

200911005B

厚生労働科学研究費補助金

創薬基盤推進研究事業

多因子疾患モデルマウスの効率的樹立法の開発

平成19～21年度 総合研究報告書

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平成22(2010)年 3月

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多因子疾患モデルマウスの効率的樹立法の開発

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疫学研究などで蓄積された遺伝学的データから、多因子疾患を再構築するシステムを意図し、任意の多重遺伝子ノックダウンマウスの高効率樹立法を開発した。具体的には、多種類の shRNA を発現する ES 細胞の樹立法を開発した。他方、試験管内 SNPs 解析システムの基盤技術を開発した。他の疾患モデルと異なり、ヒトでの多因子疾患の病因をもつ材料を提供できる。

A. 研究目的

生活習慣病など多くの疾患は多因子疾患であることが知られるが、そのモデル動物はほとんど整備されていない。現在知られている多因子疾患モデル動物は、変異導入や選抜による自然発症モデル動物であり、表現型は似ているものの、病因が同一でないためにヒトの病態を正確に反映しているとは言い難い。一方で近年、遺伝学的データは膨大に蓄積され、多因子疾患の原因遺伝子群の同定作業は急速に進んでいる。今後は、同定した変異が病因となるかを決定する目的と、ヒト多因子疾患に即したモデルマウスを作成する目的で、多種類の遺伝子を機能阻害（SNPs の大半は機能抑制的と考えられる）して病態解析する必要があるだろう。そこで、本研究では多重ノックダウンシステムを開発し、多因子疾患の再構築システムとして提案する。

従来、遺伝子の機能阻害はノックアウト

法などにより行われていたが、多種類を扱うには操作が煩雑に過ぎ、現実には多因子疾患を再構築することは不可能であった。本研究では shRNA によるノックダウン法を用いるが、一般的手法であるゲノム上へのランダム挿入では、位置効果によって発現がばらつき多種類を一定レベルに発現できないこと、多数の遺伝子座で挿入突然変異が起きるため副作用が無視できないことが問題である。

発現カセットの置換方法について

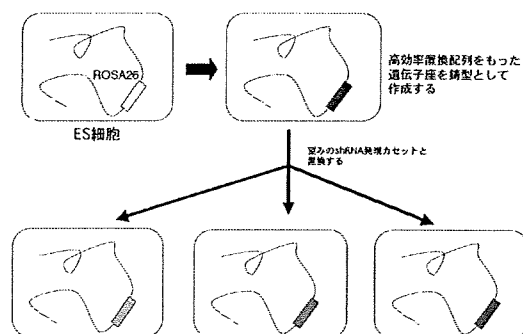


図1. 本研究のスキーム

そこで、本研究では全組織で均一に発現

する ROSA26 遺伝子座に多種類の shRNA 発現ユニットをロックインするシステムの開発を行った。そのための基盤技術として、これまでに私が ES 細胞において開発した、ROSA26 上で発現カセットを高効率で置換できるシステムを用いた (図 1 ; Nucl Acids Res. 33:e43, 2005)。19 年度に 2 種ロックダウンシステムの構築を、20 年度に 3 種ロックダウンのシステムを構築、21 年度にマウス樹立の試みと、SNPs 解析の新しいシステム開発を行った。

B. 研究方法

マウス ES 細胞 EBRTcH3、BZnTR1 は 129/Ola 由来、BAg73C2 は C57BL/6J 由来であり、ジャームライントランスミッション能が広く確認されている ES 細胞株の派生株である。ゼラチンコートシャーレを用いて、フィーダー非存在下、LIF (ESGR0, Chemicon) 1000U/ml、10%血清存在下で培養を行った。

遺伝子導入法は Lipofectoamin2000 (Invitrogen) を用いたリポフェクション法にて行った。ロックダウン効率の検証として、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)

マウスを用いた実験に関しては、厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針に従い実施する。また、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) 2 種ロックダウンシステム

