物等の現格基準」(厚生省告示，1959年）に収載されている．この内容は，「食品添加物公定書」という名称で冊子体として4～8年ごとに刊行されている．「食品添加物公定書」は1960年にはじめて作成されたが，その後，製造・品質管理技術の進歩および試験法の発達等を受け改訂され，2009年現在，第8版が刊行されている．また，食品に使用した食品添加物は，指定添加物，既存添加物，天然香料，一般飲食物添加物の区別なく，原則としてすべて表示することが義務づけられている．保存料，甘味料等の8用途で使用したものについては，その用途名も併記する必要がある．用途名としては，1）甘味料，2）着色料，3）保存料，4）増粘剤，安定剤，ゲル化剤または糊料，5）酸化防止剤，6）発色剤，7）漂白剤，8）防かび剤または防ばい剤，の8種類が定められている（厚生省生活衛生局長通知「食品衛生法に基づく添加物の表示等について」).

食品添加物のうち，香料，酸味料，調味料，乳化剤など14種類の目的に使用される食品添加物については，一括名による表示が認められている．米国では，経験や科学的な知見から専門家が判断して一般的な使用法においてリスクがないものとみなされた物質をGRAS（generally recognized as safe）物質として使用できるようにしている[7]．GRAS物質は「一般的に安全と考えられるもの」と訳されている．1958年の食品添加物規制の大幅な改訂の際に設けられた．GRAS物質の届け出には民間の科学的専門家による一定の定められた科学的手順に基づいた適切な評価があればよく，政府（FDA）の判断を除外するわけではないが要求していない点が特徴として挙げられる．米国のFlavor and Extract Manufacturers Association（FEMA）(米国食品香料製造者協会）ではFDAの了解のもとに，食品香料（フレーバー）に関するFEMA GRASと呼ばれる物質リストを作成しており，リスト中の物質は米国で自動的に食品に使用できることとなっている．

b. 食品添加物の安全性評価[5] 食品添加物の安全性評価は，物質の代謝や実験動物を用いた毒性試験結果等の科学的なデータに基づき，食品安全委員会の行う食品健康影響評価（リスク評価）によって審議され，食品添加物ごとに許容一日摂取量(acceptable daily intake：ADI）が設定される．この結果を受けて，薬事・食品衛生審議会において食品添加物としての指定の可否，成分規格，使用基準などにつき審議・決定される．ADIは，ヒトがある物質を毎日一生涯にわたって摂取し続けても，現在の科学的知見からみて，健康への悪影響がないと推定される一日あたりの摂取量であり，毒性試験から求められた無毒性量（NOAEL）を安全係数で除して算出され，通常mg/kg/dayで表される．安全係数は通常，種差と個体差の観点からそれぞれに10倍を見込み，これらを乗じた100が用いられているが，固定されたものでなく，評価に使われたデータが不足している場合や現れた毒性が，神経毒性，発がん性，催奇形性など重篤な場合には追加の係数が加えられることがあり，500など数値は変わりうる．なお，いわゆる健康食品の主成分など，食品自身に由来し薬事法・食品衛生法等の法律で規定されていない化学物質に関しては，通常，安全係数の概念は適用されずADIの算出も行われない．

毒性試験については，標準的実施方法の指示のもと，(1) 28日反復投与毒性試験，(2) 90日反復投与毒性試験，(3) 1年間反復投与毒性試験，(4) 繁殖試験，(5) 催奇形性試験，(6) 発がん性試験，(7) 1年間反復投与毒性/発がん性併合試験，(8) 抗原性試験，(9) 遺伝毒性試験，(10) 一般薬理試験，を検討することが定められている（1996年厚生省通知「食品添加物の指定および使用基準改正に関する指針について」).こうした毒性試験の整備に伴い，これまでに人体への悪影響を考慮して消除されたものだけで十数種にのぼる．例として，着色料の食用赤色1号（肝障害，肝がん），甘味料のサイクラミン酸ナトリウム（チクロ）(膀胱がん，催奇形性），甘味料のズルチン（肝障害，肝がん），保存料のAF-2（変異原性，染色体異常試験陽性），既存添加物であったアカネ色素（腎がん）がある[6]．

食品添加物の摂取状況については，厚生労働省により，マーケットバスケット方式および食品添加物の生産流通統計量に基づく一日摂取量推定調査が実施されている．マーケットバスケット方式とは，スーパーなどで売られている食品を購入し，そのなかに含まれている食品添加物量を分析して測り，その結果に国民栄養調査に基づく食品の喫食量を乗じて推定摂取量を求める方式をいう．この調査結果の一例をADIとともに表5.3.1に示す[5]．

c. 食品添加物規制の国際標準化[5] 食品添加

表5.3.2 マイコトキシンによる食品汚染[8](一部改変)

マイコトキシンの種類	主な原因カビ	主な汚染食品	毒性様式
	Aspergillus 属		
アフラトキシン (B_1, B_2, G_1, G_2, M_1, M_2など約20種類の誘導体)	*A. flavus*, *A. parasiticus*	ピーナッツ，トウモロコシ，麦，米，綿実	肝がん，肝障害
ステリグマトシスチン	*A. versicolor*	穀類	肝がん，肝硬変，血管肉腫
オクラトキシン	*A. ochraceus*, *A. carbonarius*	ピーナッツ，トウモロコシ，麦，コーヒー豆	腎がん，腎障害
	Penicillium 属		
ルテオスカイリン	*P. islandicum*	穀類（米）	肝がん，肝硬変
シトリニン	*P. citrinum*	穀類（米）	腎障害
シトレオビリジン	*P. citreoviridae*	穀類（米）	神経毒性
パツリン	*P. expansum*	麦芽根，小麦，リンゴ加工品	消化管障害，腎機能障害
	Fusarium 属		
ニバレノール	*F. culmorum*	トウモロコシ，麦，米	免疫系・造血器・消化管障害
デオキシニバレノール	*F. graminearum*	トウモロコシ，麦，米	免疫系・造血器・消化管障害
T-2トキシン	*F. sporotrichioides*	トウモロコシ，麦，米	免疫系・造血器・消化管障害
ゼアラレノン	*F. graminearum*, *F. culmorum*	トウモロコシ，麦	不妊症（エストロゲン様作用）
フモニシン	*F. moniliforme* (*F. verticillioides*)	トウモロコシ，麦，大豆，アスパラ	肝がん，腎障害，ウマの白脳軟化症
	Claviceps 属		
麦角アルカロイド（エルゴタミンなど）	*C. purpurea*	穀類（麦）	神経毒性，循環器毒性，流産

物の規格や基準については，それぞれの国の法律により定められており，各国間で相違点がある．他方で，国際的な貿易が盛んとなり，食品の輸出や輸入が増大し，食品の安全性を確保しつつ，規制を整合化することが，国際的な課題となっている．食品添加物については，国連食糧農業機関（Food and Agriculture Organization, FAO）/世界保健機関（WHO）の合同食品規格委員会（コーデックス委員会；Codex Alimentarius Commission）の食品添加物部会において検討がなされている．また，食品添加物の安全性について国際的な評価を行う機関としては，国連食糧農業機関/世界保健機関合同食品添加物専門家会議（FAO/WHO Joint Expert Committee on Food Additives：JECFA）がある．JECFAは，コーデックス委員会とは独立しているが，コーデックス委員会に対して助言を行っている．

5.3.3 食品汚染物質[2]

有害な化学物質による食品の汚染は，偶然あるいは過失で混入する場合と，環境汚染物質として食品に残留する場合が考えられてきたが，最近ではメラミン添加牛乳事件や事故米の食品への転用で明らかになったように，意図的な犯罪行為への対応も考慮する必要が出てきた．なお，メラミン混入ペットフードにより2007年に米国を中心に多数の犬や猫が腎不全等で死亡する事件が起きたが，米国獣医師会は，メラミンとその不純物シアヌル酸との反応から生じた結晶がその原因物質である可能性を報告している．

a. 加工製造中の不純物混入による汚染　この実例として，粉乳中にヒ素が混入したヒ素ミルク事件（1955年），米ぬか油にPCB（ポリ塩化ビフェニル）に混入した事件（カネミ油症，1968年）を挙げることができる．ヒ素ミルク事件では，乳質安定剤として使用された工業用リン酸水素二ナトリウムに含まれていた不純物ヒ素を摂取したために起き，乳児の死亡を招いた．カネミ油症では，油を加熱脱臭するために，熱媒体として使用したPCBが熱交換パイプから漏出し，油に混入したためとされており，塩素痤瘡，色素沈着，肝障害などを招いた．なお，この油症の原因物質は，PCBに含まれていた

ダイオキシン類の一種であるポリ塩化ジベンゾフラン（polychlorinated dibenzofurabin, PCDF）と考えられている．

その他，食品容器に由来する化学物質による汚染で問題となっているものに，プラスチック製品の可塑剤のフタル酸エステル，合成原料のビスフェノールAなどが挙げられ，これらは内分泌攪乱物質の可能性が示唆されている．同様に食器用合成樹脂合成原料である塩化ビニルモノマー，ホルムアルデヒド，アクリロニトリルなどの漏出や陶器から溶出した鉛なども食品衛生上問題となる．鉛は他にも鉛製の水道管からの溶出が問題となる．

b. 環境中に排出された重金属・化学物質による汚染　この代表的な実例として，水俣病およびイタイイタイ病が挙げられる．水俣病は，排水中の，触媒として使用した無機水銀から副生されたメチル水銀が，魚介類に蓄積し，ヒトがこれを摂取することにより起きたものとされており，四肢のしびれ，歩行障害などが起きた（熊本県水俣市，1956年）．また有機水銀は，胎盤を介し胎児に移行し，生まれた子どもに脳性麻痺等の症状が出た．その後，新潟県阿賀野川流域においても有機水銀中毒という第二水俣病（新潟水俣病）が発生した（1965年）．現在，魚類に蓄積した有機水銀量については国際的に注意が払われており，日本では自然界で水銀を蓄積しやすいサメ，メカジキ，キンメダイ，クジラ類の一部，マグロ類等の妊婦の摂取について注意が喚起されている．イタイイタイ病は，富山県神通川下流域で，鉱山廃水に含まれて排出されたカドミウムを多量に含む米を摂取したため起きたものとされており，骨が非常に脆くなり骨折しやすい患者が多く出た（1955年）．

近年，船底防汚剤，木材防腐剤などとして使用されたトリブチルスズなどの有機スズ化合物が，水生生物に対して低い濃度で作用し，内分泌攪乱物質である可能性が示唆され，海洋汚染物質として，ヒトへの生体影響が懸念されている．

