GDF3, a BMP inhibitor, regulates cell fate in stem cells and early embryos

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The TGFβ superfamily of ligands plays key functions in development and disease. In both human and mouse embryonic stem cells, a member of this family, GDF3, is specifically expressed in the pluripotent state. We show that GDF3 is an inhibitor of its own subfamily, blocks classic BMP signaling in multiple contexts, interacts with BMP proteins and is expressed specifically in the node during gastrulation in a pattern consistent with BMP inhibition. Furthermore, we use gain- and reduction-of-function to show that in a species-specific manner, GDF3 regulates both of the two major characteristics of embryonic stem cells: the ability to maintain the undifferentiated state and the ability to differentiate into the full spectrum of cell types.

KEY WORDS: GDF3, TGFβ, BMP, Embryonic stem cell, Inhibitor, *Xenopus*, Human, Mouse

INTRODUCTION

To begin to understand the molecular character of totipotent cells and the mechanisms of the primary cell fate decisions of the early mammalian embryo, we previously performed a global analysis of all genes expressed in human embryonic stem (ES) cells when cultured under undifferentiated versus differentiated conditions (Sato et al., 2003). The examination of signaling components specific to the state of pluripotency revealed a very small number of ligands belonging to the main signaling pathways involved in early embryogenesis. Notably, only three secreted factors of the TGFβ superfamily are expressed at high levels during pluripotency and decline dramatically when the cells are allowed to differentiate, suggesting a role during this process: LEFTY1, LEFTY2 and GDF3 (Sato et al., 2003). Both LEFTY proteins have been previously shown to be secreted inhibitors of TGFβ ligands (reviewed by Branford and Yost, 2004). The role of GDF3, however, is unknown, as this TGFβ family member has not been as extensively studied as the others. In this study, we dissect the cellular and biochemical functions of GDF3 in the context of embryonic development and ES cells.

GDF3 is a TGFβ superfamily member subclassified into the bone morphogenetic protein/growth and differentiation factor (BMP/GDF) branch of this family (Jones et al., 1992). GDF3 is most similar to VG1 (57% amino acid identity, 79% similarity) and has 53 and 50% identity to BMP2 and BMP4, respectively. GDF3 has only six of the classical seven cysteines present in other TGFβ superfamily members (Jones et al., 1992). It is missing the fourth cysteine – the one involved in inter-molecular interactions among TGFβ family members and with their secreted inhibitors (Groppe et al., 2002).

GDF3 is a mammalian-specific TGFβ ligand as it has not been found in the genomes of fugu, frog, zebrafish or chick. It was first isolated from a mouse embryonic day (E) 6.5 cDNA embryonic library by homology to *Xenopus VG1*, although neither GDF3 mRNA expression nor GDF3 protein has ever been detected before mid-gestation mouse development (Jones et al., 1992). Later, GDF3 is expressed in embryonic bone and adult bone marrow, thymus, spleen and fat. It is also expressed in the pluripotent mouse teratocarcinoma cell line, F9, where its expression is reduced upon retinoic acid-induced differentiation (Jones et al., 1992; McPherron and Lee, 1993). Human *GDF3* was identified through subtractive hybridization in activin-treated embryonic carcinoma (EC) cell lines (Caricasole et al., 1998).

Although very little is known about GDF3, the roles of its other subfamily members, classic BMPs, have been well characterized. In the early *Xenopus* embryo, one of the earliest known functions of BMP signaling occurs in the late blastula and leads to the inhibition of neural fate in the dorsal ectoderm and induction of epidermal fate in ventral ectoderm (Munoz-Sanjuan and Brivanlou, 2002). In mammalian embryogenesis, the first in-vivo cell fate decision of the blastocyst is between inner cell mass and trophoblast fates. Recent evidence suggests that BMP signaling also plays a role in this crucial balance, as the addition of classic BMPs to human ES cells results in differentiation to trophoblast (Xu et al., 2002). Conversely, in mouse ES cells, BMPs have been shown to promote the maintenance of pluripotency, or 'stemness', in cooperation with LIF (Ying et al., 2003).

To study the potential function of GDF3 in the undifferentiated, pluripotent state of ES cells, we characterized GDF3 expression throughout very early murine embryogenesis. We also show that GDF3 is a direct BMP inhibitor in early embryos and pluripotent cells. In addition to its embryological and biochemical characterization, we found that GDF3 acts in human ES cells to maintain markers of pluripotency, while, paradoxically, in mouse ES cells it acts to maintain the ability to differentiate in vitro into cell types representing all germ layers.

MATERIALS AND METHODS

Handling of *Xenopus* embryos

Embryos were obtained and manipulated as previously described (Hemmati-Brivanlou et al., 1989) and staged as described in Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryo explants (animal caps, ventral and dorsal marginal zones) were isolated in 0.1× MMR, washed once and transferred immediately to 0.5× MMR with gentamycin for culture. Staging was determined using sibling embryos.

Cell culture

BGN1 and Jasmine human ES cells were maintained as previously described (Sato et al., 2003). Ten ng/ml of recombinant human BMP4 (rhBMP4) (R&D) was used for treatments. P19 cells were maintained in

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MEM-α-modified media (Sigma) with 7.5% CBS, 2.5% FBS. C2C12 cells were maintained in DMEM (Sigma) with 15% FBS. For differentiation of C2C12 cells, cells were grown to confluence, changed to DMEM with 2% serum and BMP4 (100 ng/ml) or TGFβ1 (R&D) (1 ng/ml) was added. Genetrap ES cells (The Wellcome Trust Sanger Institute) parent strain 129O1a and genetrap AD0857 cells were cultured on gelatin with 1400 U LIF/ml in 1× GMEM containing 10% FBS, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 1× non-essential amino acids and 55 μmol/l β-mercaptoethanol.