RNA ポリメラーゼ II 依存性プロモーターを用いて、2 つの転写ユニットからの同時発現について、機能確認を行った。CMV 由来の最小プロモーターと、ヘルペスウイルス由来 TK 遺伝子最小プロモーターを用いて、直列方向および反対方向に連結することで EGFP および DsRed の発現を検証した。その結果、反対方向につないだもので強い同時発現を確認した (図 2)。

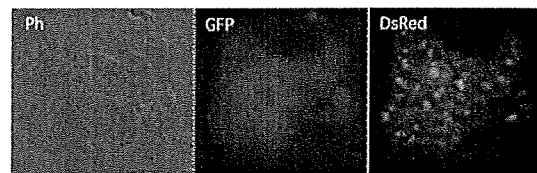


図 2. RNA ポリメラーゼ II 依存性プロモーターを用いた反対方向連結による同時発現

つぎに、ROSA26 遺伝子座において正逆両方向の転写 (すなわちロックダウン効果として得られる) が起きるかどうかを、それぞれの方向にロックインされた独立の 2 クローンを取得し、テトラサイクリン除去した場合の定量的 RTPCR 法を用いたロックダウン効果の解析を行った。その結果、いずれの方向においても同程度のロックダウン効果が得られたことから、2 種ロックダウン系が機能することがわかった。

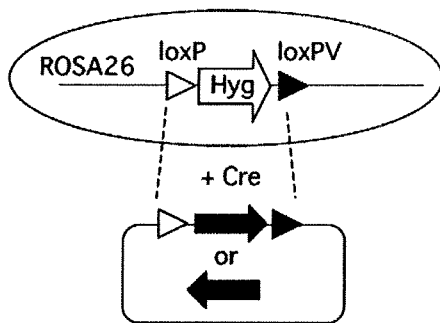
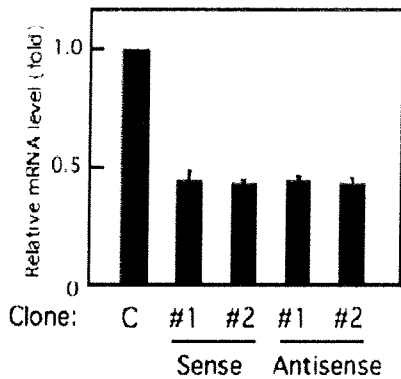


図 3. ROSA26 ではどちらの向きでもノックダウンコンストラクトが転写される

2種ノックダウン系を用いた解析例として、2型糖尿病関連遺伝子 Hnf4a と Tcf712 (Nature 445:881-5, 2007、これらのノックアウトマウスは胚性致死であるためノックダウンでのモデルマウスが必要とされる) のダブルノックダウンマウス作成を意図し、機能する shRNA の選定を行った。ここでは miRNA (生理的 shRNA) を採用しており、RNA ポリメラーゼ II により転写されるため、将来的に組織特異的プロモーターを利用した多重ノックダウンシステムへと応用できる。