その他，環境中の化学物質による汚染で問題視されるものとして，ゴミ焼却場などから排出されるダイオキシン類や残留農薬が挙げられる．ダイオキシン類は，ポリ塩化ジベンゾパラジオキシン（polychlorinated dibenzo-o-dioxin, PCDD），ポリ塩化ジベンゾフラン（PCDF）およびコプラナーポリ塩化ビフェニル（coplanar polychiorinated biphenyl, Co-PCB）の総称である（ダイオキシン類対策特別措置法）．塩素置換数と置換位置によってそれぞれ数多くの異性体が存在する．空気中で塩素源と炭素源が300℃程度の不完全燃焼することにより発生する．したがって，この発生はプラスチックの不完全燃焼だけとは限らないこととなる．ダイオキシン類の毒性の強さは異性体によって異なり，もっとも毒性が強いのは2, 3, 7, 8-テトラクロロベンゾ-p-ジオキシン（2, 3, 7, 8-TCDD）である．ダイオキシン類の毒性評価に際しては，2, 3, 7, 8-TCDDに対する毒性等価係数（TEF）がWHOから提唱され，各異性体のTEFと残留濃度の積の合計量（TEQ）を用いて検討されている．モルモットに極めて低用量で急性毒性（2, 3, 7, 8-TCDDによる半数致死量は，600ng/kg）を示した以外にも，実験動物に対し免疫毒性，発がん性や催奇形性などの毒性を示す．遺伝子改変マウスを用いた実験等から，この作用の多くが芳香族炭化水素受容体（aryl hydrocarbon receptor；Ahレセプター）（ダイオキシン受容体）を介して引き起こされるものと考えられている．

c. かびによる食品汚染[8]　かび等の真菌類による食品汚染の中では，発がん性等ヒトや動物に有害作用を有する二次代謝産物として産生される毒の総称であるマイコトキシン（mycotoxin）による健康被害がとくに問題となる．主なマイコトキシンの種類と原因かび，汚染源，毒性様式について表5.3.2に示した．アスペルギルス属（*Aspergillus*），ペニシリウム属（*Penicillium*），フサリウム属（*Fusarium*）の3属によるものがほとんどである．アフラトキシンB_1には強い肝毒性と強い発がん性がある．ピーナッツ，トウモロコシ，麦などの貯蔵，輸送の管理が不適切だった場合に発生する．マイコトキシンにより引き起される障害は，肝障害，腎障害以外にも，ゼアラレノンのように家畜で不妊を起こすもの，エルゴタミン（麦角アルカロイド）のように血管収縮作用や子宮平滑筋収縮作用を有するものなど多彩である．日本では2009年現在，アフラトキシンB_1，デオキシニバレノール，パツリンの3種について規制している．アフラトキシンB_1は，食品衛生法第6条により，全食品を対象に検出されてはならないと規制されている．パツリンについて

は，りんごの搾汁（ジュース）を対象に含有量が0.050ppmを超えるものであってはならないと規制されている（パツリン告示法）．デオキシニバレノールは，小麦を対象に暫定的な基準値1.1ppmが定められている．

なお，エルゴタミンは片頭痛治療薬として利用され，ゼアラレノンはエストロゲン活性を有し，またフモニシンはスフィンゴ脂質の生合成経路を阻害するが，このようにマイコトキシンのなかには，細胞分子機能解析用の生理活性物質としても研究用試薬として注目されているものがある．

5. 3. 4　飼料添加物

飼料添加物（feed additive）とは，「飼料の安全性の確保および品質の改善に関する法律」（飼料安全法）において，「飼料の品質の低下の防止その他の農林水産省令で定める用途に供することを目的として飼料に添加，混和，浸潤その他の方法によって用いられる物で，農林水産大臣が農業資材審議会の意見を聴いて指定するものをいう」と定義されている．法の規制対象とする家畜等は経済動物に限定することが妥当と考えられている．2003年に改正された政令で定められた動物（家畜等）は，家畜（牛，豚，めん羊，山羊およびしか）（馬は対象外），家禽（鶏およびうずら），みつばち，養殖魚（ぶり，まだい，まあじ，ひらめ，すずき，すぎ，くろまぐろ，うなぎ，あゆなど23種）の計31種となっている．飼料添加物は，飼料の品質の低下の防止（抗酸化剤，防かび剤，乳化剤等），飼料の栄養成分その他の有効成分の補給（アミノ酸，ビタミン，ミネラルなど），飼料が含有している栄養成分の有効な利用の促進（抗生物質，合成抗菌剤，生菌剤等）を目的として添加される（2004年現在で合計153種）．それぞれ対象飼料や添加量が定められている．また，BSEの新たな発生を防止するため，牛の餌については，骨肉粉などの動物由来タンパク質が混入してはいけないことが定められている．

ヒトへの健康被害との関連では，過剰摂取あるいは抗生物質における薬剤耐性などが問題となるが，抗生物質，合成抗菌剤については，「動物用医薬品の使用の規制に関する省令」によって，投与用量や出荷前の使用禁止期間が定められている．また，動物用医薬品として投与されたホルモン剤や抗生物質等の食肉への残留も問題となるが，これもこの省令により投与用量や出荷前の使用禁止期間が定められている．したがって抗菌性物質はその用途により「飼料添加物」と「動物用医薬品」の両方に含まれ，飼料安全法，薬事法により，それぞれに使用規制がある．ヒトへの健康被害防止に際しては，これらの物質のモニタリングが重要となっている．

食品中の飼料添加物と動物用医薬品の残留は，残留農薬と同様にポジティブリスト制が導入されており，基準値が設定されていないものには，一律基準が適用される．残留基準は，食品安全委員会により定められたADIに基づき，厚生労働省の薬事・食品衛生審議会において定められる．

なお，犬，猫等の愛がん動物用の飼料（ペットフード）は，飼料安全法において規制の対象とされていなかったが，この安全性の確保を図るため，2009年6月1日より「愛がん動物用飼料の安全性の確保に関する法律（ペットフード安全法；農林水産省・環境省共管）が施行された．2009年6月現在，政令により愛がん動物は犬と猫と定められている．

［北嶋　聡］

文　献

1) 内藤裕史（2007）：アマメシバ，健康食品・中毒百科7，p.35，丸善．
2) 石井秀美，杉浦隆之編著（2005）：衛生薬学，5食品衛生行政と法規（p.96），食品成分の変質と保存（p.115），8経口感染症と食中毒（p.151），9化学物質による食品汚染（p.157）の各項参照，朝倉書店．
3) 清涼飲料水中のベンゼンについて（平成18年7月）：厚生労働省　報道発表資料：http://www-bm.mhlw.go.jp/houdou/2006/07/h0728-4.html
4) 健康食品のホームページ：厚生労働省 保健機能食品・健康食品関連情報：http://www-bm.mhlw.go.jp/topics/bukyoku/iyaku/syoku-anzen/hokenkinou/index.html
5) 食品添加物に関するホームページ：厚生労働省：http://www.mhlw.go.jp/topics/bukyoku/iyaku/syokuten/gaiyo.html
6) 伊藤誉志男（2007）：日本人の食品添加物の一日摂取量調査研究　マーケットバスケット方式（25年間のまとめ）：FFI JOURNAL, Vol. 212, p. 815-p. 838, No. 10.
7) Klaassen, C.D.編（2004）：キャサレット&ドール トキシコロジー　第6版，第30章 食品のトキシコロジー，サイエンティスト社，p.1216.
8) 小西 良子，杉山 圭一（2008）：カビ毒のリスク評価と国際的な動向，食品衛生学雑誌49：1-10.

Maintenance of Embryonic Stem Cell Pluripotency by Nanog-Mediated Dedifferentiation of Committed Mesoderm Progenitors

Atsushi Suzuki, Ángel Raya, Yasuhiko Kawakami, Masanobu Morita, Takaaki Matsui, Kinichi Nakashima, Fred H. Gage, Concepción Rodríguez-Esteban and Juan Carlos Izpisúa Belmonte

Abstract Embryonic stem (ES) cells can be propagated indefinitely in culture while retaining the ability to differentiate into any cell type in the organism. The molecular and cellular mechanisms underlying ES cell pluripotency are, however, poorly understood. Here, we characterize a population of early mesoderm-committed (EM) progenitors that is generated from mouse ES cells by bone morphogenetic protein (BMP) stimulation. We further show that EM progenitors are actively dedifferentiated to ES cells by the action of Nanog, which, in turn, is directly up-regulated in EM progenitors by the combined action of leukemia inhibitory factor (LIF) and the early mesoderm transcription factor T/Brachyury. Finally, we demonstrate that this negative feedback mechanism contributes to the maintenance of ES cell pluripotency. These findings uncover specific roles of LIF, Nanog, and BMP in the self-renewal of ES cells and provide novel insights into the cellular bases of ES cell pluripotency.

Keywords Pluripotency · T (Brachyury) · Self-renewal · Mesoderm differentiation · Leukemia inhibitory factor

1 Introduction

Mouse embryonic stem (ES) cells are permanent cell lines derived from pre-implantation embryos [1, 2] that display the peculiarities of combining unlimited self-renewal and pluripotency abilities while retaining a normal karyotype. In practical terms, these peculiarities mean that mouse ES cells can be maintained in culture for indefinite periods of time while conserving their ability to differentiate into any cell type if the appropriate context is provided, either in vivo or in vitro [3].

Strict culture conditions must be followed in order to maintain the self-renewal of pluripotent mouse ES cells. Two extrinsic culture requirements, a feeder layer of fibroblasts and the addition of fetal bovine serum, have been identified to be necessary to sustain proliferation of undifferentiated mouse ES cells and their activities pinpointed to specific molecules [4]. Thus, self-renewal of mouse ES cells can be sustained in feeder-free conditions by supplementing the culture media with the cytokine leukemia inhibitory factor (LIF) [5, 6]. In the absence of LIF, ES cell colonies flatten and form epithelial-like sheets [5, 6]. More recently, the self-renewal-promoting activity of animal serum has been identified to be mediated by ligands of specific families of the transforming growth factor-β (TGFβ)superfamily, including the bone morphogenetic protein (BMP) family members BMP2 and BMP4, and the growth and differentiation factor (GDF) family member GDF6 [7]. In the absence of BMP/GDF signals, LIF is not sufficient to prevent the neural differentiation of ES cells, whereas the absence of both BMP/GDF and LIF stimulation results in a flattened cell phenotype similar to that of LIF withdrawal [7].

The intracellular signaling cascades initiated by both LIF and BMP/GDF that sustain self-renewal of mouse ES cells have been worked out in a significant degree of detail [4]. In summary, binding of LIF to its cognate LIF receptor results in the recruitment of gp130 and the formation of a ternary complex that catalyzes the tyrosine phosphorylation, dimerization, and nuclear translocation of the downstream signal transducer STAT3. BMP/GDF, in turn, promotes ES cell self-renewal by inducing the expression of members of the Inhibitor of differentiation (Id) family of negative transcriptional modulators, most likely mediated by activation of the TGFβ downstream signal transducer Smad1 [7].

In addition to extrinsic requirements, the pluripotency of mouse ES cells has been shown to depend on intrinsic determinants, such as the expression of the POU transcription factor Oct4 [8] and the divergent homeodomain-containing

J.C.I. Belmonte (✉)
Gene Expression Laboratory, Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd., La Jolla, CA 92037; Center of Regenerative Medicine in Barcelona, Dr. Aiguader 88, 08029 Barcelona, Spain
e-mail: belmonte@salk.edu

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factor Nanog [9, 10]. Both factors are absolutely required for ES cells to maintain their pluripotent identity. Thus, the lack [8] or down-regulation [11] of *Oct4* expression induces trophoectoderm differentiation, whereas ES cells lacking *Nanog* function differentiate to endoderm lineages [9]. The relationships between extrinsic and intrinsic determinants of ES cell identity are only recently beginning to be understood. The maintenance of pluripotent ES cell self-renewal by Oct4 requires functional LIF/STAT3 and BMP/GDF/Id signaling cascades [7, 11], but the function of LIF/STAT3 does not appear to be the maintenance of *Oct4* expression [11]. Overexpression of *Nanog*, in turn, circumvents the necessity of either LIF or BMP/GDF stimulation [7, 10], although synergism between Nanog function and LIF/STAT3 signaling has been noted [10].

The manipulation of mouse ES cells is currently a standard tool in many laboratories, inasmuch as it allows the generation of mice carrying targeted gene mutations for the direct analysis of a gene's function. Moreover, the ability of mouse ES cells to give rise in vitro to virtually any cell type of the organism has been exploited to gain insights into the molecular and cellular mechanisms of cell differentiation to specific lineages. However, most of the recent interest in ES cell research results from the successful derivation of human pluripotent cell lines [12, 13], which created new prospects for future cell replacement therapies. Many basic questions about the biology of these promising cells need be answered if their potential is to be realized. Human ES cells share with their mouse homonyms the peculiarities of self-renewal and pluripotency. The molecular mechanisms by which self-renewal and pluripotency are maintained in human and mouse ES cells, however, appear to differ [14]. Moreover, the cellular bases of pluripotency of either mouse or human ES cells are largely unknown. How intrinsic and extrinsic determinants of ES cell identity crosstalk to maintain cell pluripotency, whether the symmetric self-renewal of pluripotent ES cells depends on a truly symmetrical cell division, on a particular resistance of ES cells to undergo cell differentiation – yet retaining the ability to do so, or on the reversal of early steps of cell differentiation, and what kinds of mechanisms operate in ES cells so they maintain pluripotency are still open questions.

