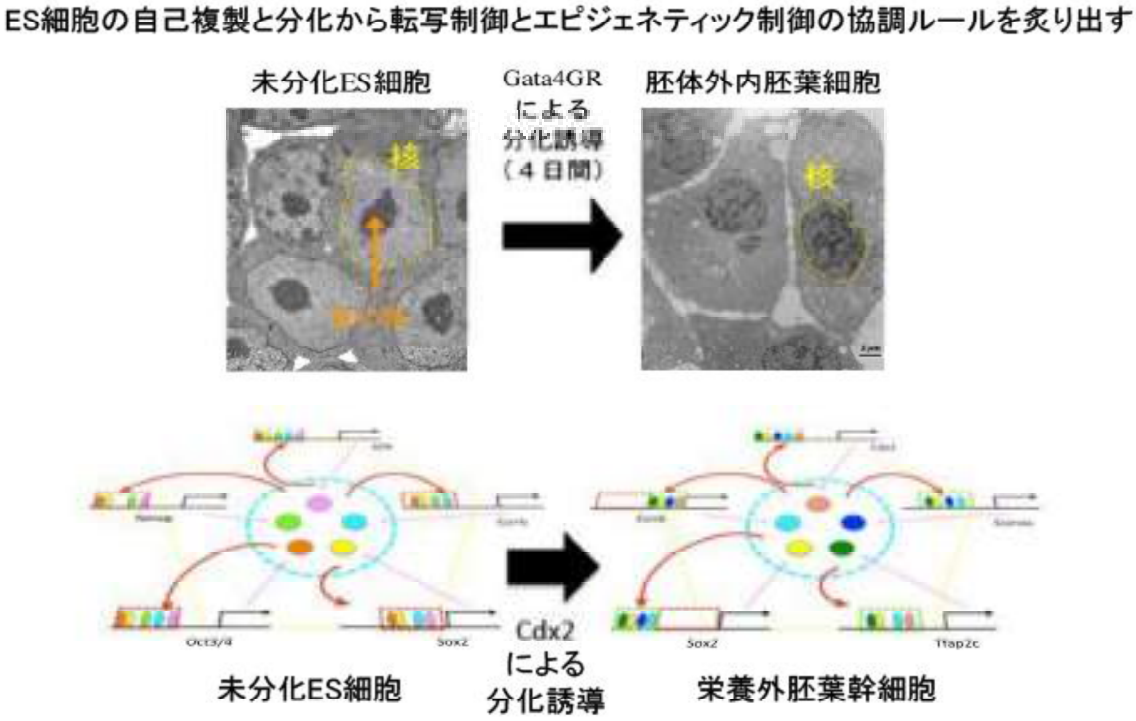
多能性幹細胞分野
Department of Pluripotent Stem Cell Biology

胚性幹細胞などが持つ分化多能性は、胚を構成する全ての種類の終末分化細胞へと分化できる能力として定義される。当分野では、この多能性を規定する分子機構の解明を目指して研究を進めている。より具体的には、（１）多能性を維持する転写因子ネットワークとエピジェネティック制御機構の解析、（２）分化に伴う多能性維持プログラムの遷移メカニズムの解析、（３）多能性を維持する実行的分子機構の解析、などのテーマで基礎研究を進めている。このような研究を通じて、細胞分化に伴う遺伝子発現プログラム制御機構の基本原理を解明することを、究極的目標としている。

Pluripotency is defined as an ability of a cell to differentiate all types of terminally-differentiated cells in an organism. We are studying about the molecular mechanisms determining cellular pluripotency in mouse embryonic stem (ES) cells. The projects are divided into 3 subjects: (1) Analysis of the functions of transcription factor network and epigenetic mechanisms to maintain pluripotency, (2) Analysis of the mechanisms governing the transition of transcription factor network during differentiation, and (3) Analysis of the molecular mechanisms that direct the maintenance of pluripotency. We aim to reveal the general principles governing the regulation of gene expression program in cellular differentiation.