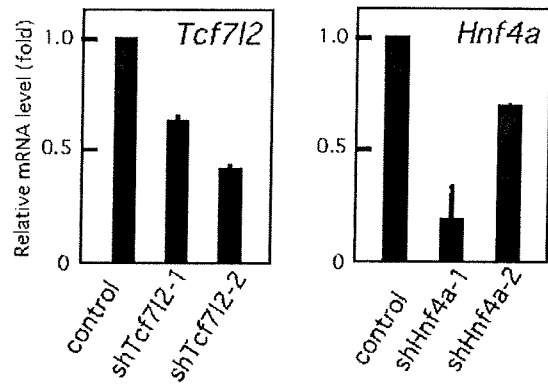


図 4. shRNA の選定。それぞれの遺伝子について4種調査した内の、効果が確認できた2種を示す。

それぞれの遺伝子について、4種の miRNA 発現ユニットを作成し、ES 細胞へ導入した。24時間後に RNA を回収し、定量的 RTPCR 解析に供した。その結果、それぞれ2種の miRNA がマイルドなノックダウン効果を示した (図 4)。これらの miRNA を反対方向に連結し、EBRTch3 細胞に導入した。ES 細胞クローンの名称は、shTch712-2 + shHnf4a-1 を RTH6-1、shTch712-1 + shHnf4a-2 を RTH6-2 とした。取得したそれぞれの ES 細胞クローンからキメラマウス作成を行ったところ、RTH6-1 から15匹、RTH6-2 から6匹のキメラマウスが得られた。野生型マウスと交配し、ヘテロマウスの取得を試みたが、得られなかった。

ii) 3種以上ノックダウンシステム

3種以上ノックダウンのために、RNA ポリメラーゼ III 依存性プロモーターを用いたノックダウンコンストラクト作成を

行った。

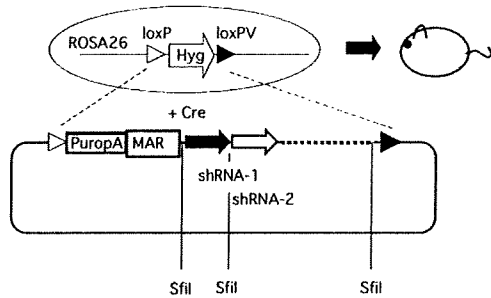


図5. 3種以上のノックダウンコンストラクトを搭載するスキーム

上記 i) の RNA ポリメラーゼ II 転写系では、直列 (タンデム) に連結した発現ユニットは転写干渉を起こして発現しないことが知られる。3種以上の転写ユニットを並べる場合、必ずどこかで直列に配置されてしまうことから、より多数の shRNA 同時発現のためにはタンデム連結でも転写干渉しないシステムが必要である。そこで、RNA ポリメラーゼ III 依存性プロモーターを採用した。MAR (Matrix Attachment Region; より確実なトランスジーン発現を保証するために挿入してある) 近傍の SfiI サイトへ、ワンステップで4断片を順列特異的に導入できる (つまり望みの4種が入ったプラスミドを選択する必要が無い) システムを構築した (図6)。これにより、ノックダウンする遺伝子の種類数が n 個に増加しても、 n 回コンストラクション操作 (1回のコンストラクションに1週間かかるとする

と、 n 週間かかることになる) を経ることなく迅速に多重ノックダウンコンストラクトが作成できる。

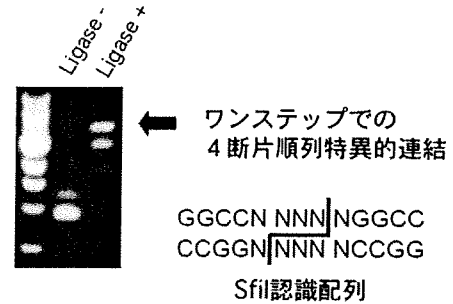


図6. SfiI断片による順列特異的連結

このシステムにおいても解析例として Tcf7l2 と Hnf4a を用いた。

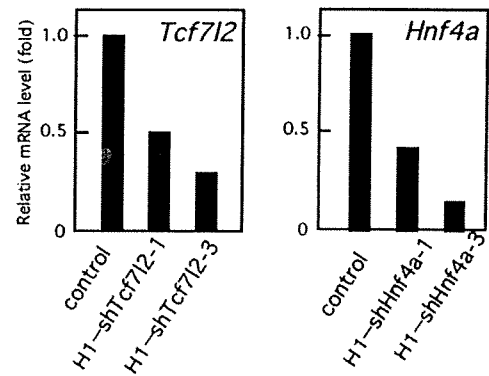


図7. H1によるshRNAの選定

両遺伝子のノックダウンコンストラクトを導入し、そのES細胞を用いてマウス作成を試みた。ブラストシストへインジェクトしてキメラマウス一匹を得た。これを野生型マウスと交配したが、ヘテロマウスは得られなかった。2種ノックダウンシステムで用いた親株細胞 EBRTcH3 を、