Here we show that mouse ES cells cultured on feeders in the presence of LIF and serum contain commited mesoderm progenitors, the number of which is dependent on the amount of LIF in the culture medium. By clonal analyses, we show that, in the presence of LIF the commitment of these cells to mesoderm fate can be reverted, so that they give rise to fully pluripotent ES cells. We further demonstrate that the process of dedifferentiation of committed mesoderm progenitors is important to maintain the pluripotency of mouse ES cells over long-term cultures, and that this process is regulated by *Nanog*. Specifically, we show that *Nanog* expression is up-regulated in mesoderm progenitor cells by the combinatorial action of STAT3 and the mesoderm-specific transcription factor T/Brachyury. Finally, we provide evidence from gain- and loss-of-function experiments demonstrating that Nanog prevents the progression of BMP-induced mesoderm differentiation of ES cells by directly binding to Smad1 and interfering with the recruitment of co-activators, thus blocking the transcriptional activation of downstream targets, including that of *T/Brachyury*.

2 Results

To characterize the early steps of mouse ES cell differentiation toward mesoderm lineages, we generated transgenic ES cell lines expressing enhanced green fluorescent protein (eGFP) under the regulatory sequences of *T/Brachyury* (*T*), which encodes one of the earliest markers of mesoderm differentiation [15, 16]. The expression of eGFP in nine independent *T-eGFP* ES cell lines faithfully recapitulated that of endogenous *T*, as assayed by the presence of *T* transcripts in *T-eGFP*-positive [T(+)] cells sorted from embryoid bodies differentiated in vitro, and by their absence in *T-eGFP*-negative [T(−)] cells (Fig. 1A). Interestingly, colonies of undifferentiated *T-eGFP* ES cells grown under standard culture conditions (on a fibroblast feeder layer in culture medium containing serum and 1000 μ/mL of LIF) contained T(+) cells. These cells were found in small numbers (1–3 cells per colony) in colonies of otherwise undifferentiated morphology (6.7%, n = 120, Fig. 1B), and no colonies formed exclusively by T(+) were ever detected under these

Fig. 1 (continued) 400 μ/mL of LIF. Bar shows mean ± SD (n = 4). (**E**, **F**, **G**) Colony formation from single T(−) or T(+) cells isolated from *T-eGFP* ES cells obtained at P20 with 1000 μ/mL of LIF (**E**), at P20 with 400 u/ml of LIF (**F**), or at P4 without LIF (**G**). Scale bar = 10 μm. *Graphs* show the percentage of colonies containing only T(+) or T(−) cells, or both types of cells (mosaic colony) formed from purified T(−) or T(+) cells. *Bar* shows mean ± SD (n = 3; 40 colonies were examined in each dish). (**H**, **I**) ES cells cultured with 400 μ/mL of LIF expressed the mesoderm marker *T*, but not ectoderm or endoderm markers. RT-PCR analyses of ectoderm (*Sox1*), mesoderm (*T*), and endoderm (*HNF4α*) marker genes in *T-eGFP* ES cells obtained at P20 (1000 and 400 μ/mL of LIF) or P4 (without LIF) (**H**). Western blotting analysis of T in *T-eGFP* ES cells obtained at P20 (1000 and 400 μ/mL of LIF) or P4 (without LIF) (**I**). (**J**) Western blotting analysis of tyrosine-phosphorylated STAT3 (STAT3-P) in *T-eGFP* ES cells obtained at P20 (1000 and 400 μ/mL of LIF) or P4 (without LIF). Total STAT3 levels are shown as a control. Panels A-B and H-J are reproduced with permission of Nature Publishing Group [48]

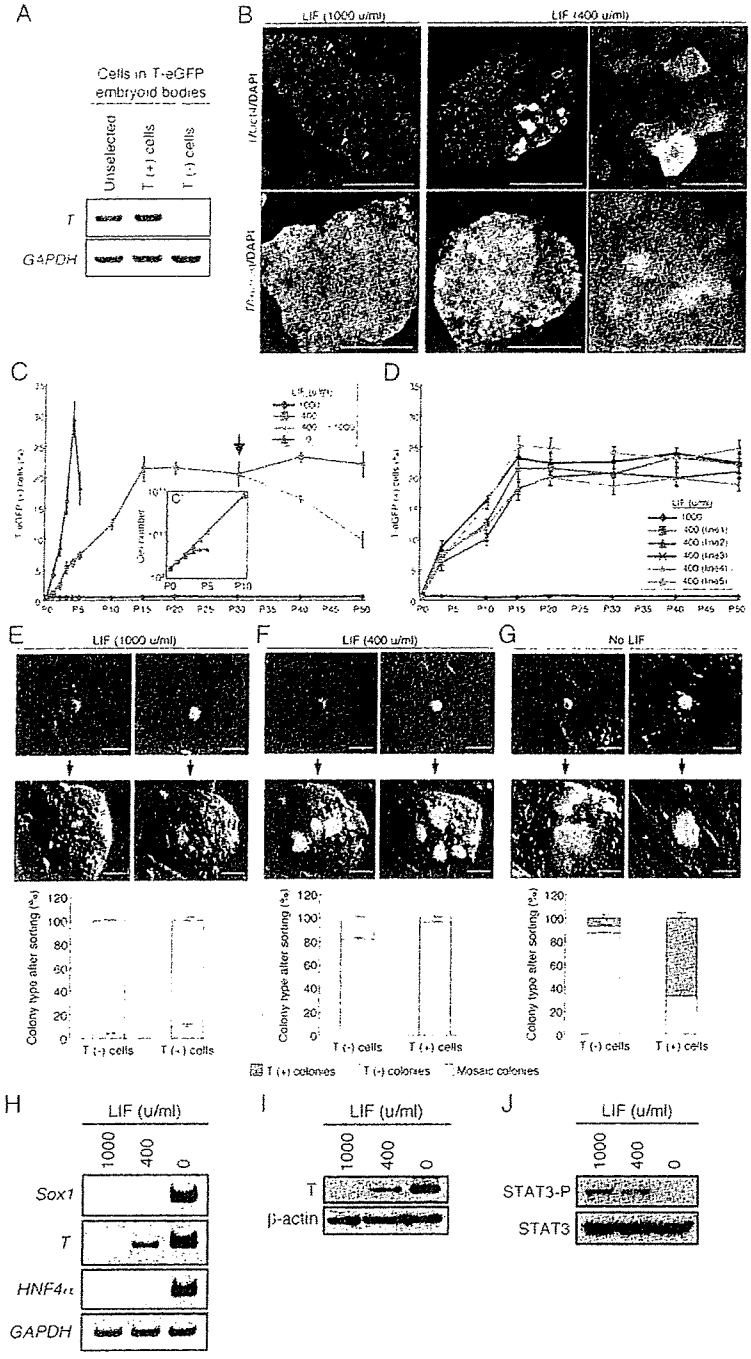

Fig. 1 LIF regulates the percentage of *T*-expressing cells in mouse ES cell cultures. (**A**) RT-PCR analysis of *T* expression in *T-eGFP* embryoid bodies (unselected cells), and in T(+) or T(−) cells isolated from *T-eGFP* embryoid bodies. *T-eGFP* expression recapitulated endogenous *T* expression. (**B**) Fluorescent images of *T* (eGFP)/Oct4 and *T* (DsRed2)/*Nanog* (eGFP) expression in mouse ES cell colonies at passage (P) 25 in culture with 1000 or 400 μ/mL of LIF. DAPI staining identifies individual cells in each field. T(+) cells were observed not only in colonies formed in culture with 400 μ/mL of LIF (*right* and *center* panels), but also in colonies formed in culture with 1000 μ/mL of LIF (*left* panels, *arrowheads*). Scale bar = 50 μm (*left* and *center* panels) or 10 μm (*right* panels). (**C**) Flow-cytometric analysis of T(+) cells in *T-eGFP* ES cell cultures with various LIF concentrations (1000, 400, and 0 μ/mL). A portion of ES cells cultured with 400 μ/mL of LIF was retreated with 1000 μ/mL of LIF from P30 (shown by *arrow*). Bar shows mean ± SD (n = 4). (**C'**) Growth of *T-eGFP* ES cells expressed by the number of cells in culture with 1000, 400, or 0 μ/mL of LIF (n = 4). (**D**) Flow-cytometric analysis of T(+) cells in five independent ES cell lines carrying the *T-eGFP* reporter, cultured with 1000 or 400 μ/mL of LIF. Cell line 1, 2, 3, 4, and 5 corresponds to J1, SAT1, SAT2, SAT6, and SAT11, respectively. Five independent ES cell lines exhibited similar dynamics of T(+) cell-accumulation in culture with

culture conditions. Flow cytometric analyses of cultures of *T-eGFP* ES cells revealed a 0.59 ± 0.05% of T(+) cells (n = 4), a fraction that remained virtually invariable after more than 50 passages (Fig. 1C'). These findings are consistent with the widely known (though frequently overlooked) fact that mouse ES cells, even when maintained under optimal culture conditions, exhibit some degree of spontaneous differentiation. Indeed, this very fact has been recognized as a hallmark of a "good" ES cell line [3]. Our observation is also consistent with the identification of *T* as a transcript selectively enriched in undifferentiated mouse ES cells [9, 17].

3 Mouse ES Cells Contain a Population of *T*-Expressing Cells

We reasoned that, if the fraction of T(+) cells remained constant at 0.5% after continuous passaging, the rate at which T(+) cells were produced by spontaneous differentiation must be in equilibrium with their disappearance. A priori, the most likely scenarios involved lengthening of the cell cycle and eventual mitotic arrest of T(+) cells, selective cell death, the progression of differentiation with loss of *T* expression, or a combination of these mechanisms. To directly address these possibilities, we analyzed the fate of individual T(+) cells isolated by fluorescence activated cell sorting (FACS) and plated at clonal density. Surprisingly, T(+) cells gave rise to colonies that contained only a few or no T(+) cells, and which were of similar size and undifferentiated characteristics as those generated by T(−) cells, as judged by their morphology (Fig. 1E) and transcription profile (data not shown). These results suggest the possibility that T(+) cells are not selectively eliminated from undifferentiated ES cell cultures; rather, they undergo a dedifferentiation process that gives rise to undifferentiated T(−) progeny.

Since the addition of LIF is critical for the maintenance of undifferentiated ES cells, we next investigated whether the percentage of T(+) cells in our cultures depended on the amount of LIF in the culture medium. *T-eGFP* ES cells cultured in the absence of exogenous LIF differentiated extensively over time and could not be grown over five passages (Fig. 1C'). Under these conditions, the percentage of T(+) cells rose exponentially (Fig. 1C) and markers of ectoderm, mesoderm, and endoderm lineages were expressed (Fig. 1H), indicating that the majority of ES cells underwent spontaneous differentiation. T(+) cells sorted from cultures maintained for three to four passages in the absence of LIF displayed a dramatically reduced ability to generate colonies containing T(−) cells, when compared with cells grown in medium containing 1000 μ/mL of LIF (Fig. 1G). In contrast, reduction of exogenous LIF to 400 μ/mL resulted in colonies of normal ES cell morphology (Fig. 1B) that could be maintained in culture for over 50 passages with no signs of differentiation or crisis. This finding is consistent with the range of exogenous LIF concentrations reported to sustain self renewal of pluripotent mouse ES cells [6, 18], with the activation of STAT3 phosphorylation under these conditions (Fig. 1J), and with the fact that ES cells cultured in medium containing 400 μ/ml of LIF for extended periods of time, when injected into blastocysts, result in degrees of chimerism and contribution to the germline not different from ES cells maintained in 1000 μ/mL of LIF (our unpublished observations).