Embryoid body formation
Cells were trypsinized to a single cell suspension, counted and diluted to 10,000 cells/ml in ES growth media with no LIF. Droplets of cell suspension (25 μl) were placed on the lid of a 10-cm bacterial culture dish and cultured inverted over media for 2 days. Embryoid bodies were then flushed into EB media (DMEM with 10% FBS) and cultured for 8 days. Percentage EB formation was assessed by counting the number of embryoid bodies formed/number of droplets per condition ×100%.

RT-PCR
For *Xenopus*, ten animal caps or one embryo were isolated. For mouse, five pre-implantation embryos, three gastrulation stage embryos or one post-gastrulation stage embryo were used. For stem cells, 0.1-1 ng RNA was used. Primer sequences are available upon request.

Luciferase assays
All luciferase assays were done in three separate experiments, each in triplicate; representative individual triplicate experiments are shown in the Results section. In *Xenopus* embryos, 20 pg of luciferase DNA construct (BRE-Lux) was injected into the animal region of two cell embryos together with the indicated RNAs: mouse BMP4 (0.5 ng/embryo), mouse GDF3 (0.5 ng or 1 ng/embryo) transcribed from constructs in pCS2++. Pools of four embryos were harvested at stage 11 in 50 μl of lysis buffer. In P19 cells, cells were transfected with 150 ng of reporter, 3.3 ng of renilla reporter, 0.25 μg of SMAD1 and SMAD4 (each), 0.1 μg of OAZ and test constructs in pCS2++ or empty vector for a total of 1.6 μg/well. After 6 hours of transfection, media was changed to MEM-α-modified media with 0.2% serum. After 32 hours, cells were lysed in 150 μl of lysis buffer and analyzed for luciferase activity. The error bars indicate standard deviation.

Immunoprecipitations and western blots
Immunoprecipitations on overexpressed proteins were performed as previously described (Yeo and Whitman, 2001) with mouse αFLAG (Sigma; 1 μl/sample) or mouse αHA (Babco; 5 μl/sample). Antibodies used in western blots were mouse αtubulin (Sigma; 1:1000); rabbit αcyclophillinB (Affinity BioReagents; 1:2000); mouse αSMAD1 (Santa Cruz; 1:750); mouse αSMAD2 (BD Biosciences; 1:750); rabbit αP-SMADs (Cell Signaling; 1:1000); mouse αOCT3 (BD Transduction Laboratories; 1:1000); mouse αTROMA1 (Dev Studies Hybridoma Bank; 1:75); goat αGDF3 (R&D; 1:10,000); mouse αFLAG (Sigma; 1:10,000); mouse αHA (Covance; 1:1000). α indicates antibody.

In-situ hybridization
Mouse embryos were obtained from wild-type Swiss Webster and C57BL/6 pregnant mice. The day of development was determined as the number of days following morning observation of a coital plug (E0.5). Embryos were dissected into cold PBS and fixed, and whole-mount in-situ hybridization was performed as described (Merrill et al., 2004). Probes were prepared from *Gdf3* in pCS2++ (anti-sense *Pst*I/T3; full-length sense *Not*I/Sp6). Sectioning of whole-mount embryos was performed with 10-μm cryosections after embedding in OCT.

Immunofluorescence
Blastocysts were fixed in 4% paraformaldehyde, then washed in PBS containing 0.25% BSA, blocked and permeabilized in 10% donkey serum with 3% Triton, and incubated in primary antibody gαGDF3 (R&D) (1:100) in 10% donkey serum for 3 hours at room temperature. Samples were then washed and incubated in secondary antibody then counterstained with SytoxGreen (Molecular Probes) for nuclear stain.

Constructs
The coding region of GDF3 was cloned into pCS2++ from pBluescript (generously provided by S. J. Lee) by PCR and was inserted into the *Eco*I/*Not* sites. For sense RNA, GDF3 was linearized with *Not*I and transcribed with SP6. BMP4 untagged is in pSP64T and RNA was produced with *Avr*II/SP6. BMP4-HA was produced with *Avr*II/T7. ActivinB-HA is in pCS2++ and was produced with *Not*I/SP6. VG1-HA (provided by S. Cheng) is in pcDNA3.1 and was produced with *Avr*II/T7. xNR1 is in pCS2++ and was produced with *Not*I/SP6.

RESULTS
GDF3 expression is associated with the undifferentiated state of embryonic stem cells
To confirm our microarray findings that GDF3 expression is associated with the undifferentiated state of ES cells, we performed RT-PCR to examine GDF3 mRNA levels in both human and mouse ES cells. We cultured H1 human ES cells and 129Ola mouse ES cells either in conditioned media (CM) or LIF, respectively, which maintain the undifferentiated states, or in the absence of these factors to allow differentiation. We found that while GDF3 was present in the human ES cells grown in CM and the mouse ES cells grown in LIF, it was significantly reduced when these ES cells were allowed to differentiate, together with expression of known stem cell markers (Fig. 1).

GDF3 acts as a BMP inhibitor and elicits secondary axis induction and direct neural induction
As our studies in human and mouse ES cells suggested that GDF3 is involved in the earliest cell fate decisions, we examined the molecular mechanism of GDF3 activity to determine whether it has classic BMP/GDF signaling activity. We chose to perform these experiments with microinjection of GDF3 mRNA into frog embryos, because the effects of TGFβ pathway signaling in *Xenopus* are well established through phenotype and marker gene expression analysis. We reasoned that if GDF3 is an agonist or antagonist of the BMP pathway, its function might be partially determined through its effect on normal frog embryogenesis. In all frog experiments, mouse GDF3
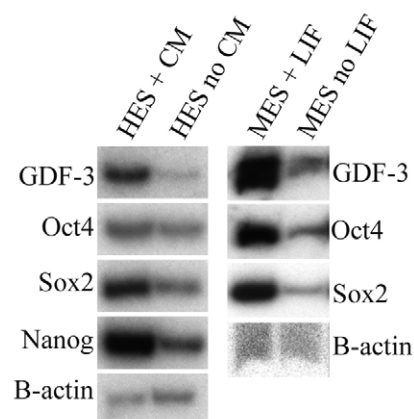