3種ノックダウンシステムでも用いた。染色体異常をもつ可能性を排除するため、染色体数解析を行ったが、異常は検出されなかった。

iii) 上記 ii) のシステムの場合、遺伝子抑制型の SNP には対応可能なのだが、遺伝子過剰発現型を反映した変異状態を作成できない。多種類のノックダウンコンストラクトとタンパク質が同時に発現できる新規システムの開発を意図し、原理的に新しいシステム (T7 フェージ転写系に基づく) の構築を行った。

T7系を使用する技術的理由:

多数の因子を同一細胞内で同時発現するシステムについては、下記の3条件を満たすことが必要である。

1. 同一ベクターに搭載する
2. 一転写単位の DNA 鎖長はできるだけ短くする
3. 一転写単位当たり翻訳タンパク質量を十分量確保できる

1の理由:

多種類の転写単位を一つ一つ別々に細胞集団へ共導入し、後から全種類導入された細胞を選択する手法では、選択後細胞がごく少数になること、およびそれぞれの因子の発現量のばらつきが大きくなることから、効果の解析が困難になる (図 10)。

2の理由:

大腸菌内において反復配列は組み換えや欠失を起こしやすい。多種類の転写単位

を同一ベクターに組み込むためには、ベクターを反復配列で不安定にすることのないようタンパク質コード領域以外の配列が出来るだけ短いことが望ましい。

3の理由:

転写単位にコードされる因子が機能を発揮する上で必要十分量発現されるよう、翻訳効率が比較的高い必要がある。ここで、複数の転写単位からの発現を測定する場合、逆転写PCR法による mRNA 検出法を用いると、terminator 配列を read-through した隣の転写単位から連続する mRNA を検出してしまい、翻訳可能な mRNA の検出にならないことが予想される。合わせると、多因子同時発現システムとしての一転写単位からの発現の確認は、或る程度の存在量以上でその効果を発揮する翻訳産物 (タンパク質) で検証すべきであろう。したがって、ここでは比較的大量のタンパク質分子数によって蛍光が確認できる EGFP を用いる。

T7系を使用する生物学的理由:

2つ以上の転写単位を同一ベクターに搭載すると、発現効率が極端に落ちることが知られており、この現象は原核生物・真核生物の由来を問わずいかなるプロモーターでも起きると考えられている (転写干渉; Shearwin et al., *TRENDS in Genetics* 21:339-345, 2005)。私は、転写が多数の基本転写因子の協調的機能によるものであり、その多数の構成因子のうちどの一つが機能阻害を受けても転写

できなくなる故であると考えた（つまり、同様の働きをするタンパク質が競合的に働くことに「もろい」）。例えば、10因子からなる転写系を考えると、それぞれの因子が競合的阻害をうける確率が0.2だとして、そのどれもが阻害を受けない／転写される確率は0.8の10乗、すなわち0.11程度になる。一方、1因子からなる転写系だと、たとえ競合阻害を受ける確率が0.3だったとしても、転写される確率は0.7となる。つまり、極めて単純なポリメラーゼとプロモーターから成る転写系を用いることで、転写干渉を回避できるはずだ。

バクテリオファージT7由来のRNAポリメラーゼは一種類の分子でT7プロモーターからの転写が可能である。そこでT7系を用いた多因子発現システムの構築を試みた。先ず転写単位の翻訳効率を至適化し、複数の転写単位を同一ベクターに搭載する順番で実験を行った。

