厚生労働科学研究費補助金

再生医療実用化研究事業

安全に移植できる細胞を誘導するためのタンパク質導入法の開発

平成２０ー２１年度　総合研究報告書

研究代表者　升井　伸治

平成２２（２０１０）年　３月

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安全に移植できる細胞を誘導するためのタンパク質導入法の開発

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転写因子セットを導入し有用細胞を誘導する知見（例；iPS 細胞）は今後急速に蓄積される。その応用過程として、遺伝子導入を介さない方法での作出証明が必要だが、従来のタンパク質導入法はトラブルが多い。本研究では、一般的に使用される融合タグを切断することでタンパク質導入法が確実に機能できる知見を得た。本知見をもとに、一旦タンパク質導入法で移植細胞が「出来る」とわかれば、臨床応用は促進されるだろう。

A．研究目的

ヒト iPS 細胞作出の成功要因は、「転写因子セット」の導入である。一般に細胞の性質は、核内の転写因子ネットワークが決定していると考えられるが、iPS 細胞作出法は ES 細胞転写因子ネットワークの人為的再現だった。同様の手法を用いた有用細胞作出の試みは報告が増えつつあり、今後数年内に「○○を作出できる転写因子セット」が次々と報告されるだろう。一方で、ウイルスなどによる多数の遺伝子導入は腫瘍化をはじめとした様々な副作用を細胞にもたらすため、臨床応用への次の段階としては、遺伝子導入を経ない転写因子セット導入法でその細胞が作出可能なことを証明する必要があるだろう。その最有力な手法はタンパク質導入法だが、従来法ではタンパク質の種類に依存して導入効率や活性が大きく異なることが障害となり、最初にクリアすべきステップである「タンパク質導入法でも分化誘導出来る」ことの証明に非常に時間がかかるのが問題である。

現在、主に２つのタンパク質導入法がある。そのうち一つである細胞膜透過性の低分子化合物との共導入法（市販キット）では、目的タンパク質を精製しておく必要があるのだが、転写因子は組み換えタンパク質としては良く発現しない場合が多く（DNA 結合能による宿主への毒性）、また精製後も活性を保つような条件検討が困難であった。もう一方の細胞膜透過ドメインとの融合法では、原理的に細胞膜透過ドメインと目的タンパク質との間でフォールディング干渉が起きやすく、様々なタンパク質と融合させたときに一定した導入効率と活性が得られていない（Pharmac Res 19:1302-9, 2002）。そこで本研究ではタンパク質精製を経ず、フォールディング干渉を起こさない簡便で確実なタンパク質導入システムを開発する。これを用いて目的細胞が誘導可能であることがわかれば、内毒素除去など

さらなる安全性を担保し臨床研究に進めるだろう。

２０年度は、COS 細胞を用いて分泌したタグ付きタンパク質の導入効率を計測した。２１年度は、タグを切断する酵素の機能確認と、正常な転写活性のためにはタグ切断が必要であることの証明を行った。本知見をもとに、一旦タンパク質導入法で移植細胞が「出来る」とわかれば、臨床応用は促進されるだろう。

Ｂ．研究方法

ES 細胞の培養：フィーダーフリー株 ZHBTc4 (Nat Genet (2000) 24:372-6)、2TS22C (Nature cell biology (2007) 9: 625-635) 、EB3 (Genes Cells (2004) 9: 471-477) を用いて、ゼラチンコート培養皿上にて以下の培地で培養した。Glasgow minimal essential medium (GMEM), 10% fetal calf serum, 1 mM sodium pyruvate, 10-4 M 2-mercaptoethanol, 1x nonessential amino acids (Invitrogen), 1000 U/ml LIF。

COS 細胞 (BMT10) およびパッケージング細胞 (Plat-E ; Experimental Hematology 31 (2003) 1007-1014) の培養：ゼラチンコート皿上、DMEM + 10%血清にて培養した。

プラスミド構築：レトロウイルスは pMXs (Experimental Hematology 31 (2003) 1007-1014) を用いた。

Nacalai 社、Hygromycin は Invivogen 社 HygroGold を使用した。

トランスフェクション： Lipofectamine 2000 (Invitrogen) を用いたリポフェクション法にてメーカー指定の方法に従ってトランスフェクションを行った。

蛍光顕微鏡観察：IX71 (オリンパス社) を用い、蛍光ミラーユニットは GFP は U-MGFPHQ、DsRed は U-MRFPHQ を使用した。

遺伝子発現解析：SYBR Premix ExTaq (Takara) を用いた定量的 RT-PCR を行い、リアルタイム PCR 装置は MyiQ リアルタイム PCR 解析システム (BioRad) を用いた。

(倫理面への配慮)

本研究では実験動物を用いる予定は無いため、倫理上の問題は無い。遺伝子組み換え実験を行うが、P2 レベルで申請は受理されている。

Ｃ．研究結果

i) タンパク質導入系の開発

細胞膜透過ドメインを含むタグ融合タンパク質を培地中に高濃度で分泌発現させ (COS 細胞を用いる)、この培養上清をヒトおよびマウス繊維芽細胞に添加する。繊維芽細胞にはタグを除去するシステムを機能させておくため、導入されたタンパク質はタグのフォールディング干渉を受けずに機能できる仕組みである。

このタグは分泌シグナル配列の Igk リーダー配列 (分泌後は切断されている)、細胞膜透過性ドメイン、およびリンカー配列を付加し、個々の遺伝子に固有の立体構造の影響を緩和する。

タグ融合タンパク質発現ベクターを、COS

細胞においてリポフェクション法を用いて導入する。血清の影響を排除するため血清置換剤 KSR ベースの培地で過剰発現させ、その培養上清を用いてタンパク質導入効率を検定する。

タンパク質導入の効率を上げるため、培養上清は容量を極力低く設定し、且つ長時間発現させて濃度を高くする。培地中に分泌されたタンパク質は比較的安定であり、VP22 では培養上清調製後４８時間でも導入効果があることを確認できた。分泌シグナル配列(Igκリーダー配列)、細胞膜透過性ドメイン(VP22, 11R)を連結させた試作タグに EGFP 遺伝子を繋いだ発現ベクターを、COS 細胞においてリポフェクション法を用いて導入したところ、導入効率は１割前後であったことを２０年度に確認した（図１）。

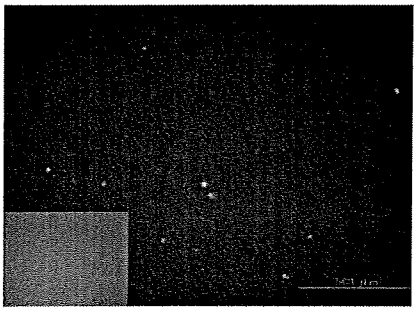

図１．培養上清にて調製された膜透過型 GFP タンパク質

導入効率の上昇を促すため、培養上清を限外濾過膜で濃縮しタンパク質濃度を高めることを試みたが、導入効率に変化は見られなかった。一方で、融合タンパク質の濃度を上げる実験を行った。細胞膜透過ドメインーＢＳＤ（ブラストサイジン耐性遺伝子）融合タンパク質の大量精製を行った。精製した 11R-BSD タンパク質を 2TS22C に加えて培養し、セレクション実験を行った。培地に 11R-BSD タンパク質を（10μg/ml，1μg/ml，100ng／ml，10ng/ml，1ng/ml ，0）加え、24 時間培養した。細胞をよく洗った後、BSD を 10 μg/ml 加えた培地で、48 時間培養した。この操作を、コロニーが大きくなるまで繰り返した。その結果、10 μg/ml の濃度でおよそ９０％の細胞がブラストサイジン耐性を示した。

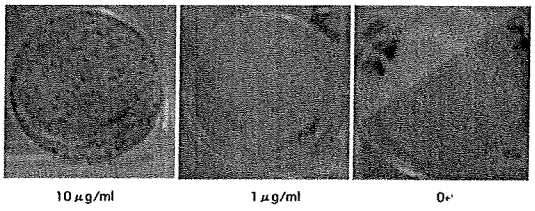

図２．11R-BSD は薬剤耐性を賦与する

タグ融合転写因子タンパク質も、10μg/ml 以上の濃度で添加する必要があるだろう。

ii）タグ切断の有効性の確認

Igk-VP22-Sox2 及び タグが切断された Sox2 のプラスミドを 2TS22C に導入し、Sox2 抑制における分化をレスキューする活性をもって転写活性とした。