Fig. 1. GDF3 is specifically expressed in the undifferentiated state of embryonic stem cells. (Left panel) RT-PCR of GDF3 expression levels in H1 human embryonic stem cells (HES) in the continued presence (CM) and absence (no CM) of conditioned media for 7 days. (Right panel) GDF3 RT-PCR in 129Ola mouse ES cells in the presence or absence of LIF for 3 days. OCT4, SOX2 and NANOG are markers of pluripotency. β-actin is shown as a loading control; no RT control showed no signal.

mRNA was used, because there is no *Xenopus* homolog of GDF3. The *Xenopus* system is suitable for the study of mammalian proteins, as all major signaling pathways are conserved, allowing us to study GDF3 function in a system with no background levels of protein.

We injected mouse GDF3 mRNA into the prospective marginal zone (MZ) of 4-cell frog embryos. An increase of classic BMP/GDF signaling in this scenario would ventralize the embryos. Instead, we found that injection of mouse GDF3 mRNA in the ventral marginal zone (VMZ) induced secondary dorsal axis formation (Fig. 2A). In six batches of embryos (*n*=151), we found this phenotype in 61% of embryos. Secondary axis induction in amphibian embryos can occur by three mechanisms: (1) induction of the canonical WNT pathway; (2) induction of the SMAD2/3 pathway by nodal/activin signaling; and (3) inhibition of ongoing BMP signals. To address how GDF3 caused a secondary axis, we performed a series of reporter assays in the frog embryo and in a pluripotent mammalian cell line, P19.

For the reporter assays we used a BMP-responsive promoter element that drives expression of luciferase (BRE-Lux) (Hata et al., 2000) in both *Xenopus* embryos and P19 cells. Fig. 2B presents the result of experiments in which BRE-Lux was co-injected with BMP4, GDF3 or both RNAs. While BMP4 increased reporter activity, this induction was strongly inhibited by the presence of GDF3.

To confirm this finding in a cell type that normally expresses GDF3, we then performed similar experiments in P19 cells into which we transfected mouse *Gdf3* with or without mouse *Bmp4*. In

order to generate a robust BMP signal, this system was complemented by co-transfection of downstream signaling components: SMAD1, SMAD4 and OAZ (Hata et al., 2000). Fig. 2C shows that in P19 cells, mouse GDF3 reduced BMP4 signaling. This evidence confirms that GDF3 is an inhibitor of the BMP-GDF subfamily of TGFβ in frog embryos and in mouse pluripotent cells.

To assess the ability of GDF3 to inhibit BMP regulation of cell fate in frog embryos, we examined cell fate changes induced in response to GDF3 in three independent contexts: ectodermal explants (animal caps) and mesodermal explants subdivided into dorsal (DMZ) and VMZ regions. Fig. 2D provides a schematic of the dissection of these explants (Hemmati-Brivanlou, 1999). When dissected and cultured alone, ectodermal explants of *Xenopus* blastula adopted an epidermal fate, and inhibition of the BMP pathway in these cells led to a direct conversion of epidermal to neural fate. Two-cell embryos were microinjected in the animal pole with mRNA encoding mouse GDF3 and allowed to develop to blastula stages. Animal caps were isolated and cultured to either late gastrula (stage 12.5) or late neurula (stage 21). The explants were then harvested and analyzed by RT-PCR for the detection of cell-type-specific markers. Our analysis of expression of early markers (Fig. 2E) and late markers (Fig. 2F) shows that, by contrast to other BMP family members, GDF3 acted as a direct neural inducer. GDF3 decreased expression of the immediate early response to BMP signaling, MSX-1, and increased expression of both early (SOX2) and late neural markers, such as OTX2 and NCAM. This conversion was direct, because it was not concomitant with