系の構築にES細胞を用いる理由：

実験に用いた細胞はマウス胚性幹細胞（ES細胞）である。下記3点の特徴をもつことから、ES細胞で転写翻訳できる系ならば他の全ての細胞で使用可能と推測できる。

i) 外来のプロモーターを抑制する強い活性をもち、限られたハウスキーピング遺伝子プロモーターが使用できるのみである。

ii) 高速で増殖するため翻訳産物が拡散

するのが速く、転写翻訳の効果を得られにくい。

iii) 高速増殖と多能性に起因する高い毒性（あるいは分化誘導刺激）感受性により、T7RNAポリメラーゼ分子が毒性（あるいはクロマチン状態や細胞の性質になんらかの影響）を示せばたちまち細胞は死滅（あるいは分化）する。

T7ポリメラーゼを強制発現したES細胞BZnTR1へ、EGFPとDsRed発現ユニットを直列および反対方向に連結したプラスミドを導入し、24時間後に蛍光を観察した。その結果、直列につないだものでも両蛍光タンパク質の発現が確認できた（図8）。

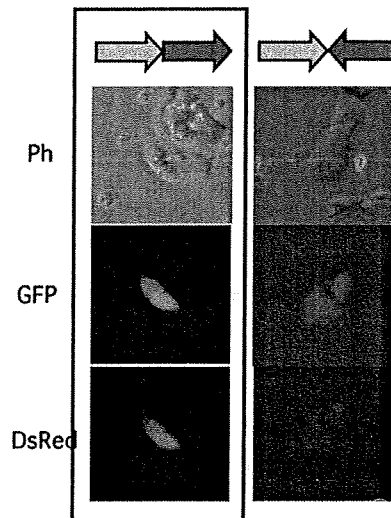


図8. 新規発現系による2種遺伝子同時発現の確認。

次に、GFPとDsRedに加えてネオマイシン耐性遺伝子、ハイグロマイシン耐性遺伝子の計4種の遺伝子を搭載し同時発

現を試みた。その結果、両蛍光タンパク質は同細胞で発現しており、また両薬剤耐性遺伝子も同時に発現していることが確認できた。したがって、原理上は無数の遺伝子を連結・発現できるシステムが構築できたといえる（図9）。

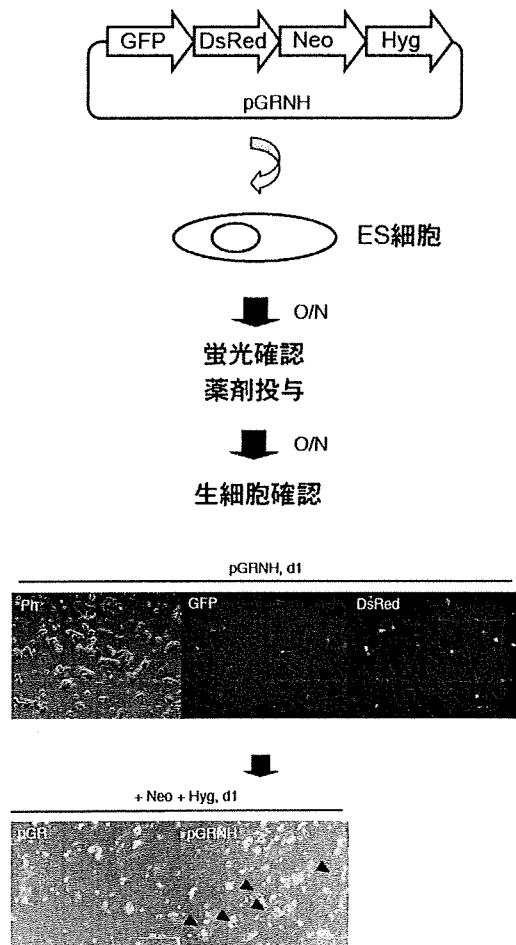


図9. 4種遺伝子の同時発現

21年度に、染色体に挿入した場合の発現量を解析した結果、大幅に減少することがわかった。したがって、一過性発現での使用を想定する必要がある。

そこで、試験管内（培養細胞）での複数SNPs相互作用の解析システムとしての応

用を目指した。具体的には、複数遺伝子座の変異によって、複数の変異型タンパク質が存在するときに現れる表現型を、簡便に再現できるシステムの基盤技術としての応用を考えた（図11）。従来の複数遺伝子発現方法は、主にレトロウイルスなどを用いて一因子ずつばらばらに導入しており、細胞集団中において全因子が導入された細胞よりも導入されなかった細胞の方が多いため、効果の解析が困難である。多因子を同時に発現できる本システムを用いた場合、一過性発現の後の薬剤選択により、全因子を発現する細胞のみを選択でき、効果の解析が可能になる（図10）。