Despite their apparently undifferentiated morphology, colonies of *T-eGFP* ES cells adapted to grow in medium supplemented with 400 μ/mL of LIF contained large numbers of T(+) cells (Fig. 1B). The adaptation process was gradual, so that the percentage of T(+) cells increased over time when the cells were switched to culture medium containing 400 μ/mL of LIF and reached a plateau of 21.2± 2.0% at 15 passages (n = 4), after which the fraction of T(+) cells remained constant (Fig. 1C). Also, the expression of *T* at both mRNA (Fig. 1H) and protein (Fig. 1I) levels was increased in cultures supplemented with 400 μ/mL of LIF when compared with *T-eGFP* ES cells grown in medium containing 1000 μ/mL. To rule out the possibility that the increased number of T(+) cells under these conditions represented a peculiarity of the ES cell line used in these experiments (J1), we established four independent ES cell lines from a different genetic background (C57BL/6 × 129/TerSv) that were used to generate additional *T-eGFP* transgenic lines. With small variations, all four lines displayed similar dynamics of accumulation of T(+) cells when cultured in medium containing 400 μ/mL of LIF (Fig. 1D).

Other than the increased numbers of T(+) cells, we could not detect any differences in cultures maintained with 400 versus 1000 μ/mL of LIF. Thus, the increase in *T* expression was not accompanied by up-regulation of other transcripts involved in mesoderm differentiation, and no markers of ectoderm or endoderm differentiation were detected in ES cell cultures supplemented with 400 μ/mL of LIF (Fig. 1H). Moreover, T(+) cells generated under these conditions showed proliferation rates similar to T(−) cells when plated at high density (0.2×10^6 cells yielded $4.18 \pm 0.27 \times 10^6$ and $4.00 \pm 0.31 \times 10^6$ cells, respectively, after 7 days in culture, n = 3; see also Fig. 1C'), displayed an ability to give rise to T(+) cells comparable to that of T(−) cells when plated at clonal density (Fig. 1F), co-expressed the pluripotency-associated markers *Oct4*, *Nanog*, and *Rex1* (Figs. 1B, 3A), and stained positive for alkaline phosphatase (not shown). Importantly, the number of T(+) cells in cultures supplemented with 400 μ/mL of LIF declined progressively after the concentration of the cytokine was increased to 1000 μ/ml (Fig. 1C). Taken together, our results indicate that, in cultures of mouse ES cells, a population of T(+) cells exists

whose size is controlled by the amount of LIF present in the culture medium. Importantly, lowering the concentration of LIF to 400 u/ml did not appear to be detrimental for the self-renewal of mouse ES cells, even though T(+) cells formed up to 20% of the cells in these culture conditions. For these reasons, and since the size of the T(+) fraction was more amenable to analysis, we continued our studies in culture medium containing 400 µ/ml of LIF.

4 Reversal of Mesoderm Commitment of ES Cells by LIF

To characterize the identity of T(+) cells, we first analyzed their ability to generate differentiated progeny. For this purpose, we performed in vitro differentiation assays of bulk *T-eGFP* ES cells as well as of sorted populations of T(+) and T(−) cells. In these assays, unsorted ES cells and T(−) cells behaved similarly, giving rise to differentiated cells that expressed markers of ectoderm, mesoderm, and endoderm fates (Fig. 2A). In contrast, embryoid bodies formed from T(+) cells differentiated exclusively into cells expressing markers of mesoderm lineages (Fig. 2A), indicating that the T(+) cells present in our cultures of *T-eGFP* ES cells were lineage-committed. Thus, we termed this population of T(+) cells "early mesoderm-committed" (EM) progenitors.

In our experiments of colony formation from sorted cells plated at clonal density (Fig. 1E, 1F), EM progenitors gave rise to large numbers of T(−) cells. In light of the mesoderm commitment of T(+) cells, two possible scenarios could account for these findings: T(−) cells generated in these conditions could represent (i) a further step of mesoderm differentiation of EM progenitors, in which *T* was no longer expressed [16]; or (ii) undifferentiated ES cells dedifferentiated from EM progenitors. To investigate these possibilities, we analyzed the differentiation potential of T(−) cells generated from EM progenitors (Fig. 2B). In vitro differentiation assays revealed that T(−) cells derived from EM progenitor cells were able to give rise to cells of ectoderm, mesoderm, and endoderm lineages (Fig. 2C). Moreover, T(−) cells readily generated beating cardiomyocytes that stained positive for myosin, albumin-positive cells after prolonged periods under differentiation-promoting conditions, and Tuj1-positive cells after treatment with retinoic acid (Fig. 2D). We also analyzed the ability of T(−) cells to colonize embryo lineages in vivo after their introduction into mouse blastocysts. To trace the progeny of the injected cells, a constitutively expressed LacZ reporter was introduced into *T-eGFP* ES cells. We recovered 11 embryos from 2 independent injections of T(+)-derived T(−) cells into mouse blastocysts, of which 5 were overtly chimeric. In these embryos LacZ-positive cells contributed to organs of ectoderm, mesoderm, and endoderm lineages (Fig. 2F–2K). As a control for our in vitro and in vivo differentiation assays of T(−) cells, we analyzed T(+) cells generated from EM progenitors. In all cases, T(+) cells behaved the same as T(+) cells isolated from bulk cultures of *T-eGFP* ES cells, displaying in vitro differentiation potential restricted to mesoderm lineages (Fig. 2C) and failing to contribute to embryogenesis in vivo (we could not detect any signs of chimerism in eight embryos recovered; Fig. 2E). Our results show that T(+) cells represent a population of EM progenitors that, in the presence of LIF, are able to recover the abilities of self-renewal, pluripotency, and chimera contribution characteristics of mouse ES cells.

5 Nanog Regulates the Dedifferentiation of EM Progenitors

In our characterization of the transcriptional profile of EM progenitors, we did not detect changes in the expression of the pluripotency-associated markers *Oct4* and *Rex1* or in the levels of *Gbx2*, *Fgf5*, or *Lif* expression (see Discussion below). However, we detected a clear up-regulation in the expression of the pluripotency-associated marker *Nanog*, when compared to that of ES T(−) cells (Fig. 3A). Indeed, aside from the expression of *T* itself, these two cell populations only appeared to differ in the level of *Nanog* expression, raising the possibility that Nanog function is mechanistically linked to the transition of ES cells to EM progenitors, or vice versa. We investigated this possibility by directly manipulating the level of *Nanog* expression in *T-eGFP* ES cells and analyzing the consequences of such manipulations in the size and characteristics of the T(+) and T(−) cell populations. The introduction of a *Nanog* expression transgene driven by a strong constitutive promoter resulted in sustained overexpression of *Nanog* transcripts in *T-eGFP* ES cells (Fig. 3B). In these conditions, the expression of *T* was down-regulated (Fig. 3B), and the transition of EM progenitors to ES cells was facilitated, as evaluated by the ~3-fold reduction of T(+) cells (Fig. 3F) and the ~17-fold increase in the percentage of colonies composed exclusively by T(−) cells generated by EM progenitors overexpressing *Nanog* (Fig. 3H, 3J), when compared to mock-transfected *T-eGFP* ES cells (Fig. 3E, 3H, 3I).

For the converse experiment, down-regulation of Nanog function, we assayed the efficiency of short-hairpin RNAs (shRNAs) to induce partial *Nanog* silencing, since complete loss of Nanog function is incompatible with the pluripotent phenotype of ES cells [9]. Introduction of *Nanog-shRNA* into *T-eGFP* ES cells resulted in a marked decrease in the levels of Nanog, as evaluated by RT-PCR (Fig. 3D) and immunoblotting with specific antibodies (Fig. 3C). In these

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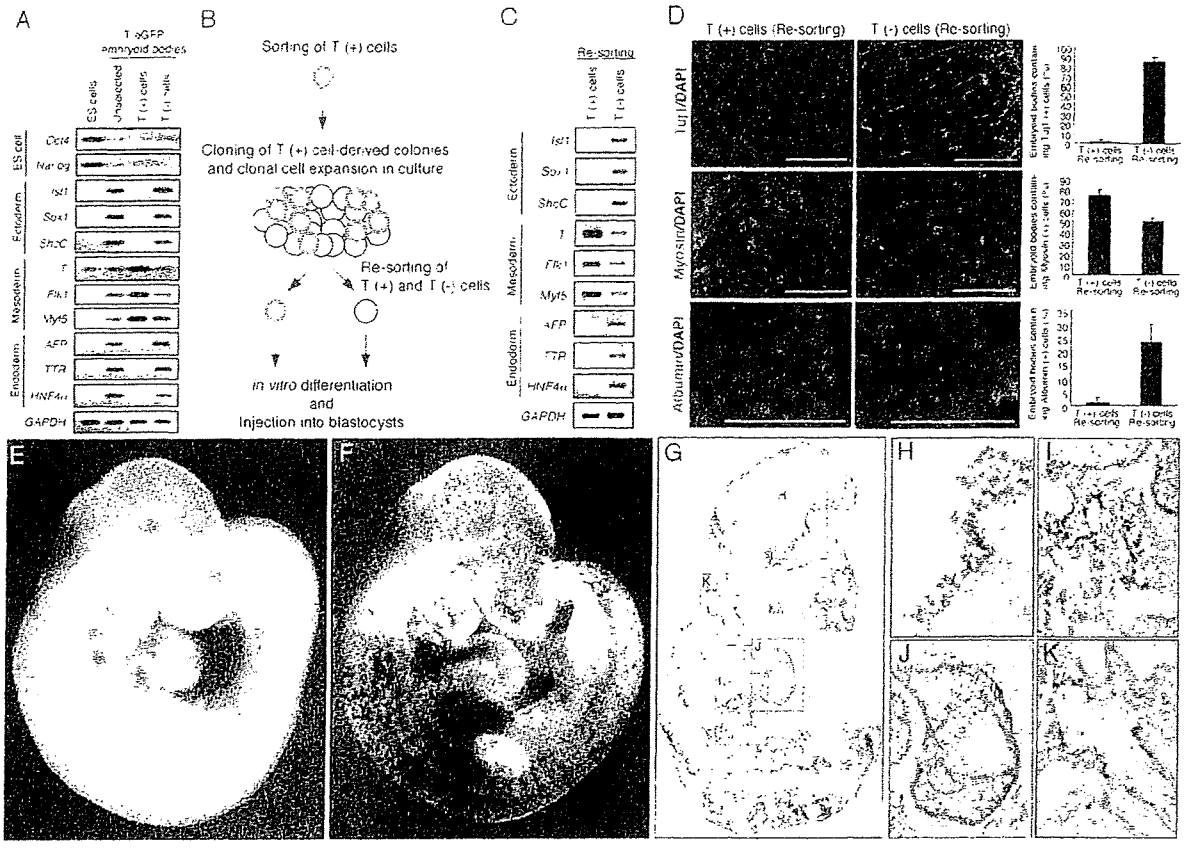