Fig. 2. GDF3 acts as a BMP inhibitor in frog embryos and mouse pluripotent cells. (**A**) Stage 41 embryos injected with 2 ng GDF3 RNA in the VMZ at the 4-cell stage (upper embryo) or uninjected sibling embryo (lower embryo). (**B**) Luciferase units of total embryo lysate from frog embryos injected with BRE-Lux and GDF3, BMP4 or both RNAs. (**C**) Relative luciferase units (luciferase:renilla) from total cell lysate of P19 cells transfected with *BRE-Lux* and the BMP signaling components *Smad1*, *Smad4* and *OAZ* and *Gdf3, Bmp4* or both. (**D**) Diagram of frog embryo showing ectodermal explant (animal cap), and VMZ and DMZ. (**E**,**F**) RT-PCR of GDF3-injected and uninjected animal caps matured to sibling stage 12.5 or stage 21 with whole embryo as positive control and whole embryo with no RT (–RT) as a negative control. ODC is shown as a loading control. (**G**) GDF3 protein produced in oocytes injected with either water or GDF3 RNA (50 ng). Oocyte CM was collected and oocytes were lysed at the end of culture (lysate) and western blot was performed with pαGDF3. The prepro form (**) of GDF3 is 45 kD and the mature form (*) is 18 kD. (**H**) VMZ and DMZ explants cultured in 0.5× MMR (–), in the presence of activin protein secreted from oocytes (diluted 1:50 or 1:500), or in the presence of GDF3 protein secreted from oocytes (diluted 1:100 or 1:1000).
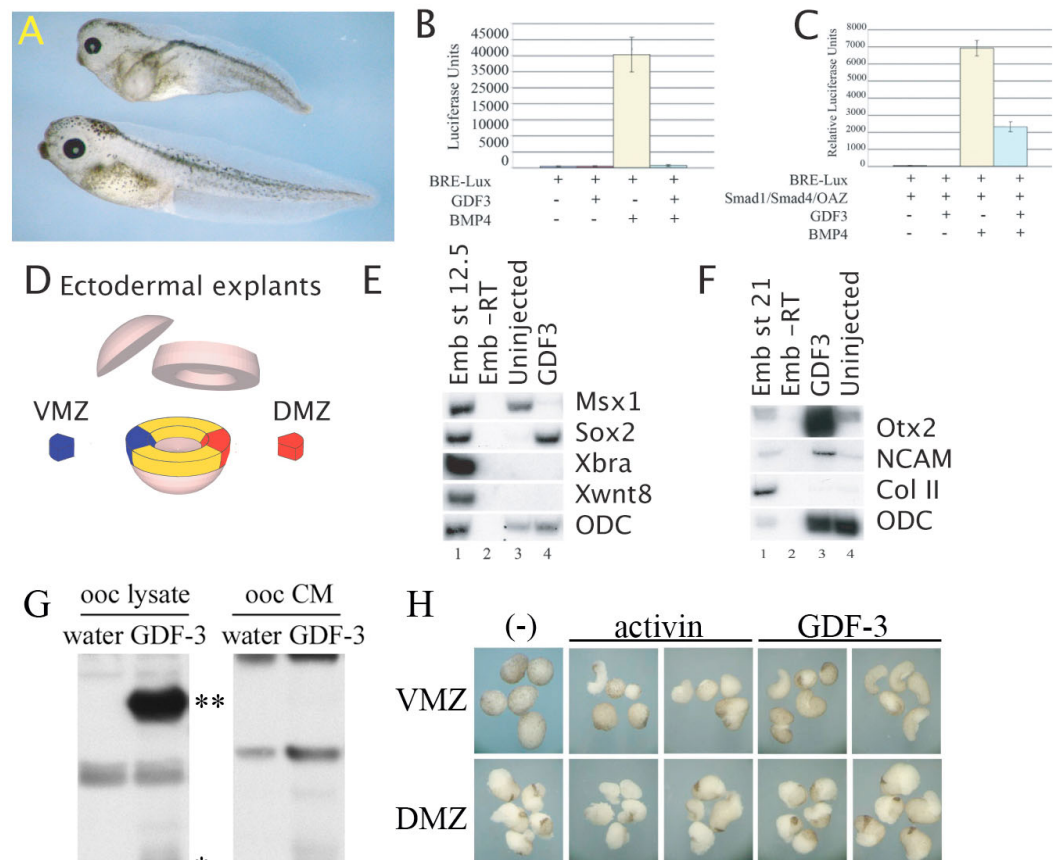
mesoderm induction, as demonstrated by the lack of induction of the pan-mesodermal marker brachyury (Xbra) and the marker of axial mesoderm, collagen type II.

To control for possible artifacts due to microinjection of RNA encoding GDF3, we performed assays with GDF3 protein in cells of the MZ, which are more sensitive to changes in BMP activity. BMPs are normally expressed throughout the early embryo, but the local secretion of BMP inhibitors in the DMZ establishes the dorsal axis in normal *Xenopus* embryos; therefore, creating a second region of BMP inhibition in the VMZ results in the induction of secondary axial structures.

GDF3 protein was obtained by microinjection of 50 ng GDF3 mRNA into *Xenopus* oocytes, followed by collection of GDF3-containing medium after 48 hours. We first examined the oocyte lysate and the conditioned media for GDF3 protein to check for processing and secretion of GDF3. In the oocyte lysate, we found both the prepro and mature forms of GDF3, but only the mature form was secreted into the conditioned media (Fig. 2G). The conditioned medium was then presented to both DMZ and VMZ, and the behavior of the explants was compared to controls. Fig. 2H shows that VMZ explants normally formed a sphere in culture, whereas DMZ explants elongated due to the formation of tissues, such as muscle and notochord, that undergo convergent extension. Activin protein can dorsalize VMZ explants (causing elongation), although through a mechanism distinct from BMP inhibition; therefore activin was used as a positive control. GDF3 protein dorsalized VMZ tissue, creating elongation and the appearance of anterior structures such as the cement gland. Analysis of molecular markers by RT-PCR in these explants agreed with the conclusion that GDF3 has dorsalizing activity in the mesoderm, as GDF3 protein decreased the expression of the ventral mesodermal marker globin and induced the expression of dorsal paraxial markers such as muscle actin in the VMZ explants (see Fig. S1 in the supplementary material). These data collectively demonstrated that GDF3 acts as a BMP inhibitor in both frog embryos and in pluripotent mammalian cells; however, its mechanism of BMP inhibition remained unknown.

Mechanism of GDF3 action

BMP inhibition may occur at the level of secretion of BMP ligands, receptor binding, SMAD phosphorylation, transcriptional activation or crosstalk with other pathways. As GDF3 is a secreted ligand, and because GDF3 mature protein can function when presented to cells in solution and is expressed in regions of the embryo that do not co-localize with BMP expression (see below), we hypothesized that GDF3 acts as an extracellular inhibitor. To determine whether GDF3 inhibition of BMPs is direct, we performed reciprocal co-immunoprecipitation assays and found that GDF3 and BMP4 protein interact (Fig. 3A). HA-tagged BMP4 (or untagged BMP4) and FLAG-tagged GDF3 were injected into the animal poles of frog embryos at the 2- to 4-cell stage, and embryos were cultured to gastrula stage. The animal caps were lysed and immunoprecipitated using anti-HA or anti-FLAG. FLAG-GDF3 immunoprecipitated prepro and mature BMP4 (60 and 26 kDa) and HA-BMP4 immunoprecipitated the prepro (45 kDa) and mature forms (18 kDa) (Fig. 3A). Similar results were obtained with FLAG-GDF3 immunoprecipitation of untagged BMP4 (data not shown).