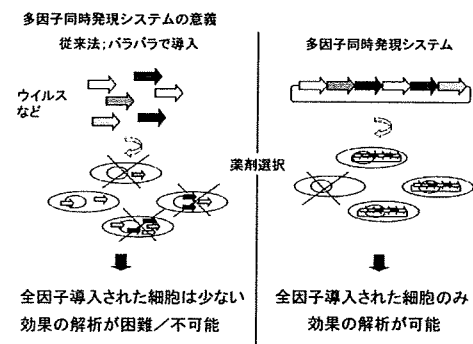


図10. 多因子発現システムの意義

本システムを用いて変異型タンパク質を同時に発現し、且つ内在性タンパク質を同時に抑制することで、SNPs機能解析システムとしての使用が可能になると期待される。

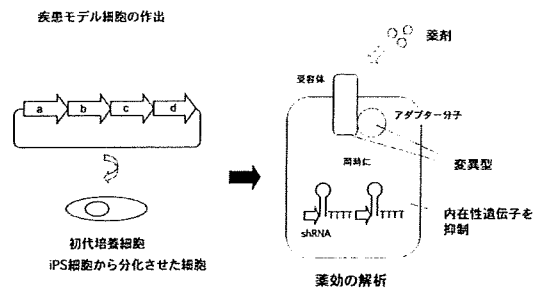


図 3. 疾患モデル細胞作出法として

内在性遺伝子の抑制のために、ES 細胞 (BAg73C2) の Rosa26 遺伝子座に、2 種・3 種ノックダウンシステムで用いたカセット交換アレルを導入した (図 4)。その結果、正しく導入された 2 クローンを得た。

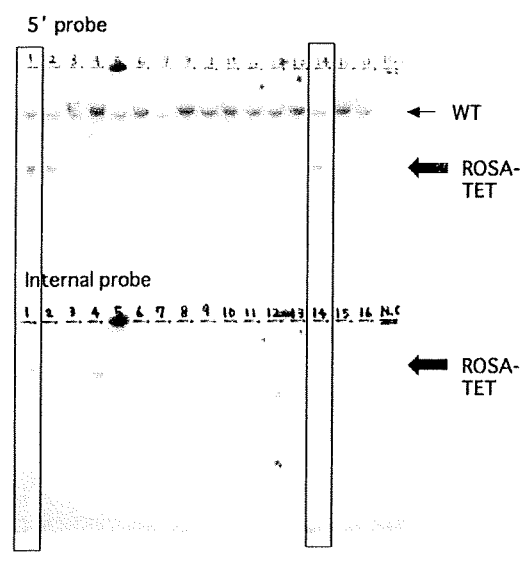


図 4. カセット交換アレルの導入 BAgRTcH1, BAgRTcH14 細胞を取得した。

BAg73C2 細胞は、遺伝的背景が C57BL/6J 由来で、ノックアウト解析などで標準系

統として使用されており、マウス個体の解析知見との照合が容易になるだろう。特に、129 系統と C57BL/6J 系統は代謝等の性質が大きく異なっていることが知られるため、使用する ES 細胞の系統はマウスのものと合致させておく必要があるだろう。将来的には両方の系統を比較することも必要になるだろう。