その結果、VP22 や 11R など、一般に膜透過性を賦与するとされるアミノ酸配列をもつ場合、Sox2 の活性は大きく低下することがわかった。

ベクター	Tc+	Tc-	転写活性
empty	22	344	6.3
Sox2	270	286	94.4
VP22Sox2	49	466	10.5
11RSov2	57	436	13.0
Cut-Sox2	162	258	62.7

図３．タグは転写活性を低下させる
CAG-IB ベクターによる強制発現。

細胞内に取り込まれたタグ融合タンパク質は、機能するためにはタグが切断されることが必須であると考えられる。
そこで、タグを切断するプロテアーゼを発現する ES 細胞（2TS22C, ZHBTc4 それぞれについて作製した）に、タグ融合タンパク質遺伝子を強制発現し、細胞内でのタグ切断による活性上昇を解析した。

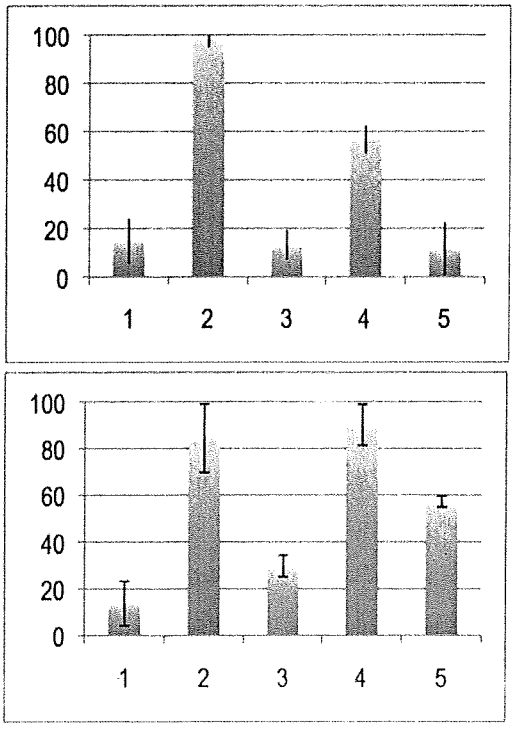

図４．細胞内でのタグ切断により転写活性は上昇する
上段；プロテアーゼ非発現 2TS22C
下段；プロテアーゼ発現 2TS22C
1, CAG-IB; 2, CAG-Sox2-IB; 3, CAG-Igk-11R-Sox2-IB; 4, CAG-cutSox2-IB; 5, CAG-Igk-11R-rs-Sox2-IB
rs；認識配列

その結果、プロテアーゼ認識配列をもつタグ融合 Sox2 について、転写活性の上昇を認めた。同様の結果は、Oct3/4 と ZHBTc4 細胞を用いても得られた。したがって、タグ切断が正常な転写活性の維持に必要であることがわかった。

iii）多因子発現システムを用いた解析
ウイルスを使わず（腫瘍化の危険が少なく）分化転換させる別のシステムとして、２０年度までに多因子発現システムを開発した。目的細胞内で多因子を同時に発現し分化転換させるシステムを意図した。導入因子数が多いと、ばらばらに導入すると全因子が入った細胞数は少なくなり、解析が困難になる。しかし、通常用いられているプロモーターでは、直列に連結すると転写が阻害される（転写干渉とよばれる）。２つまでなら逆向きに配置することで転写可能だが、３つ以上の場合は必ずどれかが直列の向きに位置することになり、転写干渉により発現しなくなる。異なるプロモーターを用いると直列でも

転写できる場合もあるが、百個以上の遺伝子のそれぞれに異なるプロモーターを用いるのは非現実的であり、少なくともどれかは転写干渉を免れない。そこで、同一のプロモーターを用いて、直列に連結したときにも発現する新しい転写システムを開発し、多数の遺伝子を同時発現させることを試みた。原理的に新しいシステムであるため、タンパク質翻訳量が十分か懸念されたが、至適化した結果、十分な量が得られた（図５の蛍光と薬剤耐性)。このシステムでは、染色体に入ると転写されないため（おそらくクロマチン因子が結合すると阻害的に働くため)、レトロウイルスより腫瘍化の危険が小さい。暫定的非ウイルス型分化転換システムとして提示できる。

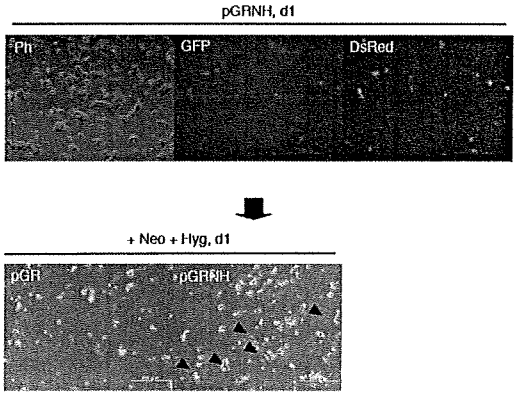

図５．４種遺伝子の同時発現

iv）分化転換のモデル系開発

分化転換法を用いた目的細胞の樹立十分条件の解析には、その細胞の排他的培養条件が判明している必要がある。iPS 細胞のケースでは、それまでの ES 細胞での解析知見から極めて排他的な培養条件と形態学的特徴がすでに明らかになっており、これを利用することで樹立したクローンを単離することに成功している。そこで、タンパク質導入法および新規プロモーターシステムを用いた分化転換が可能であることを示す解析モデル系（多能性幹細胞以外の、という意味で）の構築を試みた。体細胞同士の分化転換を可能ならしめるシステムであることが分かれば、後に任意の目的細胞を用いることができるだろう。ここでは扱いやすい（均一に未分化のまま培養できる）モデル幹細胞として、平面培養 NSC（神経幹細胞様細胞）を用いてマイクロアレイ解析を行い、まず特異的転写因子の絞り込みを行った。独立に樹立した３株を用い、コントロールには ES 細胞とマウス全身の RNA を用いた。その結果、特異的発現のみられる１５０遺伝子を同定した。後にこれらの遺伝子を新規プロモーターシステムに搭載し同時発現を試みる。次に、発生系譜の大きく異なる細胞が NSC に分化転換したイベントを鋭敏に検出できるシステムの開発を行った。胚体外内胚葉細胞 ExEnC を対象細胞として用いて、選択的培地（ExEnC が死滅して NSC のみが生育する培養条件）の検討を行うことにした。まず片方の Sox2 アレルに Hygromycin 耐性-Thymidine kinase 融合遺伝子 HygTK が導入された ESC (Elht5) に (Nat Cell Biol 9, 625-35 (2007))、ExEnC への分化誘導に十分となる転写因

子 Gata6 を導入した（BMC Dev Biol 7, 80 (2007)）。ExEnC に分化させ７日間培養し、その後さらに７日間を Gancyclovier 存在化で培養し、Thymidine kinase の作用によって残存 ES 細胞（Sox2 を発現する；すなわち HygTK を発現する）を完全除去した。XhtG5 と名付けたこの細胞は、NSC へと分化転換すれば内在性 Sox2 を発現するため Hygromycin 耐性となる。XhtG5 と NSEB5-2C を様々な培養条件下で選択的条件を探索した結果、XhtG5 は血清１０％が必須であること、一方 NSEB5-2C には N2 supplement と FGF2 および EGF が必須であることがわかった。したがって NSC 分化転換アッセイ時においては、血清存在化で XhtG5 を培養し多因子発現を行い、数日後に無血清培地（N2+FGF2+EGF）に交換すればよいだろう。Hygromycin 選択と組み合わせて、鋭敏且つ信頼度高く NSC 分化転換イベントを検出できるだろう。