We also tested whether GDF3 interacts with other TGFβ members to determine whether the interaction between GDF3 and BMP4 is specific or reflective of promiscuous binding by GDF3, and we found that GDF3 did not interact with activin (Fig. 3B). Interestingly, although the prepro form is the major form produced
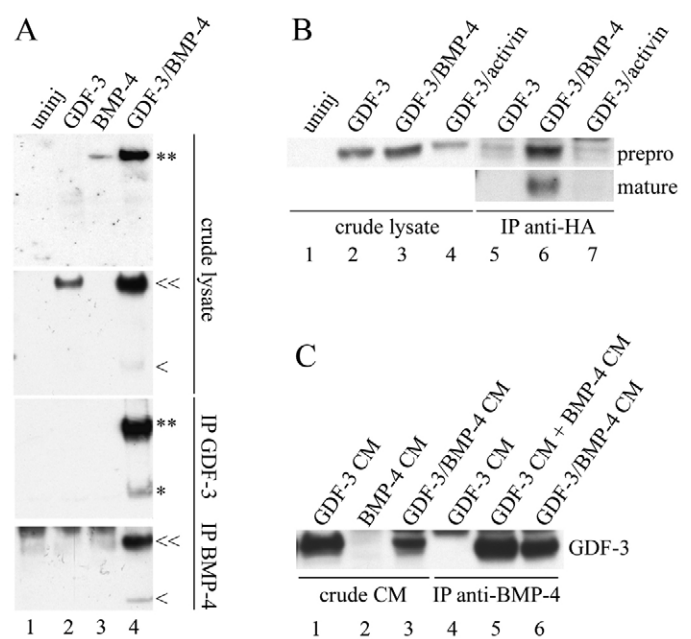

Fig. 3. Immunoprecipitation experiments. (**A**) Immunoprecipitation of GDF3 and BMP4 from animal caps of frog embryo. First and second panels show 10% crude levels input for immunoprecipitation. Third panel shows immunoprecipitation with FLAG-GDF3, blotted for HA-BMP4; fourth panel shows immunoprecipitation with HA-BMP4, blotted for GDF3. Prepro forms are indicated (**, <<) and mature forms are indicated (*, <). (**B**) Immunoprecipitation by BMP4-HA or activin-HA using mαHA antibody and blotting with goat αGDF3. The GDF3 prepro (**) form is shown in the upper panel and mature form (*) in the lower panel. (**C**) Immunoprecipitation of GDF3 by BMP4-HA from CM of COS cells. *GDF3* and *BMP4* were transfected into separate COS cells (lanes 1,2,4,5) or together in the same COS cells (lanes 3,6). In lane 5, COS CM from separately transfected *GDF3* and *BMP4* cells was mixed together for the immunoprecipitation. Only the prepro form of GDF3 is shown.

in cells and immunoprecipitated in these assays, GDF3/BMP interactions do not rely on the prepro domain. We tested this by using GDF3 to immunoprecipitate BMP4 containing the BMP4 pro-domain or the activin pro-domain and found that GDF3 immunoprecipitated both forms of BMP4, whereas it did not interact with activin (data not shown).

We sought to determine whether GDF3 can interact with BMPs extracellularly, providing a mechanism for its inhibition. We used COS cells that stably express either EYFP or *Gdf3*-FLAG and transfected with *EYFP* or with mouse *Bmp4-HA*. We performed immunoprecipitations on conditioned media or cell lysate from cells that co-expressed GDF3-FLAG and BMP4-HA as a positive control for interaction and compared these to immunoprecipitations performed on combined conditioned media or cell lysate from cells expressing either GDF3 or BMP4. We found that GDF3 and BMP4 interact whether they are co-secreted from the same cells, or expressed separately, confirming that this interaction could take place extracellularly (Fig. 3C).

GDF3 partially maintains pluripotent cell types in human ES cells

It has previously been shown that exogenous BMP signaling from other BMP/GDFs promotes extra-embryonic cell fate differentiation in human ES cells (Xu et al., 2002). To determine
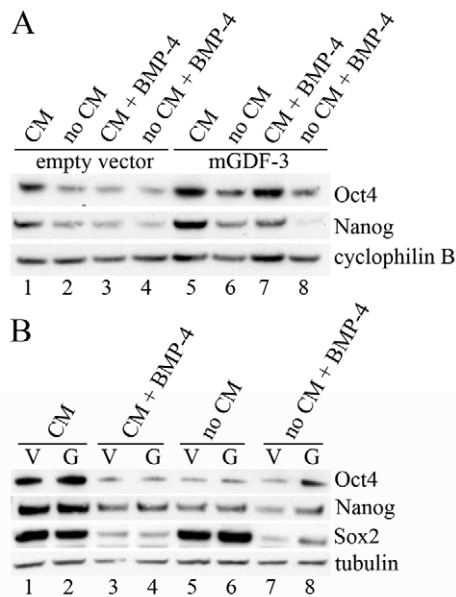
Fig. 4. Protein analysis. Western blot of BGN1 (**A**) and Jasmine (**B**) human ES cells transfected with empty vector (V) or *Gdf3* (G). Cells were either maintained undifferentiated in CM or cultured in the absence of CM (non CM), with and without rhBMP4 treatment for 3 days. OCT4, NANOG and SOX2 are markers of stemness. Cyclophilin B and tubulin are shown as loading controls.

whether, as a BMP inhibitor, GDF3 can oppose these functions, we transiently transfected BGN1 or Jasmine human ES cells with *Gdf3* plasmids and cultured these cells in CM, which maintains the undifferentiated state, or in the absence of CM, which allows heterogeneous differentiation, and in the presence or absence of BMP4 protein. Fig. 4A shows that *Gdf3*-transfected BGN1 human ES cells maintained significant levels of the pluripotency markers OCT3/4 and NANOG in conditions that normally promote differentiation but did not have increased levels of TROMA1, a marker of trophoblast (data not shown). In Jasmine human ES cells, we found that *GDF3* overexpression resulted in a more limited maintenance of pluripotency markers upon differentiation, confined to the combined treatment of BMP4 protein and the absence of conditioned medium (Fig. 4B). In addition, *GDF3* overexpressing cells had a more compact, stem-like morphology, whether cultured in the presence of CM or in differentiating conditions (data not shown). This demonstrates that GDF3 contributed to the maintenance of pluripotent gene expression in human ES cells.