Fig. 2 Dedifferentiation of lineage-committed mesoderm progenitors into pluripotent stem cells. (**A**) RT-PCR analysis of ES cell, ectoderm, mesoderm, and endoderm marker gene expression in *T-eGFP* ES cells obtained at P20 (400 μ/ml of LIF), and in embryoid bodies derived from unselected *T-eGFP* ES cells, and from purified T(+) or T(−) cells. (**B**) Schematic representation of the experimental procedure for characterizing T(+) and T(−) cells derived from a single T(+) cell (EM progenitor cell). A single T(+) cell formed a mosaic colony in clonal density cultures with 400 μ/ml of LIF. Each mosaic colony was independently picked up and expanded. Then, T(+) and T(−) cells originally derived from a single T(+) cell were re-sorted by FACS to analyze their differentiation potential in vitro (**C**, **D**), and in vivo by injecting into blastocysts (**E**, **F**, **G**, **H**, **I**, **J**, **K**). (**C**) RT-PCR analysis for ectoderm, mesoderm, and endoderm marker gene expression in embryoid bodies derived from re-sorted T(+) or T(−) cells. (**D**) Immunocytochemical analysis of Tuj1 (neuronal lineage), myosin (muscle lineage), and albumin (hepatic lineage) production in embryoid bodies derived from re-sorted T(+) or T(−) cells. Scale bar = 100 μm. *Graphs* show the percentage of embryoid bodies containing antigen positive cells. *Bar* shows mean ± SD ($n = 3$; 30 embryoid bodies were examined in each dish). (**E**, **F**) Chimeric analysis demonstrating contribution of re-sorted T(+) (**E**) or T(−) (**F**) cells in E10 mouse embryos. β-galactosidase activity was used to visualize the contribution of re-sorted T(+) or T(−) cells in chimeric embryos. Images are *right* lateral view with the anterior to the *top*. (**G**, **H**, **I**, **J**, **K**) Histological images of the embryos shown in panel (**F**). A sagittal section counter stained with eosin (**G**) and close ups of different areas (**H**, **I**, **J**, **K**): (**H**) neuroepithelium; (**I**) gut tube and hepatic primordia within septum transversum; (**J**) myocardial wall; (**K**) artery. Panels A-D are reproduced with permission of Nature Publishing Group [48]

conditions, cell colonies of undifferentiated ES cell morphology formed, and no signs of endoderm differentiation were apparent (not shown). However, the number of T(+) cells generated from EM progenitors expressing *Nanog-shRNA* more than doubled that of mock-transfected *T-eGFP* ES cells maintained under similar culture conditions (Fig. 3G). Also, EM progenitors expressing *Nanog-shRNA* generated ~20% of colonies composed exclusively of T(+) cells when plated at clonal density (Fig. 3H, 3K), indicating that the transition of EM progenitors to ES cells was impaired upon down-regulation of Nanog function. Together with the results of our gain-of-function experiments, these findings demonstrate that Nanog controls the dedifferentiation of EM progenitors to ES cells.

6 Positive Regulation of *Nanog* Expression by T and LIF/STAT3

We next investigated the mechanism by which *Nanog* expression is up-regulated in EM progenitors. Based on our observations that *Nanog* expression is found to be increased

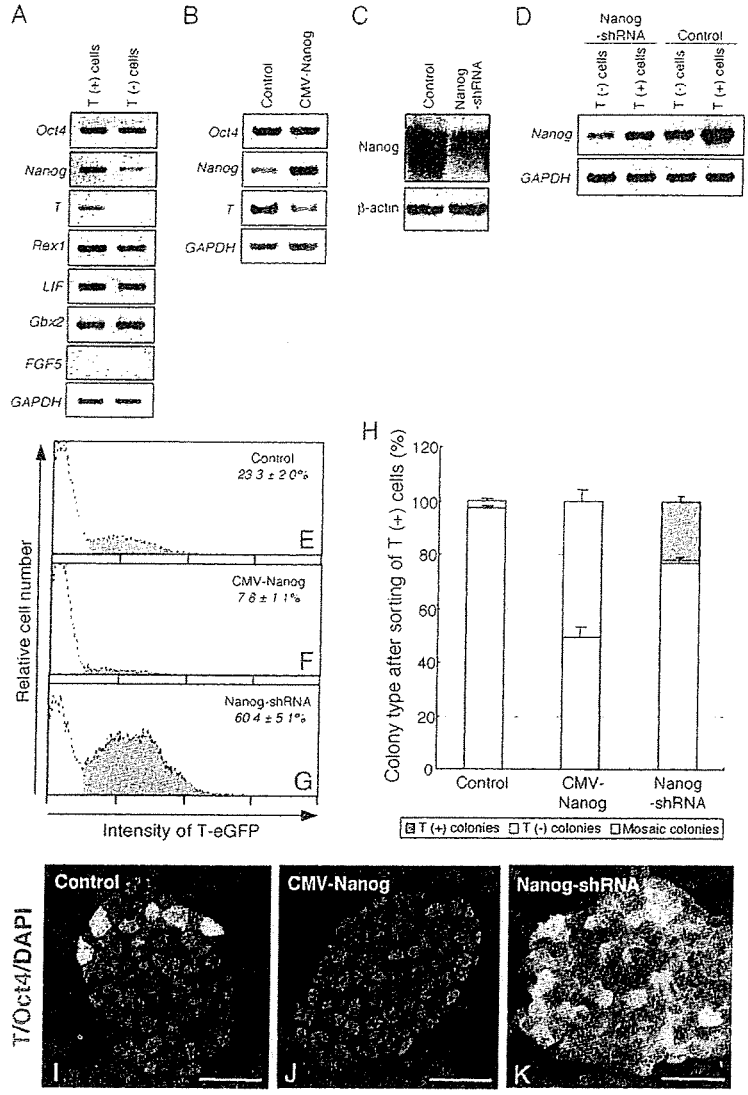

Fig. 3 *Nanog* is sufficient and necessary for dedifferentiation of EM progenitors into pluripotent stem cells. (**A**) RT-PCR analysis of gene expression in T(+) or T(−) cells isolated from *T-eGFP* ES cells. T(+) cells exhibited a higher level of *Nanog* expression. (**B**) RT-PCR analysis of *Oct4*, *Nanog*, and *T* expression in *T-eGFP* ES cells carrying control or CMV-*Nanog* constructs. Overexpression of *Nanog* is associated with down-regulation of *T* expression. (**C**) Western blotting analysis of Nanog in *T-eGFP* ES cells carrying control or *Nanog-shRNA* constructs. *Nanog-shRNA* efficiently down-regulates the level of Nanog protein in ES cells. (**D**) RT-PCR analysis of *Nanog* expression in T(+) or T(−) cells isolated from *T-eGFP* ES cells carrying control or *Nanog-shRNA* constructs. (**E–G**) Flow-cytometric analysis of T(+) cells produced from purified T(+) cells carrying control (**E**), CMV-*Nanog* (**F**), or *Nanog-shRNA* (**G**) constructs. *T-eGFP* ES cells were transfected with each construct, then T(+) cells were isolated, selected with puromycin, and cultured for 7 days with 400 μ/mL of LIF. Percentages of T(+) cells are shown in each panel ($n = 4$, mean ± SD). (**H**) Percentage of types of colonies in cultures of (**E–G**) before FACS analysis. *Bar* shows mean ± SD ($n = 4$; 40 colonies were examined in each dish). (**I–K**) Fluorescent images of *T* (eGFP)/Oct4 expression in colonies formed in cultures of experiments shown in panels (**E–G**). DAPI staining identified individual cells in each field. Scale bar = 20 μm. Panels A-D and I-K are reproduced with permission of Nature Publishing Group [48]

in cell populations with high levels of *T* expression (Fig. 3A), we first asked whether T could induce *Nanog* expression. For this purpose, we attempted to overexpress *T* in ES cells by transfecting a full-length cDNA driven by a constitutive promoter. Under these conditions, ES cells underwent massive differentiation and could not be propagated, even in culture medium supplemented with 1000 μ/mL of LIF (not shown). As an alternative approach, we used a doxycycline-inducible conditional expression system. ES cells expressing the tetracycline-inducible transcriptional activator (rtTA)

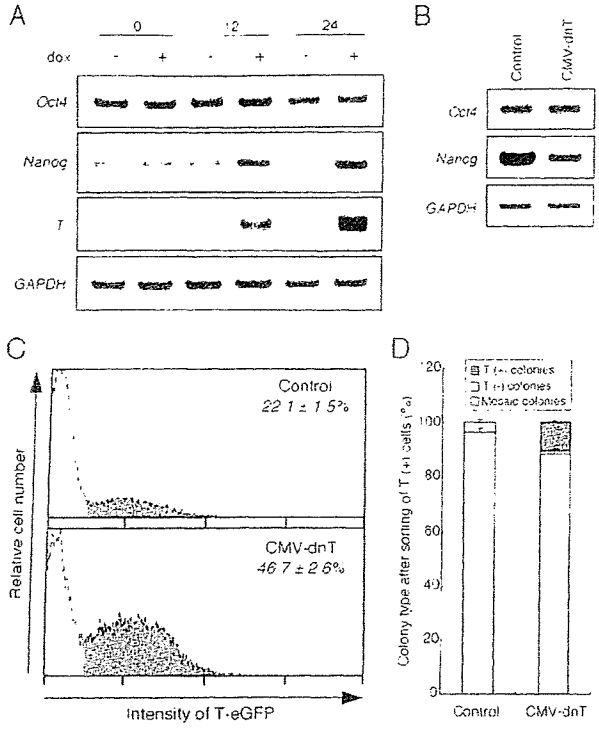

Fig. 4 T controls Nanog-dependent dedifferentiation of EM progenitors. (A) RT-PCR analysis of *Oct4*, *Nanog*, and *T* expression in doxicycline- (dox) dependent *T*-inducible ES cells cultured with 1000 μ/mL of LIF. *T* expression was induced by dox, and *Nanog* up-regulation was associated with an increased level of *T*. Numbers on the *top* indicate induction hours with dox. (B) RT-PCR analysis of *Oct4* and *Nanog* expression in *T-eGFP* ES cells carrying control or CMV-*dnT* constructs. dnT down-regulated *Nanog* expression without affecting *Oct4* expression in ES cells cultured with 400 μ/mL of LIF. (C, D) Blockage of T function impaired transition of T(+) cells to T(−) cells. Flow-cytometric analysis of T(+) cells produced from purified T(+) cells in the presence or absence of down-regulation of T by CMV-*dnT* (C). *T-eGFP* ES cells were transfected with each construct, then T(+) cells were isolated, selected with puromycin, and cultured for 7 days with 400 μ/mL of LIF. Percentages of T(+) cells are shown in each panel ($n = 4$, mean ± SD). Percentage of types of colonies in cultures of (C) before FACS analysis (**D**). *Bar* shows mean ± SD ($n = 4$; 40 colonies were examined in each dish). Panels A-B are reproduced with permission of Nature Publishing Group [48]

[19] were transfected with a construct containing the full-length *T* cDNA driven by a tetracycline-responsive promoter (T-rtTA ES cells). In the absence of doxycycline, T-rtTA ES cells cultured in medium supplemented with 1000 μ/mL of LIF formed colonies of undifferentiated ES cells, with a morphology indistinguishable from parental untransfected ES cells. Under these conditions, *T* expression in T-rtTA ES cells was negligible, demonstrating the tight regulation of the inducible system (Fig. 4A). Upon addition of doxycycline to cultures of T-rtTA ES cells, *T* expression was induced progressively and became detectable by RT-PCR after 12 h of doxycycline induction (Fig. 4A). T-rtTA ES cells cultured in the presence of doxycycline started to acquire the characteristic flattened morphology of differentiated colonies 48–72 h after doxycycline induction (not shown). Importantly, *Nanog* expression increased in T-rtTA ES cells after 12 h of doxycycline induction, paralleling that of *T* (Fig. 4A). These results indicate that the expression of *Nanog* can be regulated by T and suggest that the up-regulation of *Nanog* expression found in EM progenitors may depend on their increased levels of *T* expression.

To test this possibility, we analyzed the consequences of blocking the function of T in ES cells in the expression of *Nanog* and the generation of EM progenitors. For this purpose, we used a truncated version of T previously shown to function as a dominant-negative (dnT) [20]. *T-eGFP* ES cells stably expressing *dnT* formed colonies with undifferentiated ES cell morphology in culture medium containing 400 u/ml of LIF (see Discussion below). The level of *Nanog* expression in these cells, however, was down-regulated when compared with that of mock-transfected *T-eGFP* cells under similar culture conditions (Fig. 4B). Moreover, the blockade of T function resulted in an impaired transition of EM progenitors to ES cells (Fig. 4C, 4D), consistent with the reduced levels of *Nanog* expression found in these conditions. Thus, our results from gain- and loss-of-function experiments identify a negative feedback mechanism by which increased *T* expression in EM progenitors up-regulates the expression of *Nanog*, which, in turn, down-regulates *T* expression and promotes the regeneration of an ES cell phenotype.