構成員　Staff (2018.3)

名前	職名	Name and Position
丹羽　仁史	教授	Hitoshi Niwa, Professor
岡野　正樹	准教授	Masaki Okano, Assistant Professor
山根　万里子	特定研究員	Mariko Yamane, Posdoc
松浦　公美	技術補佐員	Kumi Matsuura, Technical Staff
市川　巧	大学院生	Takumi Ichikawa, Graduate Student
長尾　愛子	事務補佐員	Aiko Nagao, Secretary Assistant

元在籍者　Staff in the past (2012.4～2018.3)

名前	Name	在籍期間	在籍時職名	転出先
Robert Stephenson	Robert Stephenson	2016.19.1-2017.3.31	研究員	Springer Nature

研究概略　Projects

多能性幹細胞は、着床前の胚に由来し、胚を構成する全ての種類の終末分化細胞に分化する能力（多能性）と、細胞分裂を経てその能力を娘細胞に継承する能力（自己複製能）を併せ持つ。マウス胚性幹細胞(embryonic stem cells: ES 細胞)は、至適化された培養条件下でほぼ無限に自己複製を続けることができる。通常の分化細胞とは異なり、ES 細胞の多能性という性質は細胞形態上の性質としては殆ど捉えられず、それはむしろ、細胞種特異的な遺伝子発現を規定するゲノムプログラム上の特性といえる。ゲノムプログラムは、遺伝子発現を制御する転写因子と、その標的遺伝子のクロマチン状態を規定するエピジェネティック制御因子により制御され、細胞種特異的な遺伝子発現パターンを規定している。ES 細胞では、転写因子群が構成する極めて自律性の高いネットワークが、エピジェネティック制御因子による転写抑制がほぼ解除されたゲノムの遺伝子発現を安定に維持するという他に例を見ない状態が、外部シグナル依存的に維持されている。そして、このような状態は、分化に伴い、転写制御とエピジェネティック制御が協調して遺伝子発現制御を行う通常のゲノムプログラム制御パターンへと速やかに遷移する。このようなダイナミックなゲノムプログラムの変化が、シャーレ内の数日の分化過程で観察できる実験系は他にはなく、マウス ES 細胞分化誘導系はこの点において極めてユニークな実験系と言える。そこで、我々は、この実験系の特性を生かして、（1）多能性を維持する転写因子ネットワークとエピジェネティック制御機構の解析、ならびに（2）分化に伴う多能性維持プログラムの遷移メカニズムの解析、を進めている。一方で、ES 細胞が持つ細胞生物学的特性が、どのようにして転写因子ネットワークにより制御されているのかも興味深い点である。とりわけ、発生過程に存在する多能性幹細胞は一過性にしか自己複製しないのに対し、樹立された ES 細胞は、無限に近い自己複製能を獲得している。この過程の解明などを含めて、（3）多能性を維持する実行的分子機構の解析、にも取り組んでいる。

1. 多能性を維持する転写因子ネットワークとエピジェネティック制御機構の解析

ES 細胞の多能性は、複数の転写因子が構成するネットワークにより規定されていると考えられている(Niwa *Development* 2018)。転写因子ネットワークの維持は外部シグナル入力に依存し、その変化に伴い安定状態は破綻して、次の安定状態へと遷移することが、細胞分化を規定する。マウス ES 細胞においては、LIF シグナルと Wnt シグナルが、多能性維持に働くシグナルとして知られている。*Esrrb* は、これら両方のシグナル入力の標的として機能し、とりわけ Wnt シグナル入力については、その多能性維持シグナル入力に必須であることを証明した（Martello et al *Cell Stem Cell* 2012）(Fig. 1)。

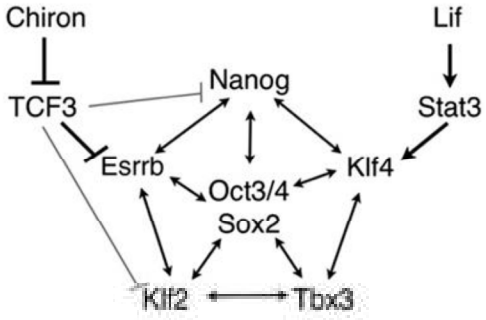


Fig 1 マウスES細胞における転写因子Esrrbの機能
(Martello et al Cell Stem Cell 2012より)