GDF3 activity is required for the full spectrum of in-vitro differentiation of mouse ES cells grown without LIF

By contrast to the reported differentiating roles of BMPs in human ES cells, it has been shown that BMPs are supportive of the pluripotent state in mouse ES cells (Ying et al., 2003). To analyze what required functions GDF3 has in mouse ES cells, we sought to perform reduction-of-function experiments and used ES cells containing the β-geo genetrap AD0857, which interrupts the GDF3 gene after exon 1 (and parent strain 129Ola), available from the Sanger Institute GeneTrap Resource. As shown in Fig. 5A, exon 1 of GDF3 contains approximately the first third of the prepro domain (red). Exon 2 contains the remaining two-thirds of the prepro domain

and all of the mature domain (green). In the AD0857 genetrap, exon 1 is fused to β-geo (Fig. 5A), and in these ES cells, GDF3 prepro and mature protein were reduced significantly (Fig. 5B).

When cultured in the presence of LIF, wild-type mouse ES cells could be maintained in the undifferentiated state, but in the absence of LIF, these cells differentiated to a flattened morphology after 4 days of culture. By contrast, AD0857 genetrap cells maintained a normal, undifferentiated morphology even in the absence of LIF (Fig. 5C). We analyzed these cells with molecular markers to determine what cell fates are formed and found that, in the absence of LIF, wild-type cells expressed high levels of brachyury, a mesoderm marker, and low levels of OCT3/4 and SOX2, markers of the undifferentiated state, and of FGF5, a marker of pluripotent epiblast; AD0857 cells had reduced levels of brachyury and maintained significant levels of OCT3/4, SOX2 and FGF5 (Fig. 5D). These findings suggest that the reduced levels of GDF3 protein in AD0857 cells precludes normal differentiation.

We tested the ability of AD0857 cells to remain functionally pluripotent even in the absence of LIF by assaying for the formation of embryoid bodies (EBs). Upon culture in hanging drops, undifferentiated ES cells form aggregates, EBs, that differentiate into many types of embryonic tissue. Wild-type and AD0857 cells grown in the presence of LIF formed EBs in 100% of the hanging drops (±0%). While wild-type cells grown in the absence of LIF rarely formed EBs (6±13%), AD0857 cells without LIF formed EBs in 79% (±25%) of the hanging drops (Fig. 5E). However, these 'EBs' were much smaller and less compact than EBs produced by cells grown in LIF (Fig. 5F).

We examined the EBs on day 8 of suspension culture by RT-PCR to determine whether a reduction of GDF3 levels alters cell fate outcomes in differentiated mouse ES cells. We studied mRNA levels of stem/primitive markers (OCT4, FGF5, SOX2), neural markers (SOX2, PAX6, RAX), mesodermal markers (NKX2.5, FLK1, SCL1) and endodermal markers (AFP, HNF3β) (data not shown) (Fig. 5G). While wild-type or AD0857 cells grown in the presence of LIF can give rise to a full profile of differentiated cells types, AD0857 cells grown in the absence of LIF retain a primitive phenotype with some neural differentiation. These cells do not form mesoderm or endoderm and do not express all neural markers tested. This evidence establishes that wild-type levels of GDF3 activity are required for normal in-vitro differentiation of the three embryonic germ layers: the signature of pluripotency.

We next sought to determine whether reduction of GDF3 levels also precludes normal differentiation in vivo by injecting stable GFP-expressing wild-type or AD0857 genetrap ES cells cultured in the presence or absence of LIF into mouse blastocysts and assessing tissue contribution at mid-gestation (E9.5). In this assay of ES cell potential, we found that neither wild-type nor AD0857 ES cells cultured without LIF gave rise to any differentiated cells within the host embryo (data not shown). Further, we did not observe any difference in tissue contribution between wild-type and AD0857 genetrap ES cultured in the presence of LIF, as both differentiated normally (data not shown). These data suggest that the effects of reduced GDF3 levels on ES cell potential are confined to in-vitro differentiation.