２１年度は、多因子発現ベクターに Oct3/4, Sox2, Klf4, c-Myc および Pac（puromycin 耐性遺伝子）を搭載したベクターpOSKMP を作製した。pOSKMP を、骨髄由来幹細胞 NRG および胚体外内胚葉細胞 XhtG5 に導入し、培養条件を検討した結果、一日おきのトランスフェクションの２回目に、１０％ＫＳＲ培地に置換することで、ES 細胞様の形態を示すコロニーが（一過性に）現れることがわかった。プレ iPS 細胞とよばれる状態であると推測できる。しかし継続して培養するとこれらのコロニーは扁平な形態に戻ることもわかった。

Ｄ．考察

膜透過ドメインを付加した転写因子による転写活性化の報告は、これまで国内外において多くあるのだが、どのシステムも任意の転写因子タンパク質をその活性化状態を保持したまま導入することはできていない。膜透過ドメイン自体の電荷の偏りやフォールディングへの影響が懸念されるため、積極的に除去されるシステムの開発を進める必要がある。本研究では、膜透過型融合転写因子におけるタグすなわち膜透過ドメインが正常な転写活性の発揮を阻害しており、細胞内におけるタグの切断によって転写活性が回復することを明らかとした。

多因子発現システムを新たに構築し、これを分化転換研究に用いることを試みた。細胞の性質を徐々に変えるには数個の機能重要転写因子の持続的発現で十分だが、迅速に変化させるには多数（百以上）の因子を同時発現する必要があるだろう。本研究で開発されたシステムは原理上無限個の因子を発現させることが出来る。ネックとなるのはコンストラクション上の問題で、プラスミドサイズであると考えられる。数百キロベースまで構築可能な BAC を用いた構築を予定しており、これが可能で有れば数百の因子を同時発現できるだろう。

他方、分化転換が可能になっても、その効率があまりにも低ければ実用化につな

がらないと予想される。本研究では一般分化転換法（モデル系）として NSC と ExEnC を用いたシステムの構築を試みた。iPS 細胞の樹立時においても培地変換のタイミングが異なると樹立効率が大きく影響をうけることが知られており、ExEnC は血清が必須だが NSC は血清存在下では分化することも同時にわかったため、培地変換のタイミングについてはさらに検討する必要がある。一方、転写因子を発現させれば確実に下流遺伝子群が発現されるわけではなく、DNA やクロマチン修飾によるエピジェネティックな要因がロック機構として働いており、分化転換の効率は低いことが予想される。エピジェネティック因子の阻害剤による iPS 細胞樹立効率の向上も報告されており、今後は転写因子とエピジェネティクス制御因子の共発現、あるいは RG109 や VPA などのエピジェネティクス阻害剤などの併用を検討する必要がある。

E．結論

細胞膜透過の際に一般的に用いられるタグ（VP22 や 11R）が、転写因子の活性を阻害すること、および細胞内においてタグを切断すると、転写活性が回復することを明らかとした。したがって、タグ融合転写因子を細胞へ添加し、細胞内でタグを切断することで、望みの転写因子をウイルス非依存的に導入し機能させることが可能であると考えられる。

他方、一過性発現で多因子を同時に発現できるシステムを開発した。本システムは染色体に挿入されると発現しないため、腫瘍化の危険が小さい。暫定的非ウイルス型分化転換システムとして提示できる。再生医療の材料としてヒト iPS 細胞や間葉系幹細胞など多くの選択肢が整備されつつある。今後は効率の良い分化誘導法の開発が急務だが、転写因子を用いた分化転換法は核内の分化プログラムに直接作用するため、成長因子や細胞外マトリクスの刺激などで分化誘導する手法と比較して分化時間が短くて済む可能性が高い。したがってドナー細胞調製コストを下げる可能性を示すことができ、医療費の抑制につながる。

安全な移植材料の調製可能証明は臨床への必須ステップである。本研究ではタンパク質導入法による転写因子導入一般法の基礎知見が得られた。今後は様々な転写因子と細胞を用いた研究が進むだろう。ひいては安全な細胞の調整法につながり、再生医療実現化に貢献する。本研究の成果は多くの患者への再生医療の実施可能性を提示し、社会保障費の抑制と労働人口の増大につながる。

F．健康危険情報

なし。

G．研究発表

1．論文発表

Masui S. Pluripotency maintenance mechanism of embryonic stem cells and

reprogramming. Int J Hematol. 91巻3号360-372, 2010

２．学会発表

なし。

H．知的財産権の出願・登録状況

１．特許取得

升井伸治・大河内仁志・浜崎辰夫　「複数の遺伝子発現を行うための発現ベクター」、出願番号2008-260553、２００８年１０月７日

２．実用新案登録

なし

３．その他

なし

別添４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Masui S	Pluripotency maintenance mechanism of embryonic stem cells and reprogramming.	Int J Hematol.	91巻3号	360-372	2010

Int J Hematol
DOI 10.1007/s12185-010-0517-9

Pluripotency maintenance mechanism of embryonic stem cells and reprogramming

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Received: 28 October 2009 / Accepted: 12 November 2009

Abstract Embryonic stem (ES) cells are derived from blastocysts and are pluripotent. This pluripotency has attracted the interest of numerous researchers, both to expand our fundamental understanding of developmental biology and also because of potential applications in regenerative medicine. Systems biological studies have demonstrated that the pivotal transcription factors form a network. There they activate pluripotency-associated genes, including themselves, while repressing the developmentally regulated genes through co-occupation with various protein complexes. The chromatin structure characteristic of ES cells also contributes to the maintenance of the network. In this review, I focus on recent advances in our understanding of the transcriptional network that maintains pluripotency in mouse ES cells.

Keywords Pluripotency · ES cells · Transcriptional network

1 Introduction

Pluripotency is defined as the capability of a cell to differentiate into all the types of cells that make up an individual [1]. Tests for pluripotency include the formation of embryoid bodies in vitro that will differentiate into the three germ layers and in vivo contribution in chimeric mice and confirmation of germline transmission. Another test is teratoma formation, in which the cells are injected into nude mice to determine whether such injection leads to the development of tumors containing cells of all three germ layers. Embryonic stem (ES) cells, first reported in 1981 from the inner cell mass (ICM) of mouse E3.5 blastocysts [2, 3], can be stably cultured in vitro while maintaining pluripotency, and the properties of ES cells have allowed detailed analysis of the molecular mechanism maintaining pluripotency. Because the area is progressing rapidly, in this review, I focus on recent advances related to the transcriptional network that maintains pluripotency in mouse ES cells.

2 ES cells and other types of pluripotent stem cells

Before looking into the mechanism in detail, I briefly introduce other types of pluripotent stem cells that have recently been reported, and summarize their points of difference from mouse ES cells. Epiblast stem cells (EpiSCs) are derived from post-implantation embryos (E5.5–7.75), which contain the epiblast, a pluripotent derivative of the ICM [4, 5]. EpiSCs require fibroblast growth factor 2 (Fgf2, Mouse Genome Informatics, also known as bFgf) for growth. They have been shown to rely on Activin/nodal signaling, but do not show dependency on leukemia inhibitory factor (Lif) [4, 5], which is essential to maintain pluripotency in ES cells (see below). These features of EpiSCs are similar to those of human ES cells [6], and there may be a correspondence between the two.

Although EpiSCs can form teratomas, they provide only a very limited germline contribution in chimeric mice, and

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Published online: 16 February 2010

the gene expression pattern of EpiSCs is slightly different from that of ES cells, suggesting that the transcriptional network maintaining EpiSC pluripotency is regulated by a different mode [4, 5]. In fact, the expression of the central transcription factor gene *Oct3/4* is regulated by different regulatory regions in ES cells and EpiSCs (see below) [5]. Upon stimulation by Activin and Fgf2, ES cells can convert/differentiate to EpiSCs, while EpiSCs are unable to revert/dedifferentiate to ES cells with the addition of Lif. This suggests that EpiSCs are in a more advanced or committed developmental stage than are ES cells [7].

FAB-SCs, another form of pluripotent stem cell, are derived from blastocysts (E3.5) by culturing with three designated factors: Fgf2, Activin, and the GSK3β inhibitor BIO, which is an agonist of wingless-related MMTV integration site (Wnt) signaling [8]. Although common molecular markers for pluripotency are expressed, FAB-SCs do not differentiate. However, upon stimulation by Lif and by bone morphogenetic protein 4 (*Bmp4*) and through cell–cell interaction including E-cadherin signaling, these cells can acquire differentiation capability similar to that of ES cells. This suggests that the FAB-SCs are in a "latent" state of pluripotency [8], which is probably maintained by somewhat different transcriptional network from that of the ES cells. To avoid confusion by integrating information derived from different types of pluripotent stem cells, in the following sections, I focus on mouse ES cells, and particularly on the transcriptional network in those cells, to understand the molecular mechanism maintaining pluripotency.