D. 考察

RNA ポリメラーゼ II 依存性プロモーターシステムを用いた 2 種遺伝子ノックダウン、および RNA ポリメラーゼ III 依存性プロモーターを用いた 3 種以上遺伝子のノックダウンマウスを作成できるめどがついた。ジャームライントランスミッションが起きなかったが、フィーダーフリー ES 細胞 (操作が簡便なシステムとして開発するためあえて用いた) はフィーダー依存性 ES 細胞と少し性質が異なるため、施設によってはジャームライントランスミッションが得られないケースも知られる。ES 細胞からはクローンマウスが得られやすいことが知られているため、核移植を行ってヘテロマウスを直接得ることも対処手段の一つだろう。

ノックダウンと強制発現が両方同時に可能な原理的に新しい T7 系システムも開発できた。一過性発現に限定した使用方法として、SNPs 機能解析システムとしての応用を考案した。C57BL/6J 由来の ES 細胞にカセット交換アレルを導入し、多因子発現システムと組み合わせることで、

内在遺伝子座を抑制しながら変異型タンパク質を多種類同時発現し、薬剤への反応性などを解析できるだろう。GWAS で得られた SNPs 一つ一つのマウスでの機能解析は C57BL/6J 系統で進められるが、細胞系についての標準系統は現在想定されていない。特定の細胞における薬効の解析などについては、試験管内での詳細な解析知見が必要とされるが、同系統であれば個体と細胞の知見の照合がスムーズに行える。今後は多因子発現システムを用いた SNPs 機能解析の例を提示することが必要だろう。一旦上手くいくとわかれば、ヒト iPS 細胞に応用するなど、広く使用されるようになるだろう。

E. 結論

多重遺伝子ノックダウンマウスの効率的樹立法が確立されるめどがついた。多因子疾患の再構成モデルマウスの必要性を感じながらも作成できなかった多くの研究者が利用するようになるだろう。

大規模 SNP 解析で疾患との相関が発見された多数の SNPs/遺伝子については、それが機能的かどうかを簡便に検証する方法が必要であった。本システムを用いてマウス個体あるいは試験管内において簡便に SNPs の機能確認を行えば、複数の SNPs の相互作用から生じる表現型を多数同定できるだろう。ヒト iPS 細胞を用いたシステムとしての応用も期待できる。波及効果として、ヒト多因子疾患に即したモデルマウスや解析系が構築されれば、

薬剤のスクリーニングや治療法の開発に多大な貢献を果たすことが期待でき、国民の健康増進に寄与するだろう。

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

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

Pluripotency maintenance mechanism of embryonic stem cells and reprogramming

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Received: 28 October 2009 / Accepted: 12 November 2009
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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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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 GSK3 β 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

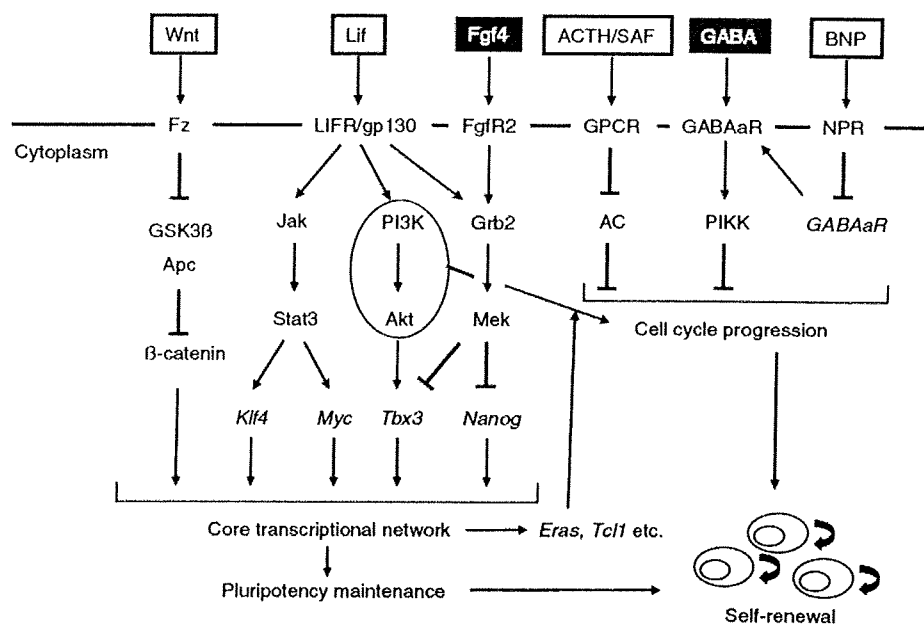
Several soluble factors have been identified that exert either positive or negative effects on ES cell self-renewal (cell proliferation while maintaining pluripotency) (Fig. 1). ES cells secrete an unidentified stem cell autocrine factor (SAF) to promote proliferation [9]. Presumably, this activity can be mimicked by high concentrations of adrenocorticotrophic 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 cyclase (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 (*GABA_AR*) [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 (TGF β) 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 *Rev1*) [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 *Tcf1*. 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 3 β (GSK3 β) 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 GSK3 β , 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], *Rarb* (retinoid \times 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*, *Ulf1*, *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-neuronal 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 *Cttnb1* 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].