GDF3 expression
We analyzed the expression of GDF3 during early mouse embryogenesis to gain a greater understanding of the role it may play in development. Temporal analysis of GDF3 mRNA expression by RT-PCR revealed that GDF3 was present at blastocyst and gastrula stages, strongly upregulated at E8.5, and significantly
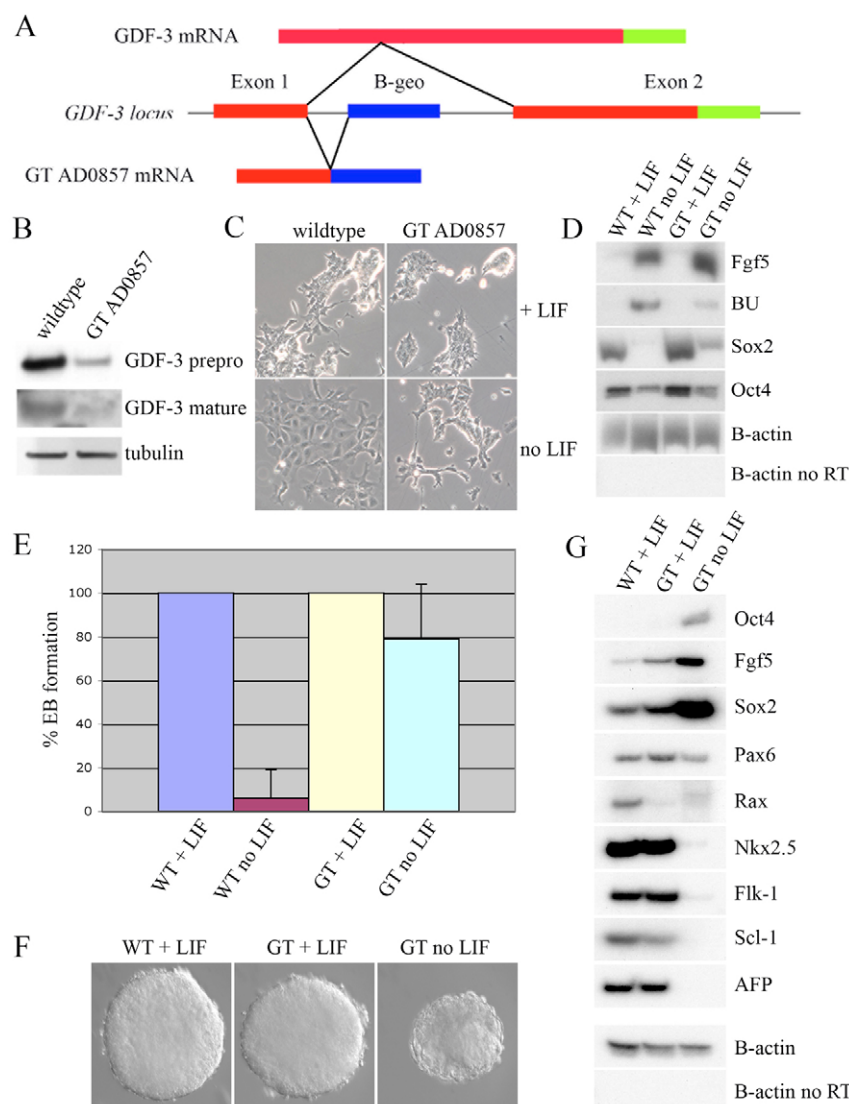
Fig. 5. GDF3 reduction of function through AD0857 genetrap in mouse embryonic stem cells. (**A**) Diagram of mouse *Gdf3* genomic locus containing the AD0857 genetrap. The *Gdf3* prepro domain (exon 1 and most of exon 2) is shown in red, *GDF3* mature (exon 2) is shown in green, and the genetrap insertion is shown in blue. The RACE Tag for the genetrap contains most of exon 1 of GDF3. (**B**) Western blot of wild-type (WT) and AD0857 genetrap (GT) ES cells with pαGDF3 (top panel, prepro GDF3; middle panel, mature GDF3) and mαtubulin (bottom panel). (**C**) Morphology of WT and GT ES after 4 days of culture in the presence or absence of LIF (×10). (**D**) RT-PCR of WT and GT ES after 4 days of culture in the presence or absence of LIF. FGF5 is a marker of pluripotent epiblast. BU is a marker of mesoderm. SOX2 and OCT3/4 are markers of the undifferentiated state. β-actin is used as a loading control. (**E**) Graph showing the percentage of hanging drops containing cells of each condition (WT and GT with and without LIF) that formed EBs. (**F**) Day 2 EBs for wild-type cells cultured in the presence of LIF, and genetrap AD0857 cells cultured in the presence or absence of LIF (×10). (**G**) RT-PCR of markers for early embryonic cell fates on day 7 suspension culture EBs. The following markers were used: OCT4 (stem/epiblast), FGF5 (epiblast), SOX2 (stem/epiblast/neural precursor), PAX6 (neural), RAX (anterior neural), NKX2.5 (cardiac mesoderm), FLK1 (endothelial mesoderm), SCL1 (blood), AFP (endoderm), β-actin (loading control). No RT control is shown for β-actin.

reduced by E10.5 (Fig. 6A). Using in-situ hybridization to localize GDF3 expression, we found that GDF3 was expressed in the blastocyst embryo within the inner cell mass, from which ES cells are derived (data not shown). However, we could not exclude staining in the trophoblast cells of the blastocyst, so we performed immunofluorescence to localize GDF3 protein. We found that GDF3 protein is expressed throughout the blastocyst embryo and is either membrane-associated or extracellular (Fig. 6B).

During gastrulation stages of development, GDF3 mRNA was confined to the node and the tissue immediately anterior to the node (Fig. 6C,D). Shortly after gastrulation, GDF3 was expressed in the forming cranial neural crest and in the ventral neural tube in a ventral-to-dorsal gradient extending through the ventral two-thirds of the neural tube (Fig. 6E,F). In E9.0 embryos, GDF3 was present in the notochord and floor plate of the neural tube for most of the length of the neural tube and was also expressed throughout the upper trunk region of the neural tube (where the tube is fully closed).

DISCUSSION

Here, we provide the first functional and mechanistic description of GDF3. We found that in stem cells GDF3 plays important, species-specific roles in the earliest cell fate decisions of embryonic cells in a manner that opposes BMP functions. As such, in human ES cells,

overexpression of GDF3 maintained markers of pluripotency even in differentiation conditions. This demonstrates that GDF3 activity supports the maintenance of pluripotency and thereby highlights the fact that, in human ES cells, BMP signals are inhibitory to 'stemness'. By contrast, in mouse ES cells, reduced GDF3 levels helped to support pluripotency and prevented normal in-vitro differentiation. This is consistent with suggestions that BMPs support stemness in mouse ES cells, and also confirms that BMP activity may be opposite in human and mouse ES. We characterized the mechanism of GDF3 activity and found that it inhibits its own subfamily, the BMP-GDF subfamily of TGFβ ligands, in frog embryos and in mouse pluripotent cells and interacts physically with BMPs. In addition, early embryonic GDF3 expression is consistent with its role as a BMP inhibitor.