To gain insights into the regulation of *Nanog* expression by T, we analyzed the mouse *Nanog* gene in the search of regulatory sequences. At 4.91 kb upstream of the translation start site of *Nanog*, we identified a 20-bp sequence forming an imperfect palindrome that shared homology with the proposed binding site for T [21] (Fig. 5A). We tested the ability of T to bind to oligonucleotides representing this sequence, but not to mutated versions thereof, by performing

Fig. 5 (continued) maintained with 400 μ/mL of LIF (**E**). Both the T- and STAT3-binding sites were required for activation of the *Nanog* EM enhancer activity in ES cells cultured with 400 μ/mL LIF (**F**). WT: −5203 to −4192 bp. MUT/T, MUT/S and MUT/TS indicate mutation in T-, STAT3- or both T- and STAT3-binding sites, respectively, in the *Nanog* EM enhancer. Bars show mean ± SD ($n = 4$). (**G**) T and STAT3 physically interact inside cells. T and STAT3 were co-immunoprecipitated when STAT3 was activated by LIF. (**H**) Fluorescent images of *T*-eGFP and *Nanog* EM enhancer DsRed2 expression in ES cell colonies formed in culture with 400 μ/mL of LIF. Co-expression of eGFP with DsRed2 in wild-type, but not when T- and/or STAT3-binding sites are mutated, indicates that the activity of the *Nanog* EM enhancer in EM progenitor cells is regulated by T and STAT3. Modified figure reproduced with permission from ref. [49]

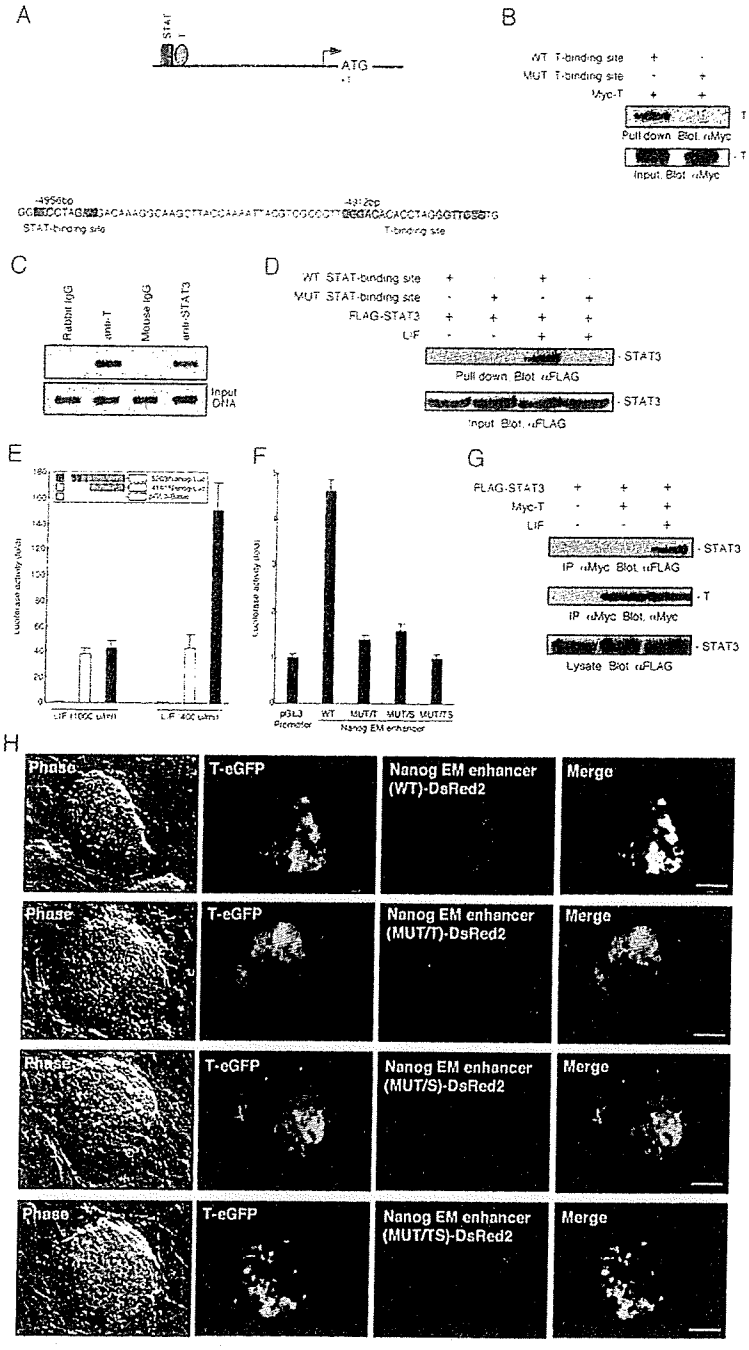

Fig. 5 Binding of STAT3 and T on *Nanog*EM enhancer is required for up-regulation of *Nanog*expression in EM progenitors. (**A**) Schematic representation of the 5'-upstream regulatory region of the mouse *Nanog* gene. Putative STAT- and T-binding sites are indicated. (**B**) T bound to the putative T-binding site in the *Nanog* regulatory region, as shown by pull-down assays. Wildtype (WT) and mutated (MUT) versions of double-strand oligonucleotides representing the putative T-binding site were used as probes. Input lysates were also blotted with anti-Myc antibody. (**C**) ChIP assay for the putative T- and STAT-binding sites in the *Nanog* regulatory region showed specific binding of T and STAT3 to the regulatory region. The *lower panel* is a PCR-amplification of input DNA prior to immunoprecipitation. (**D**) LIF-dependent binding of STAT3 to the putative STAT-binding site in the *Nanog* regulatory region. Wild-type and mutated versions of double-strand oligonucleotides for the putative STAT-binding site were used as probes. Input lysates were also blotted with anti-FLAG antibody. (**E**, **F**) Analysis of transcriptional activities of the *Nanog* regulatory region by luciferase reporter assay in mouse ES cells (1000 or 400 μ/mL of LIF). Both −5203Nanog-Luc and −4191Nanog-Luc showed a similar activation with 1000 μ/mL of LIF, whereas −5203Nanog-Luc activity was further increased in cultures

in vitro pull-down assays of biotin labeled oligonucleotides incubated with lysates of NIH3T3 expressing Myc-tagged T (Fig. 5B). We also investigated the ability of endogenous T to bind the region of interest in the *Nanog* promoter in vivo by chromatin immunoprecipitation (ChIP) assays of ES cells with a T specific antibody (Fig. 5C). Our search for putative regulatory elements in the *Nanog* promoter also identified a predicted STAT-binding site 44 bp upstream of the T-binding site (Fig. 5A). We tested the ability of STAT3 to bind to this site in vitro (Fig. 5D) and in vivo (Fig. 5C) using experimental approaches similar to the ones used to characterize the T-binding site. These results uncover the presence of functional binding sites for T and STAT3 in the mouse *Nanog* promoter.

We next analyzed the significance of the T- and STAT3-binding sites in the *Nanog* promoter for the biology of EM progenitors. We generated two constructs driving the expression of luciferase, one comprising 5.2 kb of the *Nanog* genomic sequence upstream of the translation start (–5203Nanog-Luc, which included both STAT3 and T-binding sites), and the other lacking the 5'-most 1 kb (and thus, both STAT3 and T binding sites, –4191Nanog Luc). Transient transfection of ES cells with either reporter construct resulted in a similar ~40-fold transcriptional induction (compared to a promoterless luciferase construct) when ES cells were cultured in medium containing 1000 μ/mL of LIF (Fig. 5E), a condition in which EM progenitors are generated at very low frequency (Fig. 1C). These results indicate that the regulatory elements responsible for the constitutive expression of *Nanog* in ES cells are located in the first 4.2 kb of the mouse *Nanog* gene upstream of the translation start. Importantly, the transcriptional activity of the –5203Nanog-Luc was increased by ~4-fold with respect to that of –4191Nanog-Luc in ES cells adapted to grow in medium supplemented with 400 μ/mL of LIF (Fig. 5E), in which the EM progenitor population represents ~20% of the culture (Fig. 1C). These findings suggest that the enhancer element responsible for *Nanog* up-regulation in EM progenitors (*Nanog* EM enhancer) is located between –5203 and –4192 bp upstream of the translation start of the mouse *Nanog* gene, a region containing the functional STAT3- and T binding sites.

We then generated a luciferase reporter construct driven by the *Nanog* EM enhancer and a minimal promoter. This enhancer element increased transcription levels by ~4.5-fold when transiently transfected into ES cells cultured with 400 μ/mL of LIF (Fig. 5F). Moreover, the activity of the *Nanog* EM enhancer was lost when either or both the STAT3- and the T-binding sites were mutated (Fig. 5F). To visualize the activity of the *Nanog* EM enhancer in specific cells, we used it to drive the expression of a red fluorescent protein (DsRed2) reporter in ES cells. *T-eGFP* ES cells stably expressing this reporter showed activity of the *Nanog* EM enhancer only in EM progenitors, as evaluated by the co-localization of eGFP and DsRed2 signals in these cells (Fig. 5H). Consistent with the results of the luciferase reporter assays, mutation of either or both STAT3- and T-binding sites in the *Nanog* EM enhancer abrogated the activity of this reporter in EM progenitors (Fig. 5H). Our results so far demonstrate that the up-regulation of *Nanog* expression in EM progenitors depends on the binding of STAT3 and T to specific sites in the EM enhancer in the mouse *Nanog* gene. Since the binding sites for STAT3 and T are located in close proximity to one another in the *Nanog* EM enhancer, and since both T-box transcription factors [22–24] and STAT3 [25, 26] have been described to physically interact with other transcription factors for the regulation of specific promoters, we decided to analyze whether T and STAT3 could interact inside the cell. We tested this possibility in NIH3T3 cells by co-transfecting expression vectors encoding tagged versions of STAT3 and T (FLAG-STAT3 and Myc T) and carrying out immunoprecipitation assays. Interestingly, we found an association of T with STAT3 only when nuclear translocation of STAT3 was activated by stimulation with LIF (Fig. 5G).

7 Nanog Directly Blocks Mesoderm Induction by BMPs

Our findings so far demonstrate that *Nanog* expression is up regulated in EM progenitors in the presence of LIF by the combined action of activated STAT3 and T, and that increased Nanog function promotes the transition of EM progenitors to ES cells. We reasoned that a likely mechanism of Nanog action in this process could be preventing the generation or blocking the effects of pro-differentiation factors. BMPs are potent inducers of mesoderm differentiation in the context of embryo development [27, 28], as well as in mouse ES cells [29–31]. EM progenitors, in turn, are generated rapidly during the first ~15 passages after reducing the LIF supplement in the culture medium from 1000 to 400 μ/mL (Fig. 1C). Thus, we tested whether the generation of EM progenitors in these conditions was modified by increasing or decreasing BMP signaling in cultures of ES cells. After three passages in medium containing 400 μ/mL of LIF, the size of the EM progenitor population reached ~6% in cultures of *T-eGFP* ES cells (Fig. 6A, see also Fig. 1C). This percentage almost doubled when cells were incubated in the presence of recombinant BMP2, BMP4, or BMP7 and was reduced by half upon incubation with noggin (Fig. 6A), a secreted factor that blocks BMP signaling [32, 33]. We then tested whether BMP signaling was also regulating the maintenance of EM progenitors. When pure populations of EM progenitors were plated in culture medium containing 400 μ/mL of LIF, ~75% of the resulting cells underwent a transition