また、LIF シグナル入力は、Jak-Stat3 経路の活性化により多能性維持に働くと同時に、MAPK 経路の活性化により、分化誘導シグナルも活性化する。これら２つのシグナル入力のバランスは、マウスの遺伝的背景により大きく異なり、129 系統では LIF は Jak-Stat3 経路を優位に活性化することにより多能性維持に働くが、NOD 系統では MAPK 経路を優位に活性化してしまい、LIF のみでは多能性を維持できないことを見出した(Ohtsuka and Niwa *Development* 2015)。また、これまでの報告で多能性維持への関与が示唆された転写因子ならびに転写補助因子 *Trp53, Xpc, Zscan10* についてノックアウト ES 細胞を作成し、これらの機能が多能性維持には必要でないことを証明した(Shigeta et al *Sci Rep* 2013; Ito et al *FEBS Lett* 2014; Yamane et al *BBRC* 2015)。

DNA メチル化は、遺伝子発現を抑制するエピジェネティック制御機構として機能するが、ES 細胞の自己複製には必須ではない。DNA メチル化の分化能制御への関与を検討するために、分化誘導４日目の ES 細胞の、転写因子 Gata4 の人為的活性化への応答能を、野生型 ES 細胞と、*Dnmt3a/3b* 欠損 ES 細胞で比較した。その結果、前者は Gata4 活性化に応答しなかったが、後者は一部が未分化 ES 細胞の場合と同様に、原始内胚葉へと分化した。これは、DNA メチル化が、転写因子の応答性の制御を介して分化能を規定するバリアーとして働きうることを機能的に示した最初の例である(Oda et al *PLoS Genet* 2013)。

2. 分化に伴う多能性維持プログラムの遷移メカニズムの解析

マウス ES 細胞は、転写因子 *Oct3/4* の発現抑制により、栄養外胚葉幹細胞(trophoblast stem cells: TS 細胞)へと分化する。この過程で、多能性維持に必須な転写因子の一つである Sox2 の発現は、減少はするものの機能的レベルに維持される。我々は、この過程における Sox2 の機能を解析し、*Sox2* が TS 細胞においては FGF シグナル伝達に関与し、その自己複製に必須であることを見出した。また、ChIP-seq 法を用いた解析から、Sox2 は ES 細胞と TS 細胞で異なるパートナー分子と協調し、異なる標的遺伝子群に結合してその発現を制御していることを明らかにした(Adachi et al *Mol Cell* 2013)(Fig. 2)。これらの発見は、分化に伴う転写因子ネットワークの遷移について、新たな知見を与えるものである(Niwa *Development* 2018)。

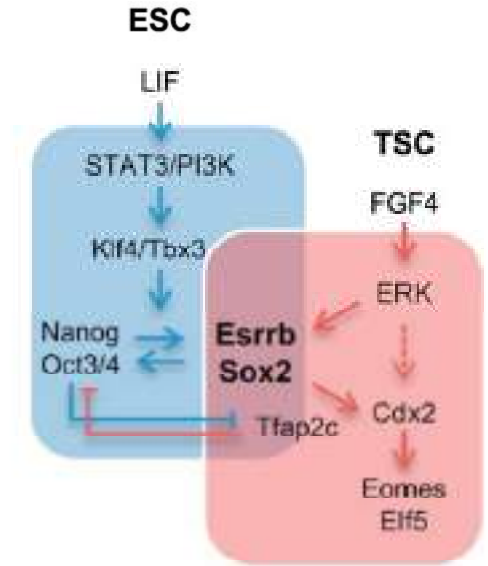


Fig. 2 ES細胞からTS細胞への移行に伴う転写因子ネットワークの再構成 (Adachi et al Mol Cell 2013より)

Sox2 の機能ドメインを検討するために、誘導型 *Sox2* ノックアウト ES 細胞を用いた機能相補実験系を用いて、Sox family 転写因子ならびにそれらの変異体の自己複製維持能を検討した。その結果、Sox2 の機能は DNA 結合ドメイン保存された数個のアミノ酸に依存し、ショウジョウバエ由来の Sox family 転写因子であっても、これらが保存されてさえいれば、内在性 *Sox2* を置換して多能性を維持できることを証明した(Niwa et al *BMC Evol Biol* 2016)。