3 Extracellular signaling that regulates self-renewal of ES cells

Several soluble factors have been identified that exert either positive or negative effects on ES cell self-renewal (cell proliferation while maintaining pluripotency) (Fig. 1). ES cells secrete an unidentified stem cell autocrine factor (SAF) to promote proliferation [9]. Presumably, this activity can be mimicked by high concentrations of adrenocorticotropic hormone (ACTH), which is known to induce proliferation in some types of cells. ACTH promotes proliferation in ES cells, probably through the inhibition of adenylyl cylase (AC) activity regulated by G-protein coupled receptors (GPCR). Alternatively, the SAF could be BNP known to be involved in cellular proliferation. In ES cells, BNP promotes proliferation through the activation of the E26 avian leukemia oncogene 1, 5′ domain (*Ets1*) and through repression of gamma-aminobutyric acid receptor A (*GABAαR*) [10], which negatively regulates proliferation by activating the phosphatidylinositol-3-OH kinase-related kinase (PIKK) family including ataxia telangiectasia mutated homolog (Atm) [11]. The above soluble factors might contribute to the characteristic cell cycle profile of ES cells, with few cells in the G1 phase and most of the cell population in the S phase. In this mode of cell cycle, a pivotal role is played by phosphatidylinositol-3-OH kinase-Akt (PI(3)K/Akt). That pathway is activated by Ras (Eras), a constitutively active form of Ras-family small GTPase that is expressed specifically in ES cells [12]. Several factors are essential in ES cell self-renewal, including T-cell lymphoma breakpoint 1 (Tcl1) [13–15] and myeloblastosis oncogene-like 2 (*Mybl2*, also known as *b-Myb*) [16–18], known to be involved in the regulation of the PI(3)K/Akt pathway (for details, see the excellent available reviews) [19, 20].

Lif, a member of the IL-6 family, is known to strongly promote self-renewal in ES cells [21]. Lif binds to leukemia inhibitory factor receptor (LIFR) to dimerize with interleukin 6 signal transducer (*Il6st*, also known as *gp130*), resulting in the phosphorylation of signal transducer and activator of transcription 3 (Stat3) via Janus kinase (Jak) activation [22]. Phosphorylated Stat3 dimerizes and translocates to the nucleus to activate a variety of downstream genes, including myelocytomatosis oncogene (*Myc*, also known as *c-Myc*) and Krüppel-like factor 4 (*Klf4*) [23, 24]. Repression of Stat3 results in differentiation [25], whereas artificial activation of Stat3 is sufficient to maintain pluripotency without Lif in the media [26]. In addition to *Stat3*, Lif signaling pathways are transduced to the PI(3)K/Akt pathway through which T-box3 (*Tbx3*) is activated, while activity is repressed by the mitogen-activated protein kinase (MAPK) pathway which is also activated by the Lif signal [24, 27]. Repression of *Tbx3* results in differentiation [28]. Forced expression of *Tbx3* blocks differentiation in the absence of Lif, by maintaining Nanog homeobox expression (*Nanog*, see below) [24].

In combination with the Lif-Stat3 pathway, the pluripotency of ES cells is modulated by transforming growth factor β (TGFβ) superfamily members. These include Bmp and Activin, which generally play diverse roles in cellular homeostasis [29]. In the ES cells, Bmp4 activates the MAD homolog 1 (Smad1). This upregulates the expression inhibitor of DNA-binding genes (*Id*), which suppress differentiation in combination with the Lif signal [30]. Activin/nodal signaling, which is known to have generally the opposite effect on Bmp4 [29], also contributes to promote the growth of ES cells [31]. In the latter study, Bmp4 signaling was shown to be dispensable for maintaining pluripotency. This finding, which appears contradictory, may reflect differences in the media components. That would affect the composition of the ES cell subpopulation based on the gene expression profile, as represented by the expression of the zinc-finger protein 42 gene (*Zfp42*, also known as *Rex1*) [32].

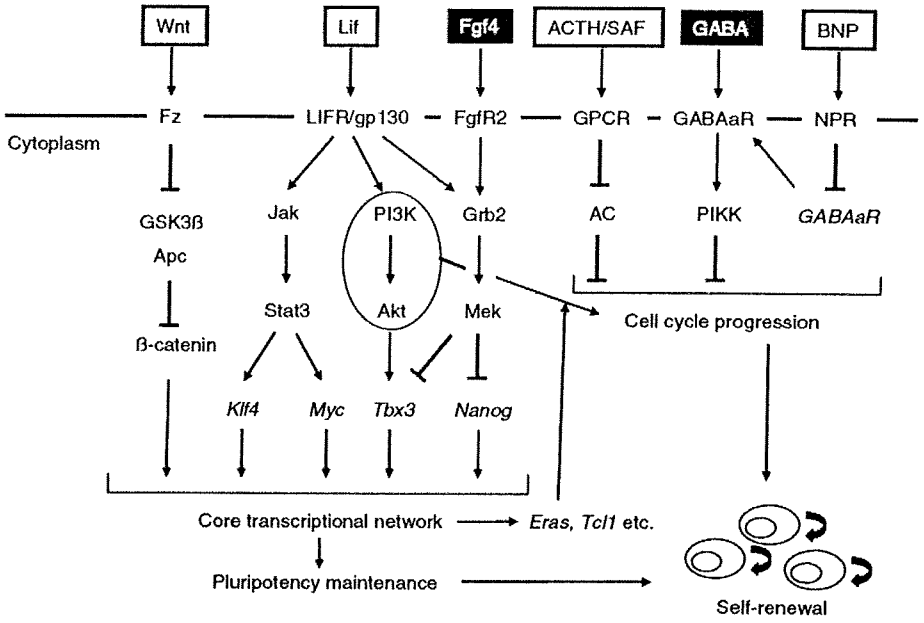

Fig. 1 External signals contributing to self-renewal of ES cells. Wnt, Lif, and Fgf4 act to modulate the transcriptional network regulating pluripotency/differentiation, as well as to modulate cell cycle progression through the regulation of genes including *Eras* and *Tcl1*. The molecules shown in *white* and *black boxes* represent, respectively, positive and negative regulation (as a main function) for self-renewal. Popular terms for these factors have been used here for the ease of understanding

Wnt signaling also contributes to the maintenance of pluripotency. In the canonical Wnt pathway, the Wnt receptor Frizzled (Fzd) transduces the signal to glycogen synthase kinase 3β (GSK3β) and adenomatosis polyposis coli (Apc). This enables catenin beta 1 (Ctnnb1, also known as β-catenin) to translocate into the nucleus to form the Ctnnb1/Tcf complex, which in turn activates the downstream genes [33]. Repression of *Apc* in ES cells causes resistance to differentiation in the media in the absence of Lif [34]. Administration of Wnt proteins, such as Wnt3a, Wnt5a and Wnt6, or BIO can contribute to maintaining the pluripotency of ES cells [35–37]. Inhibition of GSK3β, in combination with Fgf and Erk signaling inhibitors (see below), enhances the self-renewal of ES cells in a defined culture system [38]. In fact, in the presence of Wnt signaling, transcription factor 3 (Tcf3) activates the downstream genes that promote pluripotency maintenance by collaborating with the pivotal transcription factors *Oct3/4*, *Sox2* and *Nanog* (see below).

ES cells secrete fibroblast growth factor 4 (Fgf4) in an autocrine manner, which stimulates a Ras-Erk signaling cascade to induce differentiation. ES cells lacking *Fgf4* show resistance to differentiation [39]. The tyrosine kinase receptor of Fgf4 transduces the signal to Ras and Grb2, which are necessary to induce differentiation [40]. Loss of *Erk2* suppresses differentiation in ES cells [39]. Moreover, ES cells can be maintained in a pluripotent state in the absence of growth factors or cytokines through the inhibition of differentiation cues. This is done using inhibitors that target the Fgf receptor tyrosine kinases and the MAPK kinase 1/2 (Mek1/2, Erk activating enzymes) [38]. The downstream genes for Fgf signaling are largely unclear, as the target molecules of the activated Erk show considerable divergence, while *Tbx3* might be involved in the negatively regulated targets [24].