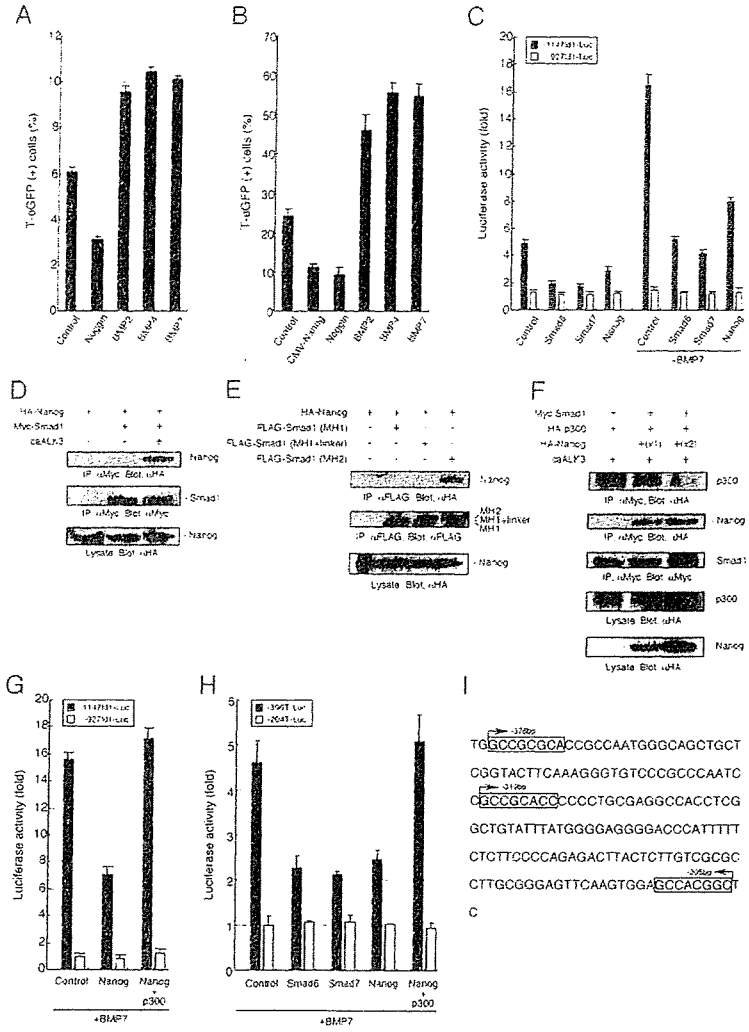

Fig. 6 Nanog interferes with BMP signaling at the level of transcription activity of Smad. (**A**) Flow-cytometric analysis of T(+) cells in *T-eGFP* ES cells cultured for three passages with 400 μ/mL of LIF under conditions of inhibition (noggin) or activation (BMP2, BMP4, and BMP7) of BMP signaling. *Bar* shows mean ± SD (n = 4). (**B**) Flow-cytometric analysis of T(+) cells produced from purified T(+) cells cultured with 400 μ/mL of LIF under conditions of inhibition or activation of BMP signaling. Inhibition of endogenous BMP signaling by noggin decreased the percentage of T(+) cells at a similar level of Nanog overexpression, whereas BMP activation increased the percentage of T(+) cells. *Bar* shows mean ± SD (n = 4). (**C**) A reporter construct of −1147Id1-Luc containing the Smad-binding sites, but not −927Id1-Luc, was activated in a BMP-dependent manner in ES cells cultured with 400 μ/mL of LIF. Nanog and inhibitory Smads (Smad6 and Smad7) down-regulated −1147Id1-Luc activity in a similar manner. Bars show mean ± SD (n = 4). (**D–F**) Co-immunoprecipitation assays of the physical interaction between Nanog and BMP-responsive Smad1. Nanog interacted with activated Smad1 (**D**). Nanog interacted with the MH2 domain of Smad1 (**E**). Nanog interfered with the interaction between activated Smad1 and p300 by competitively binding to Smad1 in a dose-dependent manner (**F**). The relative p300:Nanog ratio was 1:1 or 1:2. (**G**) Overexpression of p300 rescued the down-regulation of −1147Id1-Luc activity induced by *Nanog*. Bars show mean ± SD (n = 4). (**H**) The −396T-Luc reporter construct, but not −204T-Luc, was activated in a BMP-dependent manner in ES cells cultured with 400 u/ml of LIF. Nanog and inhibitory Smads (Smad6 and Smad7) down-regulated −396T-Luc activity in a similar manner. The down-regulation of −396T-Luc activity induced by Nanog was rescued by overexpression of p300. Bars show mean ± SD (n = 4). (**I**) Sequence of the 5'-upstream regulatory region of the mouse *T* gene. Three putative BMP-responsive Smad-binding sites are indicated with boxes. Please, add the following text: Panels A-C and G-I are reproduced with permission of The National Academy of Sciences of the United States of America [49]

to ES cells, whereas the remaining ~25% maintained EM progenitor identity (Fig. 6B, see also Figs. 3E, 3I, 4C). When the cultures were supplemented with BMPs, the maintenance of EM progenitors increased by ~2-fold, whereas it was decreased by half upon incubation with noggin (Fig. 6B). Interestingly, overexpression of *Nanog* in EM progenitors resulted in a decrease in their maintenance similar to that induced by noggin (Fig. 6B). These results indicate that

the generation and maintenance of EM progenitors depends, at least in part, on the differentiation promoting activity of BMPs and suggest that Nanog's ability to reduce the numbers of EM progenitors may depend on the blockade of BMP signaling.

Signaling by BMPs is intracellularly transduced by receptor-regulated Smads (Smad1, 5, and 8) and the co-mediator Smad4 and is antagonized by inhibitory Smads (Smad6 and 7) [34]. To characterize the mechanism by which Nanog blocks BMP signaling, we first analyzed the effects of *Nanog* overexpression in the BMP-induced transcriptional activation of *Id1*. *Id1* is a well-characterized transcriptional target of BMP signaling [35], for which the Smad-binding elements have been mapped to a specific region in the *Id1* promoter [36]. We used luciferase reporter constructs containing (−1147Id1-Luc) or lacking (−927Id1-Luc) the Smad-binding sites [36] and analyzed their activity in ES cells. Transient transfection of these reporters in ES cells resulted in a ~4.5-fold activation of the −1147Id1-Luc reporter when compared to −927Id1-Luc (Fig. 6C), indicating the existence of a significant level of endogenous BMP signaling associated with our culture conditions (see Discussion below). Addition of BMP to the culture medium resulted in a strong up-regulation of the −1147Id1-Luc reporter compared to −927Id1-Luc (Fig. 6C). That the activation of the −1147Id1-Luc reporter was due to BMP signaling was further confirmed by the fact that co-transfection of ES cells with cDNAs encoding inhibitory Smads drastically reduced the transcriptional activity of the reporter induced by endogenous or exogenous BMPs (Fig. 6C). Interestingly, *Nanog* overexpression in ES cells closely mimicked the effect of inhibitory Smads (Fig. 6C), suggesting that Nanog may block BMP signaling by interfering with the formation of activated Smad complexes.

Inhibitory Smads negatively regulate BMP signaling by binding to activated receptor-regulated Smads, hence limiting their availability to form transcriptionally active complexes with Smad4 and/or other nuclear cofactors [34]. To address whether Nanog blocked BMP signaling by a similar mechanism, we first analyzed its ability to interact with the receptor-regulated Smad1 inside the cell. Co-immunoprecipitation assays in NIH3T3 cells revealed that Nanong was indeed able to bind Smad1 only when the latter was activated by co-transfection of a constitutively active ALK3 (caALK3, Fig. 6D). Next, we mapped the interaction domain of Smad1 with Nanog. The different Smads contain two conserved domains, the N-terminal Mad homology (MH) 1 and the C-terminal MH2 domain, separated by a poorly conserved linker. The interaction of receptor-regulated Smads with Smad4 and other transcription factors and cofactors, as well as with inhibitory Smads, occurs through the MH2 domain [34]. In cells co-transfected with Nanog and expression constructs encoding the individual MH1, MH1+linker, or MH2 domains of Smad1, interaction with Nanog was found exclusively with the MH2 domain (Fig. 6E). These results are consistent with a negative role of Nanog on BMP signaling by interfering with the interaction of receptor activated Smads with Smad4 and/or additional nuclear factors.

The paralogous transcriptional coactivators CREB-binding protein (CBP) and p300 are nuclear cofactors important for TGFβ signaling, including that of BMPs, that interact with the MH2 domain of receptor-regulated Smads and Smad4 [37–39]. To gain further insights into the mechanism of Nanog-mediated down regulation of BMP signaling, we tested whether Nanog interfered with the recruitment of p300 to the complexes of activated Smads. For this purpose, Myc-tagged Smad1, HA-tagged p300, and caALK3 were expressed in NIH3T3 cells with or without HA-tagged Nanog. Immunoprecipitations of cell lysates were performed with anti-Myc antibodies followed by Western blotting utilizing anti-HA antibodies. In the absence of Nanog, Smad1 efficiently co-immunoprecipitated p300 (Fig. 6F). In the presence of co-expressed Nanog, the amount of p300 bound to Smad1 decreased in a Nanog dose-dependent manner (Fig. 6F). The functional significance of these findings was further verified by the fact that overexpression of p300 completely rescued the down-regulation in the transcriptional activity of the *Id* promoter induced by Nanog (Fig. 6G). These results indicate that Nanog negatively regulates BMP signaling by interfering with the recruitment of the co-activator p300 to the Smad transcriptional complex.

Finally, the finding that the expression of the *Xbra*, the homologue of *T* in *Xenopus*, is regulated by TGFβ signals [40] prompted us to investigate whether *T* could be a transcriptional target of BMP signaling in ES cells, and, if so, whether Nanog could directly block the induction of *T* by BMPs. In a preliminary analysis, we identified a BMP-responsive element in the ~1.2-kb region upstream of the translation initiation site of the mouse *T* promoter (data not shown). We then generated a series of luciferase reporter constructs covering this region. We transfected these constructs into ES cells cultured in medium containing 400 μ/ml of LIF and supplemented with BMP7, and further mapped the BMP-responsive element to a region located between −396 and −204 bp of the mouse *T* gene (Fig. 6H). Under these conditions, the activity of the −396T-Luc reporter was ~5-fold that of −204T-Luc and decreased by half upon co-expression of inhibitory Smads or Nanog (Fig. 6H). Interestingly, the down-regulation of −396T-Luc activity induced by Nanog could be completely rescued by co-expression of p300 (Fig. 6H). The analysis of this region in the mouse *T* promoter detected three motifs with homology to the reported consensus of BMP-responsive Smad-binding sites [41]. These results indicate that *T* is a direct transcriptional target of BMP signaling, and

that Nanog down-regulates *T* expression by inhibiting BMP signaling at the level of the formation of active Smads/p300 complexes.

8 Discussion

Mouse ES cells, consistent with their developmental origin in the embryo epiblast, have the ability to give rise to derivatives of all three primary germ layers. However, unlike cells in the epiblast, in which pluripotency is very transient, mouse ES cells can be maintained in culture indefinitely in a pluripotent state. The mechanism(s) whereby the adaptation to culture conditions releases epiblast cells from the loss of pluripotency remain an outstanding question in the biology of ES cells.

9 The Transient EM Progenitor Population

In this study, we identify a population of EM progenitors normally present in cultures of mouse ES cells. The commitment of EM progenitors to mesoderm fates is evident upon LIF withdrawal, which results in differentiation restricted to mesoderm lineages and by their failure to contribute to embryogenesis in vivo (Fig. 2A, 2C, 2E). In the presence of LIF, however, EM progenitors are phenotypically indistinguishable from ES cells, as both populations co-exist in colonies of undifferentiated morphology and both maintain pluripotency over extended periods of time in culture (Fig. 1C). Moreover, EM progenitors maintain the expression of pluripotency-associated markers such as *Oct4*, *Nanog*, and *Rex1* and have high levels of alkaline phosphatase activity (Figs. 1B, 3A, and data not shown). Indeed, in the presence of LIF, EM progenitors and ES cells interchange their identities at a rate that depends, precisely, on the amount of LIF.