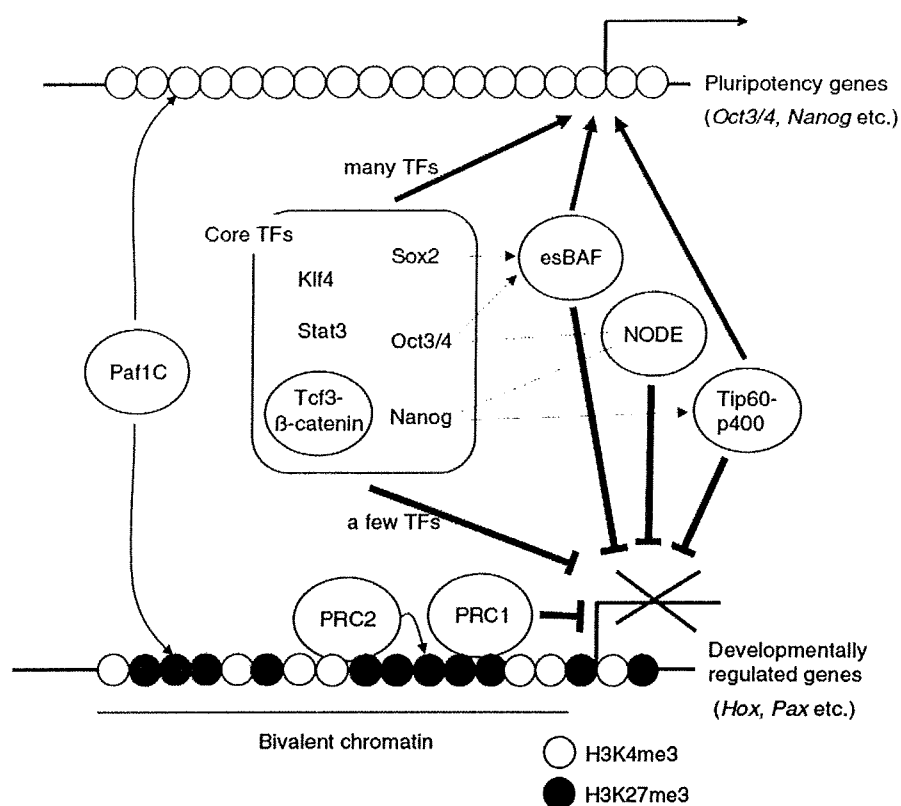
C2H2-type zinc-finger protein *Zfx* is involved in the proliferation of ES cells and hematopoietic stem cells [95]. In ES cells, repression of *Zfx* compromises proliferation while retaining differentiation capability. Forced expression of *Zfx* promotes ES cell proliferation by antagonizing differentiation 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 Ash21 (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 *Smarca2* 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*,

coordinate 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 $\beta 4$) 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 chromatin 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 effector 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].