4 Transcription factors

As described above, extracellular signaling is transduced to the nucleus through transcription factors including Stat3 and Tcf3. Those factors listed below are part of the transcriptional network and play a central role in maintaining pluripotency.

Oct3/4, also known as *Pou5f1*, was initially described as either *Oct3* or *Oct4* by three different research groups [41–43], so the official designation includes both names. Oct3/4 consists of a POU domain as a DNA-binding domain, and two transactivation domains which lie on the N terminus and the C terminus, respectively [44]. The expression of *Oct3/4* is restricted in pluripotent cell lineages such as ICM and germ cells in vivo and undifferentiated ES cells in vitro, where it plays an indispensable role in maintaining pluripotency [41–43, 45, 46]. The regulatory regions of *Oct3/4* described to date consist of the proximal promoter (PP), proximal enhancer (PE), and distal enhancer (DE). DE activity is prominent in ICM, germ cells and ES cells, whereas in the post-implantation epiblast, EC cells and EpiSC, there is a decrease in DE activity and PE activity is dominant [5, 47]. DE activity depends on both the Oct–Sox motif and another motif bound by unknown ubiquitous factor(s) [48]. PE is activated by *Nr5a2* (also known as liver receptor homolog 1, *Lrh1*), and *Nr5a2* knockout embryos show loss of *Oct3/4* expression in the epiblast

[49]. PP is regulated by a variety of nuclear receptors. The activators include *Nr5a1* (also known as steroidogenic factor 1, *Sf1*) [50], *Rxrb* (retinoid × receptor beta) and *Nr5a2* [49, 51]. The repressive factors include *Nr2f1* (also known as chicken ovalbumin upstream promoter-transcription factors 1, *Coup-tf1*) [51], *Nr2f2* (also known as *Coup-tfII*) [51], and *Nr6a1* (also known as germ cell nuclear factor, *Gcnf*) [52]. *Nr6a1* repression in the embryo results in de-repressed *Oct3/4* expression in somatic lineages of that embryo, indicating that *Nr6a1* is necessary to restrict *Oct3/4* expression in pluripotent cell lineages [52].

Oct3/4 regulates a broad range of target genes. From a developmental perspective, the main target is caudal type homeo box 2 (*Cdx2*); repression of *Oct3/4* in ES cells results in differentiation into trophectoderm through upregulation of *Cdx2*, and forced expression of that gene induces differentiation into trophectoderm [53]. Oct3/4 has been known to activate downstream genes by binding to enhancers carrying the octamer–sox motif (Oct–Sox enhancer), for synergistic activation with the SRY-box containing gene 2 (Sox2).

Sox2 expression is detected in pluripotent cell lineages and in the nervous system [54]. Sox2 consists of a DNA-binding HMG domain and a transactivation domain that can be divided into three subdomains [55]. The involvement of *Sox2* in pluripotency maintenance was first suggested by the occupation of an enhancer consisting of octamer and sox binding motifs (Oct–Sox enhancer) in the regulatory region of *Fgf4* [56]. Oct–Sox enhancers are found in the regulatory region of most of the genes that are specifically expressed in pluripotent stem cells, such as *Oct3/4*, *Sox2*, *Nanog*, *Utf1*, *Lefty* and *Fbx15* [48, 57–62]. Repression of *Sox2* in vivo will result in early embryonic lethality due to the failure of ICM maintenance, indicating that *Sox2* is necessary for the maintenance of pluripotent stem cells [54]. On the other hand, repression of *Sox2* does not affect the activity of the Oct–Sox enhancer in ES cells, suggesting that Sox2 and other Sox members expressed in these cells, including Sox4, Sox11 and Sox15, might be redundantly involved in Oct–Sox enhancer activation [63, 64]. Because the differentiation induced by repression of *Sox2* can be rescued by forced expression of *Oct3/4*, the primary role of *Sox2* in ES cell self-renewal is to maintain *Oct3/4* expression [64].

Nanog, named after Tir Na Nog (land of the ever young), was discovered through digital differential screening based on the expression patterns confined to ES cells [65], and by functional screening based on the capability to maintain pluripotency in the absence of Lif [66]. Nanog consists of three domains: a homeodomain similar to the NK-2 family, which acts as a DNA-binding domain, and the transactivation domains at the N-terminal and C-terminal, through which Nanog dimerizes to exert its full activity [67, 68]. Loss of *Nanog* in vivo results in early embryonic lethality [65]. However, *Nanog*-null ES cells can be established and still maintain pluripotency, albeit with an increasing tendency to differentiate, indicating that *Nanog* is not absolutely required to establish the pluripotency transcriptional network [69]. Nevertheless, because forced expression of *Nanog* increases the tendency toward undifferentiation under conditions that would normally favor differentiation, the function of *Nanog* can be seen as stabilization of pluripotency [69]. From a developmental point of view, one major role of *Nanog* might be the repression of GATA-binding protein 6 (*Gata6*), the forced expression of which induces differentiation into ExEn cells [70]. In the blastocyst, *Gata6* is first expressed in some cells of the ICM, most of which differentiate into extra-embryonic endoderm cells. Nanog expression and Gata6 expression are mutually exclusive, suggesting that *Nanog* suppresses differentiation into extraembryonic endoderm cells [71].

Klf4, containing C2H2-type zinc-finger motifs, regulates numerous processes including proliferation and differentiation in general [72]. In ES cells, Klf4 participates in the activation of an Oct–Sox enhancer in cooperation with Oct3/4 and Sox2 [62], and forced expression of *Klf4* maintain pluripotency in the absence of Lif. *Klf4* might regulate transition between different states of pluripotency. *Klf4* is strongly expressed in ES cells, but not in EpiSC, and forced expression of *Klf4* in EpiSC can convert/reprogram some (although not many) of these cells to ES cells [7]. The Klf4 function in pluripotency may be supported by redundant Klf family members. Although loss of *Klf4* does not seem to cause any defects in the pluripotency of ES cells or in the early embryo [62, 73], differentiation is induced by the simultaneous repression of *Klf2*, *Klf4*, and *Klf5*. This outcome suggests that these three molecules act redundantly to maintain pluripotency [74]. In fact, each of the three *Klf* factors in combination with *Oct3/4*, *Sox2*, and *Myc* can reprogram somatic cells into pluripotent stem cells (see below) [75]. On the other hand, *Klf4* and *Klf5* have slightly different functions in the proliferation of ES cells. *Klf4* suppresses that proliferation, while *Klf5* promotes it via the Tcl1–Akt pathway [15].

Myc is a potent oncogene, and is known to strongly promote proliferation [76]. In ES cells, *Myc* expression is regulated by Lif–Stat3 signaling. Stat3 activated by Lif signaling binds directly to the regulatory region of *Myc* to activate expression [23]. The sustained expression of a constitutive active form of Myc is sufficient to maintain pluripotency in the absence of Lif, whereas its dominant negative form antagonizes the maintenance of pluripotency and promotes differentiation [23].

The sal-like 4 (*Sall4*) gene belongs to the spalt family (C2H2 zinc-finger protein genes), conserved from

drosophila to human and playing a variety of important roles in development [77]. Repression of *Sall4* in the early embryo results in lethality due to ICM/epiblast defects [78, 79], although in rare cases ES cells can be established from the homozygote blastocysts [80]. Although *Sall4* may promote *Oct3/4* expression [81], *Sall4* might not be absolutely necessary for the maintenance of pluripotency, since *Sall4*-null ES cells are still pluripotent [79, 82]. In ES cells, repression of *Sall4* prolongs the G1 phase and thus retards proliferation [79, 82]. In the nucleus, *Sall4* localizes in the heterochromatin region, implying a genome-wide repressive function [79]. In fact, *Sall4* is involved in the NuRD complex (see below) to repress developmentally regulated genes including *Cdx2* [82, 83]. Collectively, *Sall4* promotes self-renewal of ES cells through promoting proliferation and repression of the target genes.