In this sense, the regeneration of a pluripotent ES cell phenotype from EM progenitors is reminiscent of the reversion to ES cells of early primitive ectoderm-like (EPL) cells [42]. EPL cells are generated in vitro by culturing ES cells in medium conditioned by HepG2 cells with or without LIF [42]. Similar to the population of EM progenitors characterized in this study, EPL cells express markers of pluripotency at levels comparable to ES cells, do not contribute to embryonic lineages upon injection into mouse blastocysts, can be reverted to an ES cell phenotype in the presence of LIF upon withdrawal of HepG2-conditioned medium [42], and differentiate in vitro preferentially (though not exclusively) into mesoderm-derived lineages [43]. EPL cells can be differentiated from ES cells based on their characteristic expression profile, which includes high levels of *Fgf5* expression and down-regulation of *Rex1* and *Gbx2* expression [42]. In contrast, EM progenitors display expression levels of *Rex1* and *Gbx2* comparable to ES cells and do not express *Fgf5* (Fig. 3A). Thus, despite some similarities, EM progenitor cells are distinct from EPL cells and are more closely related to pluripotent ES cells, at least based on their respective transcriptional profiles. In addition, two important characteristics of the EM progenitor population make its analysis especially relevant for our understanding of ES cell pluripotency: (i) EM progenitors are generated from ES cells under standard culture conditions, not by addition of ill-characterized conditioned media, and (ii) in the same culture conditions, EM progenitors undergo a dedifferentiation process that gives rise to pluripotent ES cells.

10 Differentiation-Promoting Activity of BMPs

The results from our analyses indicate that the generation of EM progenitors from ES cells depends on the direct mesoderm-inducing ability of BMP stimulation (Fig. 6). This finding is consistent with the reported roles of BMP signaling during embryo development [27, 28], and with previous studies of ES cell differentiation in vitro [29–31]. However, the mesoderm-differentiating activity of BMPs seems to be at odds with their role in maintaining the self-renewal of pluripotent ES cells [7]. Indeed, BMP signaling appears to have contrasting effects in the maintenance of ES cell pluripotency. On the one hand, BMPs are necessary to prevent ES cell differentiation toward neural fates [7, 30, 44]. On the other hand, signaling by BMPs results in loss of ES cell pluripotency by promoting their differentiation toward non-neural fates such as mesoderm-derived lineages [29, 31] (and this study). These opposing effects of BMPs can be partially explained by differences in the experimental conditions used in those studies. Thus, in the absence of LIF, low concentrations of BMPs (~0.25–10 ng/mL) promote mesoderm differentiation [29–31] at the expense of neural fates [30]. In the presence of LIF, however, similar low concentrations of BMPs prevent neural differentiation of ES cells [7, 44] and maintain their pluripotency with no signs of mesoderm differentiation [7]. Consistent with this notion, we did not detect increased generation of EM progenitors with BMP concentrations below 100 ng/mL in the presence of LIF (data not shown). Thus, LIF appears to render ES cells refractory to the mesoderm-inducing activity of BMPs. Our studies demonstrate that this resistance is, at least in part, dependent on the negative feedback mechanism mediated by Nanog.

11 A Negative Feedback that Blocks Mesoderm Differentiation

While investigating the role of LIF in the maintenance of mouse ES cell pluripotency, Rathjen and colleagues [45] found that the expression of *Lif* itself is up-regulated in the

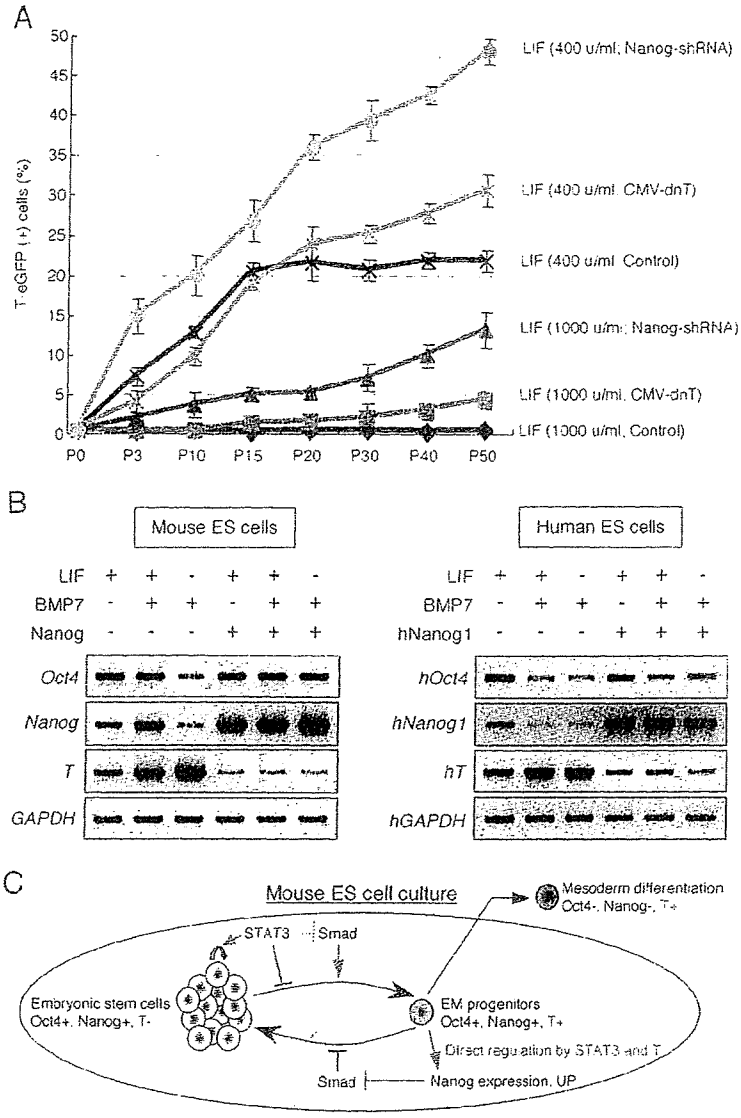

Fig. 7 Nanog-mediated dedifferentiation of EM progenitors is required for maintaining mouse ES cells. (**A**) Flow-cytometric analysis of T(+) cells in *T-eGFP* ES cells (1000 or 400 u/ml of LIF), after down-regulation of T or Nanog activities with *dnT* or *Nanog-shRNA*. (**B**) Comparison of expression profiles of mouse and human ES cells. Mouse and human ES cells were transfected with mouse *Nanog* and *hNanog1*, respectively, or a control vector, then treated with LIF and/or BMP7. In human ES cells, *hNanog1* was down-regulated in response to BMP7 stimulation, whereas *T* expression increased. In contrast, in mouse ES cells, these genes were up-regulated by LIF and BMP7. (**C**) Schematic representation of the mechanism for the maintenance of pluripotent mouse ES cells. EM progenitors are generated in ES cell cultures depending on the balance between the level of LIF/STAT3 and BMP/Smad activation. *Nanog* expression increases in EM progenitors by the combinatorial action of T and activated STAT3. As a result, Nanog inhibits the differentiation signal of BMP by interfering with the formation of activated Smad/p300 complexes, and promotes the dedifferentiation of EM progenitors into pluripotent ES cells. See main text for details

early phases of ES cell differentiation. This mechanism provides a negative feedback that may limit the progression of ES cell differentiation and contribute to the self-renewal of pluripotent ES cells [45]. The transition of EM progenitors to ES cells does not appear to depend on such a mechanism, since *Lif* expression is not noticeably up-regulated in EM progenitors (Fig. 3A). In contrast, EM progenitors do up-regulate the expression of *Nanog* (Fig. 3A). Our results also show that, in the presence of LIF, *Nanog* overexpression is sufficient to accelerate the transition of EM progenitors to ES cells (Fig. 3F, 3H, 3J). More importantly, down-regulation of Nanog function results in impaired dedifferentiation of EM progenitors to ES cells (Fig. 3G, 3H, and 3K). Thus, we identify Nanog as a critical component of

a negative feedback mechanism that blocks the progression of ES cell differentiation toward mesoderm fates (Fig. 7C). In this mechanism, mesoderm differentiation of ES cells is initiated by BMP signaling. Possible sources of BMP activity in our culture conditions include fetal calf serum [29], fibroblast feeder layer, and/or ES cells themselves [7]. Consistent with this, we detect a significant activation of the -1147Id1-Luc reporter even in the absence of exogenous BMP supplements (Fig. 6C). ES cells that initiate mesoderm differentiation express the early mesoderm marker *T*. In the presence of LIF, activated STAT3 cooperates with T to directly up-regulate the expression of *Nanog* (Fig. 5), which, in turn, provides a negative feedback that down-regulates *T* expression and eventually leads to the regeneration of ES cells from EM progenitors. In the absence of LIF/STAT3 signaling, T is not sufficient to up-regulate the expression of *Nanog*, and mesoderm differentiation proceeds. The relevance of this negative feedback mechanism for maintaining the pluripotency of ES cells is evident in long-term cultures. Thus, the size of the EM population cannot be maintained in ES cells in which either Nanog or T function is experimentally down-regulated (Fig. 7A). In such conditions, the cultures progressively accumulate EM progenitors and eventually lose pluripotency (not shown).

The existence of such a negative feedback mechanism in mouse ES cells contributes to explaining previous observations on the function of Nanog. For instance, the fact that overexpression of *Nanog* bypasses the need for LIF/STAT3 signaling [10] and BMP stimulation [7] to maintain self-renewal of pluripotent ES cells is easily understood in light of this mechanism. Since the final outcome of BMP activity is the up- regulation of *Nanog* expression, which is mediated by LIF/STAT3 signaling, the experimental up-regulation of Nanog would obviate the need for both BMP and LIF. It is clear, however, that the functions of LIF and Nanog in the maintenance of ES cell pluripotency are not restricted to participating in the negative feedback mechanism characterized in this study. Thus, the complete lack of Nanog function promotes differentiation of ES cells to endoderm lineages [9], indicating the existence of additional roles of Nanog other than that of preventing mesoderm differentiation. Indeed, the up-regulation of *Nanog* expression by T and STAT3 only takes place in EM progenitors, whereas the constitutive expression of *Nanog* in ES cells is regulated by more proximal regions of the *Nanog* promoter (Fig. 5E).

The requirement of LIF/STAT3 signaling for the maintenance of mouse ES cells has been related to the ability of pre-implantation mouse embryos to arrest development when implantation is prevented (a phenomenon known as dispause) [46]. In keeping with this idea, it appears reasonable that cells in the inner cell mass of mouse blastocysts evolve specific mechanisms to prevent unwanted cell differentiation during dispause. The mechanism described in this study could very well serve this purpose. In contrast, ES cells derived from human embryos, in which dispause does not occur, do not depend on LIF/STAT3 signaling to maintain pluripotency [47]. Interestingly, the Nanog-mediated negative feedback mechanism characterized in this study does not appear to be operative in human ES cells. First, the overall conservation of the mouse *Nanog* EM-enhancer in the human *Nanog* gene is very poor, and no T-binding site is present (not shown). Second, unlike mouse ES cells, human ES cells do not up-regulate *Nanog* expression in response to LIF and BMP stimulation, even though *T* expression is induced under these conditions (Fig. 7B). The absence of a functional negative feedback mechanism mediated by T, LIF/STAT3, and Nanog in human ES cells provides additional mechanistic insights into the reasons why LIF is dispensable for the self-renewal of human ES cells.

Taken together, our results uncover a mechanism underlying mouse ES cell pluripotency, by which committed mesoderm progenitors undergo an active process of dedifferentiation mediated by the combined action of the extrinsic cytokine LIF and the intrinsic pluripotency factor Nanog. These findings contribute to unravel the complex network of molecular interactions required to maintain the self-renewal of ES cells and shed light on the cellular bases of ES cell pluripotency. Furthermore, the possibility of reverting the differentiation status of committed cells offers new ways to approach the generation of pluripotent cells for future therapeutic interventions of regenerative medicine.

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References

1. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981;78:7634–8.
2. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154–6.