The expression of GDF3 in multipotent cell types such as ES cells, neural crest and teratomas suggests that GDF3 has a significant role in pluripotency (Sato et al., 2003; Ramalho-Santos et al., 2002). Being expressed in the blastocyst, GDF3 is therefore one of the earliest BMP inhibitors expressed in the mammalian embryo. In fact, recent studies profiling genome-wide expression in early mouse embryos describe expression of only one other possible BMP inhibitor, LEFTY, in preimplantation mouse development (in addition to GDF3) (Hamatani et al., 2004; Wang et al., 2004). Later in development, GDF3 is expressed in the node, a region known to
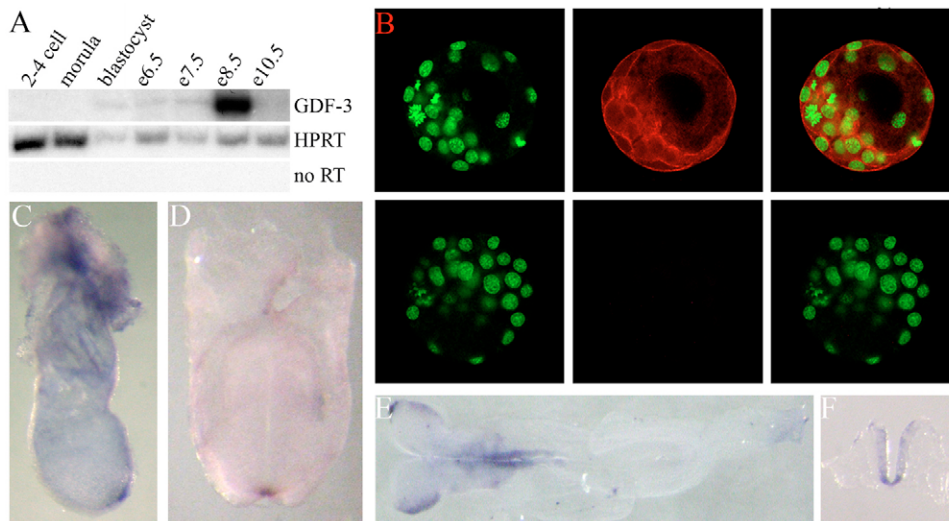
Fig. 6. GDF3 expression during development. (**A**) RT-PCR of GDF3 throughout early mouse embryo development. HPRT is shown as a loading control and HPRT no RT as a negative control. (**B**) Immunofluoresence (IF) of GDF3 protein [red (middle)] as detected by goat αGDF3 and counterstained with Sytox Green nuclear stain [green (left)], merged in the right panels. The lower panels show a no primary antibody control. Magnification is 10×. (**C-F**) E7.5 (C), E8.0 (D) and E8.5 (E) embryo in-situ hybridization with GDF3 anti-sense probe. F shows 10-μm sections of whole-mount in-situ hybridizations. In all in-situ experiments, a sense probe showed no specific staining.

be associated with BMP inhibition, and in the ventral neural tube. As BMP signaling from the roof plate promotes the dorsalization of neural cell types (Liem et al., 1997), GDF3 in the ventral neural tube may function to restrict these effects.

The role of GDF3 in ES cells was species-dependent, in that higher levels of GDF3 supported stemness in human cells, while lower levels of GDF3 supported stemness in mouse ES cells. While surprising, these results are consistent with previous observations that increased BMP signaling in human ES cells promotes differentiation (Xu et al., 2002; Pera et al., 2004) while promoting the undifferentiated state in mouse ES cells (Ying et al., 2003; Qi et al., 2004). We present three possibilities to explain these phenomena.

First, it is possible that distinct signaling profiles regulate the early cell fate decisions of human and mouse embryos. Second, it is possible that human and mouse ES cells correspond to distinct stages of in-vivo development, as human ES cells can form trophoblast (the first cell fate decision in the embryo) while mouse ES cells do not, but upon differentiation can form primitive endoderm (the second cell fate decision in the embryo). A third possibility is that human and mouse ES cells possess different sensitivities to BMP signaling and that different levels of effective BMP signaling in these cells produces their disparate phenotypes. In support of this model, BMPs can act as morphogens, creating distinct cell fates based on different concentrations of the ligand in a given time window (Wilson et al., 1997). Thus, it is probable that GDF3 expression helps to establish a BMP activity gradient. Although its activities may be species-specific, GDF3 regulates both the ability of stem cells to maintain the undifferentiated state and the ability to differentiate to the full spectrum of cell types.

Despite the fact that our genetrap experiments provided a reduction, not a loss, of GDF3 function, we detected a dramatic outcome on the state of in-vitro pluripotency of mouse ES cells, as shown by the qualitative and quantitative differences displayed by the embryoid bodies. This observation highlights the critical importance of the regulation of thresholds of BMP activity in the establishment of discrete fates in the mammalian embryo. The role of BMP morphogens as instructive signals in the establishment of early embryonic cell fates is therefore maintained throughout evolution from *Drosophila* to humans. As each threshold of BMP activity can lead to a completely different cell fate outcome, we expect that a complete loss of GDF3 function (in progress) will elicit a distinct result.

While we have shown that GDF3 inhibits BMP signaling, we cannot rule out that GDF3 has additional functions, either as an inhibitor or as a ligand. For instance, GDF3 shares the distinct lack of the fourth cysteine with GDF9, a TGFβ ligand that has been shown to act as a SMAD2/3 activator (Mazerbourg et al., 2004). In support of this possibility, we found that very high levels of mouse GDF3 mRNA injection into frog embryos produced an activin-like response.

We have previously shown that inhibition of GSK3 through BIO, a small molecule, is sufficient to maintain stemness (Sato et al., 2004), and here we show that GDF3 also plays an important role in both mouse and human ES cells. BIO maintains the expression of GDF3, which we show regulates thresholds of BMP activity. This represents the beginning of our understanding of the signaling network involved in the maintenance of the basic aspects of pluripotency in ES cells. For this picture to be complete, the pathways that mediate the pluripotency of the inner cell mass and other stem cell types in vivo must be determined to appreciate the relationship between primitive cell fates and how they differentiate to create the entire repertoire of cells in an organism.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/2/209/DC1

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