Estrogen-related receptor beta (*Esrrb*) has been known to play an essential role in placenta formation [84]. The *Esrrb* function is also necessary in ES cells, as differentiation is induced by the repression of *Esrrb* [28]. Esrrb directly interacts with Oct3/4 to activate various downstream genes, including *Nanog*, in which the regulatory region carries the Esrrb-binding site, the degenerated 9-bp estrogen-related receptor response element (ERRE). The expression of *Esrrb* is in turn regulated by *Oct3/4* and *Nanog*.

RE1 silencing transcription factor (*Rest*, also known as *NRSF*) is a transcriptional repressor that targets a group of neuronal genes by binding to the RE1 element in non-neural cells [85]. *Rest* may be essential for pluripotency maintenance in ES cells, as repression of *Rest* reduces the expression level of transcription factors such as *Oct3/4* and *Nanog*, derepressing the expression of several microRNAs including miR21 to suppress the pluripotency maintenance mechanism [86]. However, other researchers have reported that *Rest*-null ES cells can be established and that those cells express normal levels of pluripotency markers including *Oct3/4* and *Nanog* [87], hence, the contribution of *Rest* within the core transcriptional circuit is still controversial [88, 89].

Tcf3 belongs to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, which binds to Wnt-response elements (WRE: C/T-C-T-T-T-G-A/T-A/T) via their HMG domain [90]. In the absence of Wnt signaling, or where such signaling is only present at low levels, TCF/LEFs acts as a component of a repressive complex, while in the presence of Wnt, TCF/LEF forms an activating complex with *Ctnnb1* to promote the expression of downstream genes [90]. This is the canonical Wnt signaling pathway, in which *Tcf3* positively or negatively regulates pluripotency in ES cells. *Tcf3* repression derepresses *Nanog*, producing resistance to differentiation, while stimulation of Wnt signaling upregulates the expression of genes for pivotal transcription factors such as *Oct3/4* and *Nanog* [91–94].

C2H2-type zinc-finger protein Zfx is involved in the proliferation of ES cells and hematopoietic stem cells [95]. In ES cells, repression of *Zfx* compromises proliferation while retaining differentiation capability. Forced expression of *Zfx* promotes ES cell proliferation by antagonizing differentiation through activation of *Tbx3* and *Tcl1*, both of which play essential roles in pluripotency maintenance and proliferation [95].

Ronin, whose products contain a DNA-binding THAP domain for epigenetic silencing of gene expression [96, 97], is expressed primarily during the earliest stages of embryonic development, and its absence is lethal at the peri-implantation stage in knockout embryos due to ICM defects [98]. *Ronin* repression in ES cells induces proliferation defects, while forced expression of *Ronin* allows ES cells to maintain pluripotency in the absence of Lif via an unknown pathway independent of *Stat3* [98]. Ronin also acts on pluripotency maintenance through a pathway independent of the core transcription factors, as forced expression of *Ronin* partially rescues the differentiation induced by *Oct3/4* repression, and the regulation of *Ronin* expression does not depend on *Oct3/4*, *Sox2* or *Nanog* (thus the designation *Ronin*, named after masterless Japanese samurai) [98]. Although the Ronin function is largely unclear, it involves the repression of global transcription, probably through the formation of a complex with HCF-1, a transcriptional regulator that has a variety of functions including transcriptional repression and cell proliferation [98, 99].

5 Protein complexes regulating transcription

The functions of the transcription factors discussed above are controlled by associated co-activators and co-repressors (Fig. 2). Nanog physically interacts with transcription factors such as Oct3/4, Sall4, the dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X-chromosome gene 1 (Nr0b1, also known as Dax1), the BTB-domain containing protein related to *Drosophila* bric-a-brac/tramtrack (Nacc1) and Zfp281 (also known as the mouse homolog of human zinc-finger protein ZBP99), each of which are involved in distinct protein complexes contributing to transcriptional regulation by Nanog [100]. *Nacc1*, *Nr0b1*, and *Zfp281* are necessary to maintain pluripotency [100, 101]. Nacc1 and Sall4 interact with the nucleosome remodeling and histone deacetylation (NuRD) complex to repress the downstream genes [82, 83, 100, 102]. On the other hand, NuRD is not necessary for the self-renewal of ES cells, since repression of the gene for a NuRD component, methyl-CpG binding domain protein 3 (*Mbd3*), results in failure to commit to developmental lineages [103]. Nanog (and Oct3/4) interact with another

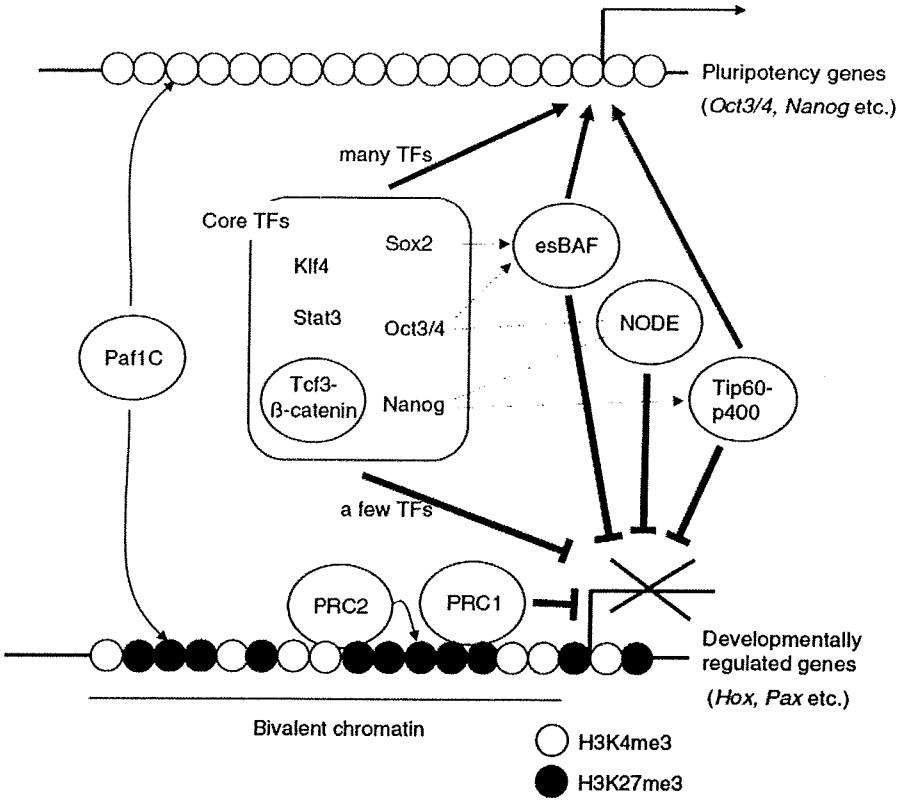

Fig. 2 Transcriptional network maintaining pluripotency while keeping differentiation capability. Core transcription factors including Oct3/4, Sox2, and Nanog co-operated with various protein complexes to positively or negatively regulate the target genes. Many core transcription factors co-operate to activate genes for pluripotency, whereas in the developmentally regulated genes only a few of these factors are used for the repressing mechanism. This presumably includes the recruitment of PRC2, which is responsible for H3K27me3 modification where PRC1 is recruited to repress the target genes. *Curved arrows* indicate modification on histones. Protein complexes are shown in *circles*. *Thin broken lines* with and without *arrowheads* indicate association with and involvement with protein complexes, respectively. Transcriptional regulation is represented by *thick lines*. *TF* transcription factor

Hdac1/2- and Mta1/2-containing complex, NODE (for Nanog and Oct3/4 associated deacetylase), to co-occupy and repress Nanog-target genes [104]. Repression of *Mta1* derepresses the expression of developmentally regulated genes and induces ES cell differentiation [104].

