Cerb-S overexpressing hESCs showed increased expression of neuroectoderm markers Sox1, Sox3, and Nestin. Conversely, they were negative for a definitive endoderm marker (Sox17) and did not generate beating cardiomyocyte structures in conditions that allowed mesendoderm differentiation from WT hESCs. EBs derived from either Lefty or Cerb-S expressing hESCs also contained a greater abundance of neural rosette structures as compared to controls. Differentiating EBs derived from Lefty expressing hESCs generated a dense network of β-tubulin III positive neurites, and when Lefty expressing hESCs were grown as a monolayer and allowed to differentiate, they generated significantly higher numbers of β-tubulin positive neurons as compared to wildtype hESCs. SB431542 treatments reproduced the neuralising effects of Lefty overexpression in hESCs. These results show that inhibition of Nodal signaling promotes neuronal specification, indicating a role for this pathway in controlling early neural development of pluripotent cells.

Keywords: Human embryonic stem cells; Lefty; Nodal; Activin; Neuroectoderm; Neurons

Introduction

Embryonic stem cells (ESCs) derive their pluripotency from their embryonic origin in the inner cell mass of blastocysts (Evans and Kaufman, 1981; Thomson et al., 1998). They are unique among mammalian cells in being able to grow indefinitely *in vitro* while maintaining a capacity to differentiate into all adult cell types. Interestingly, recent studies have shown that mouse ESCs (mESCs) and human ESCs (hESCs) maintain their pluripotent status using different signaling pathways. LIF signaling is essential for mESC self-renewal (Nichols et al., 1990; Yoshida et al., 1994), but it is not active in hESCs (Daheron et al., 2004; Humphrey et al., 2004). Instead, Activin/Nodal signaling is essential for the maintenance of hESC pluripotency and self-renewal, with FGF-2 acting as a competence factor (Vallier et al., 2004a, 2005; James et al., 2005; Beattie et al., 2005; Xiao et al., 2006).

Activin and Nodal, members of the TGF-β superfamily, are also responsible for inducing mesendoderm, the precursor of the definitive mesodermal and endodermal lineages during gastrulation (reviewed in Schier, 2003). Nodal signaling is regulated by Cripto, an extracellular GPI-linked protein that

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acts as a co-factor. Endogenous inhibitors of Nodal signaling including Lefty1, Lefty2 and Cerberus, are capable of modulating early vertebrate development (Perea-Gomez et al., 2002; Meno et al., 1999; Belo et al., 1997). Recent evidence suggests that Lefty2 binds to Cripto and prevents the formation of the Nodal/Cripto/receptor complex, thereby inhibiting Nodal signaling (Chen and Shen, 2004; Cheng et al., 2004). A truncated form of Cerberus, Cerb-S, has been found to block Nodal specifically by direct binding to the ligand (Piccolo et al., 1999). Lefty expression at peri-implantation stages of mouse development further suggests an early role for it in anterior–posterior patterning in the mouse embryo (Takaoka et al., 2006). In each of these cases the probable mechanism of inhibitor action is the disruption of signaling through the Activin/Nodal pathway.

Although numerous studies have shown the importance of diminished BMP signaling for neural induction during vertebrate development (reviewed in Munoz-Sanjuan and Brivanlou, 2002), less is known about the role of other members of the TGFβ superfamily. Nodal$^{-/-}$ embryos show enhanced neuroectoderm differentiation (Camus et al., 2006), suggesting that Nodal acts as an inhibitor of neuroectoderm specification *in vivo*. In addition, Cripto$^{-/-}$ mESCs show a propensity for neuronal differentiation (Liguori et al., 2003; Sonntag et al., 2005). Furthermore, recent studies suggest a role of Nodal inhibition in neural induction in mouse and human ESCs *in vitro* (Vallier et al., 2004a; Watanabe et al., 2005). Lefty expression has also been characterised in hESCs during their differentiation and has been implicated in ectoderm specification (Dvash et al., 2007). Thus, Nodal signaling not only controls mesendoderm differentiation during gastrulation, but may also have a reciprocal developmental role in preventing cells from differentiating into ectodermal or neural fates both *in vivo* and *in vitro*.

In this study, we investigated whether the inhibition of Nodal signaling in hESCs would promote their specification into neural progenitors *in vitro*. Nodal inhibition was initially accomplished by overexpressing full-length Lefty2 (Lefty) or a truncated form of Cerberus (Cerb-S) in hESCs grown in Chemically Defined Medium (CDM), where BMP signaling is quiescent (Vallier et al., 2005). We found that overexpression of either Lefty or Cerb-S resulted in a pronounced increase in neuroectoderm development. Furthermore, similar effects could be achieved with the specific pharmacological inhibitor of type I Activin/Nodal receptor signaling, SB431542. These data strongly support the hypothesis that Nodal signaling inhibits neuroectoderm specification during early hESC differentiation (Vallier et al., 2004a; Sonntag et al., 2005; Camus et al., 2006), and provide a robust means of achieving neural differentiation from hESCs.