Nanog and Oct3/4 co-occupy the downstream genes with Pol II-associating factor 1 complex (Paf1C) [105]. Paf1C is generally known to be involved in multiple processes, such as transcription initiation and elongation, transcript start site selection, and histone modification [106]. The components of Paf1C, including Ctr9, are expressed at higher levels in ES cells, and are downregulated during differentiation. Repression of Ctr9 causes expression changes similar to Oct3/4 or Nanog depletions, and induces differentiation, whereas forced expression of Ctr9 blocks ES cell differentiation. In ES cells, Paf1C binds to the promoters of pluripotency genes, where it is required to maintain a transcriptionally active chromatin structure through the maintenance of H3K4me3. On the other hand, in the lineage-control genes being repressed in ES cells, Paf1C is required not for the maintenance of H3K4me3, but instead for the maintenance of histone H3 lysine 27 trimethylation (H3K27me3, repressive chromatin mark), indicating that Paf1C supports pluripotency by maintaining the chromatin structure characteristic of ES cells (see below) [105].

Nanog recruits the Tip60-p400 histone acetyltransferase (HAT) and nucleosome remodeling complex (Tip60-p400) [107], which is normally involved in DNA repair and proliferation [108]. Localization of E1A-binding protein p400 (Ep400, also known as p400) to the promoters of both silent and active genes is dependent on histone H3 lysine 4 trimethylation (H3K4me3, active chromatin mark) and the presence of Nanog, since depletion of either Ash2l (a component of the H3K4me3 catalytic complex) or Nanog reduces Ep400 binding to target promoters. Repression of Ep400 deregulates the genes for developmental regulators, which significantly overlap with the downstream genes of *Nanog*, so that Tip60-p400 is necessary to maintain pluripotency. At the target promoters, Tip60-p400 acetylates histones including H4 for the proper regulation of gene expression [107].

ES cells express specific components of BAF (Brg/Brahma-associated factors), also called SWI/SNF ATP-dependent chromatin-remodeling complexes, to form esBAF [109]. The components of esBAF include Smarca4 (also known as Brg1) and Smarcc1 (also known as BAF155). The repression of these components induces differentiation in ES cells, indicating that esBAF is essential to maintain pluripotency. Although BAF complex is also found in differentiated cells, its composition in those cells differs from that in esBAF. Smarcc2 (also known as

BAF170) is found in the BAF complex in differentiated cells, but not in esBAF, and the forced expression of *Smarcc2* induces differentiation, suggesting that a specific subunit composition is required for pluripotency maintenance [109]. Smarca4 physically interacts with Oct3/4 and Sox2, and they co-occupy promoter regions of the pluripotency genes and developmentally regulated genes. Depending on the genetic context, esBAF functions as either a repressor or an activator in controlling the ES cell transcriptional circuit [109, 110].

6 Epigenetic factors

To initiate the differentiation process in precise timing, the developmentally regulated genes need to be poised for transcription in ES cells. Most transcriptionally silent developmentally regulated genes, such as the Hox family are repressed by polycomb repressor complexes (PRCs). In general, PRC2 catalyses trimethylation of H3K27, which is thought to provide a recruitment site for PRC1 (Fig. 2) [111]. In ES cells, PRC1 and PRC2 components co-occupy most of the target genes, where PRCs may be recruited by the core transcription factors such as *Oct3/4* [112, 113]. Repression of both *Ring1A* and *Ring1B*, the components of PRC1, results in complete differentiation of ES cells [112], whereas repression of either *Suz12* or *Eed*, the components of PRC2, derepresses the expression of the target genes and compromises differentiation capability [114, 115], indicating that PRCs are necessary for maintenance and execution of pluripotency [20]. Target regulation by PRCs involves the histone H2A variant H2AZ, which in general is implicated from yeast to human in many DNA-mediated processes, including gene regulation [116]. In ES cells, H2AZ and PRCs co-occupy the promoter regions of developmentally regulated genes, which are derepressed in the H2AZ-repressed ES cells, as seen with either *Suz12* or *Eed*, resulting in failure to undergo differentiation [117]. These target genes often have a "bivalent" chromatin structure, consisting of both H3K4me3 and H3K27me3 and poised for the initiation of expression [113, 117–119]. In fact, at the promoter regions, the bivalent gene RNA polymerase II (RNAP) complexes are assembled and phosphorylated on Ser 5, which marks inactive or poised genes, and are actually transcribed at low levels [120]. The mechanism of the repression involves histone H2A ubiquitination by PRC1, as repression of *Ring1A* and *Ring1B* results in the sequential loss of H2A ubiquitination, the release of poised RNAP, and subsequent gene derepression [120].

CpG methylation is also known to play important roles in epigenetic gene silencing and development. CpG DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, coordinately regulate CpG methylation in the genome [121]. In ES cells, CpG methylation is dispensable to the maintenance of pluripotency, as triple knockout ES cells (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) are capable of self-renewal [122]. However, a subset of developmentally regulated genes that are methylated at promoters in wild-type mES cells are derepressed in the triple knockout ES cells, while these genes lack either bivalent chromatin structure or association with PRC components or core transcription factors. These findings suggest that, within the promoter region of ES cells, CpG methylation represents a distinct epigenetic program that complements other regulatory mechanisms to ensure appropriate gene expression [123]. On the other hand, in distant regions (outside the promoters) such as the highly conserved non-coding elements (HCNE), which tend to be regulatory regions of developmentally regulated genes carrying a bivalent chromatin structure [118], the CpG methylation level is differentially regulated during cellular differentiation, probably regulating the accessibility of transcription factors to ensure the transcriptional network [124].

In addition to regulation in genic regions, transcription in ES cells is regulated at a genome-wide level. Intergenic sequences are broadly transcribed to express large intervening non-coding RNAs (lincRNAs), evolutionally conserved multi-exonic RNAs [125]. There are more than a thousand lincRNAs among different cell types. In ES cells, the core transcription factors regulate the expression of some lincRNAs, which play a role in pluripotency maintenance and differentiation [125]. Moreover, the synthesis of global mRNA is enhanced in ES cells. Transcriptional hyperactivity is accomplished by the elevated expression of chromatin-remodeling genes, such as *Smarca4* and the general transcription machinery including TATA box-binding protein (*Tbp*) [126]. In fact, normally silent repeat regions are active in ES cells, and tissue-specific genes are sporadically expressed at low levels [126]. The mechanism for regulation of these normally repressed regions may involve the proteasome system. Inhibition of proteasome activity by either chemical (MG132) or siRNA targeting to 26S proteasome components (such as ß4) increases the binding of transcription factors and RNAP in regulatory regions of normally repressed genes in ES cells, resulting in the activation of cryptic promoters in intergenic regions. This suggests that these factors restrict permissive transcriptional activity while keeping the genes in a potentiated state, ready for activation at later stages via assembly of the 26S proteasome [127].

This hyperactive transcription in ES cells might be supported by the hyperdynamic chromatic structure, which is potentially permissive of transcription. There is loose binding of the heterochromatin component HP1, the linker histone H1, and core histones, and these factors display

highly dynamic movement in ES cells as compared to differentiated cells [128]. Repression of histone cell cycle regulation defective homolog A (Hira), the nucleosome assembly factor, results in elevated levels of unbound histones, and differentiation is accelerated. In contrast, differentiation arrest is displayed in ES cells expressing either mutant H1 with increased chromatin-binding capacity or shRNA targeting *Chd1* that is required for maintain euchromatin, indicating that the hyperdynamic chromatin structure is essential to pluripotency maintenance [128, 129]. Widespread in ES cells, transcription becomes restricted as differentiation proceeds. This may involve the formation of large organized chromatin K9 modifications (LOCKs), the histone H3 lysine 9 dimethylation (H3K9me2, repressive histone mark) enriched regions encompassing up to several mega bases [130]. The formation of LOCKs is a function of euchromatic histone lysine *N*-methyltransferase 2 (Ehmt2, also known as G9a), which catalyzes the methylation of H3K9 [130, 131].

7 Regulations at post-transcriptional level

Translation of transcripts is regulated in ES cells. At the global level, ribosome loading on transcripts increases during differentiation, thereby enhancing translational efficiency through signaling pathways including the mammalian target of rapamycin (mTOR; a downstream effecter for PI3K/Akt-mediated regulation). This suggests that the protein synthesis capacity in undifferentiated ES cells is poised to allow rapid elevation of translation rate in response to differentiation signals [132]. At specific mRNA levels, the expression of the core transcription factor proteins is regulated by microRNA (miRNA). The expression of *miR-134*, *miR-296* and *miR-470* is upregulated upon differentiation induction, to target the coding sequence of *Oct3/4*, *Sox2*, and *Nanog* mRNA leading to the downregulation of the protein (and/or mRNA) levels, probably to ensure the progress of the differentiation process [133]. The expression of these developmentally regulated miRNAs is repressed by PRCs and the core transcription factors, as seen in the transcription factors that are developmentally regulated. The core transcription factors also promote a subset of miRNA expression, such as *miR-290* and *miR-302*, through which they fine tune the expression level of components of pluripotency maintenance and proliferation [134]. The developmentally regulated miRNAs include let-7, which regulates a diverse process including differentiation and proliferation through translational regulation of the various target mRNAs [135]. The processing/maturation of let-7 is blocked by *Lin28* [136, 137], which is capable of establishing human induced pluripotent stem (iPS) cells in conjunction with *Oct3/4*, *Sox2*, and *Nanog* [138], suggesting that let-7 negatively regulates pluripotency maintenance.

The activity of *Oct3/4* and *Sox2* is regulated by the nuclear import pathway. Nuclear proteins are known to be selectively imported into the nucleus by transport factors, such as karyopherin (importin) [139]. In ES cells, *Oct3/4* is selectively imported into the nucleus by *Kpna1* (karyopherin alpha 1), whereas *Xpo4* (exportin 4), another member of Ran-regulated nuclear transport receptors including karyopherin, facilitates nuclear import of *Sox2*, contributing to pluripotency maintenance [140].

The pluripotency machinery consisting of the core transcription factors should disappear precisely on schedule during the course of differentiation to ensure the establishment of a transcriptional network of differentiated cells. Upon induction of differentiation, the activity of Caspase-3 is upregulated to cleave the substrates including *Nanog* and presumably *Ronin* [98, 141]. Forced expression of the constitutive active *Casp3* (the gene of Caspase-3) induces differentiation, whereas Casp3 knockout ES cells become refractory to differentiation stimulus, indicating that the regulation of caspase activity is essential in both pluripotency maintenance and initiation of differentiation [141].

8 Transcriptional network to maintain pluripotency

How do these factors maintain pluripotency in the system as a whole? Genome-wide studies have revealed that *Oct3/4*, *Sox2*, *Nanog*, *Klf4*, *Tcf3*, and *Stat3* tend to co-occupy the target genes associated with cellular differentiation status, whereas genes involved in the proliferation process seem to be co-occupied by a distinct group of transcription factors including *Zfx* and *Myc* [92, 142, 143]. With greater binding of the transcription factors categorized in the former group, the target genomic regions tend to carry enriched H3K4me3 to function as an ES cell-specific enhancer, possibly corresponding to those substances previously identified as Oct–Sox enhancers, while binding of a single transcription factor marks the repressive transcriptional state, H3K27me3 (Fig. 2) [142, 143]. This mechanism may involve recruitment of PRCs and/or several other protein complexes that contribute to activating/repressing activities of the core transcription factors as described above. The expression of these epigenetic factors, including PRC components and histone modification enzymes, is regulated by the core transcription factors to self-stabilize an entire molecular network consisting of transcriptome and epigenome. The example studied in detail includes *Jmjd1a* and *Jmjd2c*, histone H3K9me2 and H3K9me3 (repressive marks) demethylase genes, whose expression is regulated by *Oct3/4*, thereby promoting the expression of the target genes including *Tcl1* and *Nanog*, respectively [13, 14].

9 Reprogramming

To date, several different approaches can be applied to the reprogramming of somatic cells to a pluripotent state [144, 145]. Briefly, a somatic cell nucleus transferred to an unfertilized egg acquires pluripotency and develops into a blastocyst, allowing cloned ES cells to be established. Alternatively, somatic cells can be reprogrammed by cell–cell hybridization (cell fusion). The disadvantages of these techniques are the destruction of the embryo (i.e. ethical problem) and the tetraploid karyotype of the fused cells (i.e. risk problem in transplantation), respectively. Because these problems can be avoided using iPS cells, most recent reprogramming studies have utilized iPS cell systems.

In iPS induction, *Oct3/4*, *Sox2*, *Klf4*, and *Myc* (4 factors) are simultaneously introduced into somatic cells, and the reprogrammed cells are selected by marker gene expression and/or morphology [146]. Retroviruses were initially used to introduce the four factors, but the resulting iPS cells showed numerous insertions in the chromosome, raising safety issues. Currently, iPS cells can be established by a DNA-free method, in which the proteins of the four factors are tagged with an arginine stretch that confers membrane permeability [147, 148]. The cell types in which successful reprogramming have been reported include fibroblasts, primary hepatocytes, and completely differentiated B lymphocytes, suggesting that all the cell types in the body can be reprogrammed using the iPS technique [149–151]. The progression of reprogramming in iPS induction is relatively slow when compared with other techniques. In cell fusion, the upregulation of *Oct3/4* in completely differentiated cells is observed within 2 days [152], whereas in the iPS process this upregulation is first detectable 16 days after induction [153].

The molecular mechanism of this reprogramming is not clear at present. The efficiency of iPS establishment is low, less than a few percent of cells treated [154], indicating that in most cells the reprogramming is aborted even in the presence of the four factors. The function of Myc for iPS induction is dispensable, although it enhances the efficiency of the iPS establishment, probably through repressing the expression of differentiated cell-specific genes while promoting binding of Oct3/4, Sox2, and Klf4 (OSK) to their target genes [75, 155]. In fact, in partially reprogrammed cells OSK does not bind to the target genes (which are thus not expressed), suggesting that the cellular environment ensuring access of these factors to the target genes is rate limiting in reprogramming [155]. In addition, administration of chemical inhibitors targeting epigenetic factors that are associated with transcriptional repression is effective for enhancing iPS cell induction. These inhibitors include BIX-01294 (G9a inhibitor) [156], AZA (5-aza-cytidine, Dnmts inhibitor) [154, 157], VPA (valproic acid, Hdac inhibitor) [158], and TSA (trichostatin A, Hdac inhibitor) [158], and such findings suggest that target accessibility accompanying the global transcriptional activation seen in ES cells is critical to the initiation of the pluripotency transcriptional network. During iPS induction, OSK activity is enhanced by the transcription factors known to co-regulate with OSK, which include Esrrb, Sall4 and Tcf3 (via Wnt signaling), since forced expression of those factors can enhance the efficiency of iPS induction [80, 142, 159–161].

Collectively, the mechanism of reprogramming in iPS induction can be hypothesized as follows. Upon introduction of the four factors, the endogenous genes for the transcription factors necessary to pluripotency are primed and gradually induced to express through the regulatory region targeted by OSK. Subsequently, the transcriptional circuit begins to self-stabilize via increasing expression of the endogenous core transcription factors, through a positive-feedback loop, while repressing the developmentally regulated genes through recruitment of epigenetic factors and various protein complexes such as Paf1C and NODE. Once the stable transcriptional network is established/self-stabilized, exogenous cDNA expression is no longer necessary, and iPS cells indistinguishable from ES cells can be obtained after selection based on the expression of endogenous *Oct3/4* and/or *Nanog*. This outcome is the result of concerted action by a group of molecules that play a central role in pluripotency.

10 Conclusion

Pluripotency of ES cells is externally regulated through several molecules, including Wnt and Lif, whose signaling pathway activates transcription factor genes such as *Klf4* and *Nanog* in the nucleus. The core transcription factors, including Oct3/4, Sox2, and Klf4 positively self-regulate while also repressing developmentally regulated genes by co-occupation with a variety of protein complexes. Introduction of *Oct3/4*, *Sox2*, and *Klf4* into the somatic cells gradually reconstitutes the above transcriptional network with the aid of Myc and epigenetic modifiers, which might allow the regulatory regions of the target genes more access to these transcription factors.

Acknowledgments I thank Dr. Mitsuhiro Endoh for his helpful suggestions. This work was supported in part by the PRESTO program of the Japan Science and Technology Agency and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. This work is also supported in part by the Grants-in-Aid for Scientific research from the Ministry of Health, Labor, Welfare of Japan, and by that of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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