Materials and methods

Human embryonic stem cell culture

H9 (WiCell Inc., Madison, Wisconsin, USA) and hSF-6 (UCSF, San Francisco CA, USA) hESCs were routinely cultured as described (Schatten et al., 2005) in KSR medium containing KO-DMEM supplemented with Serum Replacement (Invitrogen), glutamate (1 mM) and β-mercaptoethanol (0.1 mM). Every 4 days, cells were harvested using 1 mg/ml collagenase IV (Gibco) and then plated into 60 mm plates (Costar) pre-coated with 0.1% porcine gelatin (Sigma) and containing irradiated mouse embryonic fibroblasts. For serum replacer-free culture, hESCs were grown in Chemically Defined Medium (CDM) (Johansson and Wiles, 1995), supplemented with Activin (10 ng/ml, R&D systems) and FGF2 (12 ng/ml, R&D systems). The composition of CDM was 50% IMDM (Gibco) plus 50% F12 NUT-MIX (Gibco), supplemented with 7 μg/ml of insulin (Roche), 15 μg/ml of transferrin (Roche), 450 μM of monothioglycerol (Sigma) and 5 mg/ml bovine serum albumin fraction V (Sigma). To enable hESCs to adhere in CDM, plates were pre-coated with foetal bovine serum (FBS)(Hyclone) or Fibronectin 10 μg/ml (Chemicon) for 24 h at 37 °C and then washed twice in PBS.

Karyotypic analyses were performed on H9 and hSF-6 cells at various passages. Abnormalities involving chromosomes 9, 5, and 19 were occasionally observed at late passages (p80–p115) confirming that hESCs can incur genetic alterations under certain conditions (Draper et al., 2004). Consequently only hESCs from earlier passages (p50–p70) were used for these experiments.

Generation of expression constructs and stable transfection

Plasmid pTP6 (Pratt et al., 2000) containing the CAGG (hCMV/Chicken β-Actin chimeric promoter) driving the expression of GFP-IRES-Puromycin, was used as the basis for constructing Lefty and Cerb-S expression vectors. pTP6 was digested with EcoR1, removing the GFP open reading frame (ORF), and either mouse Lefty2 or Xenopus Cerb-S ORFs were cloned into this site to generate pTP6-Lefty2 and pTP6-Cerb-S respectively. For stable expression with vectors encoding Lefty2 or Cerb-S, 3 confluent 60 mm plates containing around 2000 hESC colonies each were plated onto one 6 well gelatin-coated plate containing mouse feeders. After 48 h the cells were transfected using Lipofectamine 2000 (Invitrogen) as described (Vallier et al., 2004b). Three days after transfection, the cells were passaged onto 60 mm gelatin-coated tissue-culture plates containing puromycin-resistant mouse feeders. After 3 additional days, puromycin (1 μg/ml final concentration) was added. Puromycin-resistant colonies that appeared by 12 days of selection were picked, dissociated and plated onto 24-well gelatin-coated, feeder containing plates and expanded for further analysis as described above.

Differentiation of hESCs as EBs

Lefty or Cerb-S expressing hESCs (as well as wildtype H9 and hSF-6 hESCs) were grown in 6 cm dishes (Corning), on mouse feeders. When confluent, hESCs were passaged using collagenase 1 mg/ml, as previously described (Schatten et al., 2005) and cultured in non-adherent conditions in CDM supplemented with either SU5402 (Calbiochem; 10 μM) or SB431542 (Tocris; 20 μM). Alternatively, hESCs were incubated in DMEM supplemented with 10% FCS and 1 mM glutamine (MEF medium) to promote mesoderm formation. EBs were then grown for 12–16 days at 37 °C at 5% CO_2 before being harvested for histological or molecular marker analysis. For some experiments, EBs grown in CDM were plated directly on fibronectin.

Differentiation of hESCs as a monolayer

Wildtype and Lefty expressing hESCs were grown on fibronectin in FGF(12 ng/ml)/Activin (20 ng/ml) as previously described as a monolayer in the absence of a feeder layer (Vallier et al., 2005). When cells were 70–80% confluent medium was changed to either CDM only, CDM/FGF (12 ng/ml), CDM/Noggin 200 ng/ml, CDM/SU5402 and differentiated for 24 days.

RT-PCR and real-time PCR

Total RNA was extracted from EBs using the Qiagen RNeasy Micro kit according to manufacturer instructions. For RT-PCR, 50–100 ng of RNA were