3. 多能性を維持する実行的分子機構の解析

マウス ES 細胞が持続的に自己複製し多能性

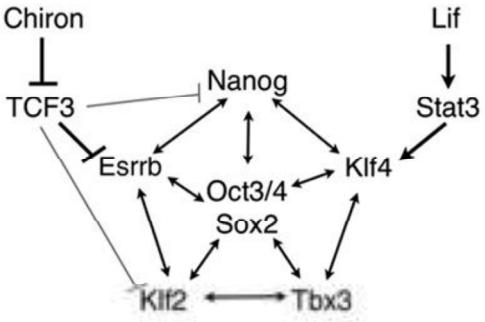


Fig 1 マウスES細胞における転写因子Esrrbの機能 (Martello et al Cell Stem Cell 2012より)

Nr0b1 は核受容体ファミリーに属する転写因子で、ES 細胞では未分化状態特異的に高発現している。そこで、*Nr0b1* の誘導型ノックアウト ES 細胞を作成して、その機能を検討した。その結果、*Nr0b1* 欠損は、多能性維持には影響を与えなかったものの、ES 細胞集団の増殖速度を顕著に抑制した。遺伝子発現解析から、これらの *Nr0b1* 欠損 ES 細胞では *Zscan4* 発現細胞が増加していた。これより、Nr0b1 は *Zscan4* の発現を抑制する転写因子であると考えられた(Fujii et al *Sci Rep* 2015)。

Zscan4 の発現制御をさらに詳細に解析するために、*Zscan4* promoter で制御される蛍光タンパク遺伝子を ES 細胞に導入し、その発現をタ

イムラプス顕微鏡で継時的に観察することにより、発現と相関する生物学的パラメーターの検討を行った。その結果、ES 細胞の分裂周期の長さが、*Zscan4* 活性化と強く相関することを見出した。ES 細胞は平均１２時間で分裂を繰り返すが、その分裂周期は分裂ごとに長くなる傾向を示した。そして、その分裂周期が２０時間に達すると、*Zscan4* が活性化される頻度が有意に高くなった。このことは、細胞分裂を繰り返すことによるテロメアの短縮が Zscan4 活性化シグナルとして機能し、テロメア伸長を誘導することにより、ES 細胞集団の自己複製が維持されていることを示唆している(Futatsugi-Nakai and Niwa *Stem Cell Rep* 2016)(Fig. 3)。

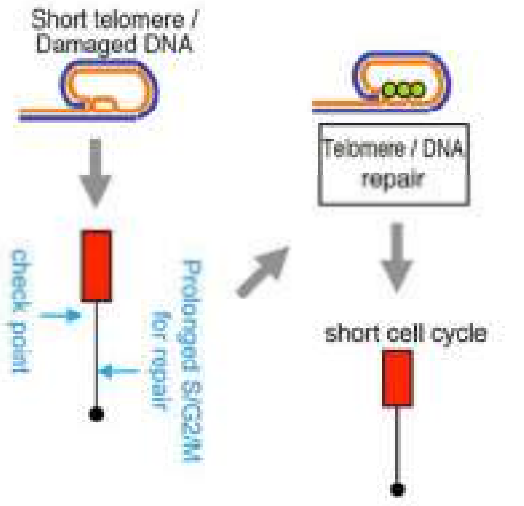


Fig 3. ES細胞におけるZscan4の発現タイミングの制御
(Futatsugi-Nakai and Niwa Stem Cell Rep 2016より)

Pluripotent stem cells are derived from pre-implantation embryos. They possess abilities to differentiate all terminally-differentiated cells in an organism (pluripotency) and to self-renew. Mouse embryonic stem (ES) cells continue self-renewal in an optimized culture condition in vitro. Pluripotency is not a character that is morphologically obvious but quite unique in the character of genome programming. Genome programming is achieved by the combination of the transcriptional regulation by the transcription factors and the epigenetic regulation of the genome, and defines cell-type-specific gene expression pattern. In ES cells, the pluripotency-associated transcription factor network with strong autonomous regulation governs gene expression of the genome with very limited epigenetic regulation in the extracellular signal-dependent manner. The pluripotency-specific genome program undergoes transition toward the differentiation-coupled (somatic) genome program that is regulated by the transcription factor network coupled with epigenetic regulation. Such dynamic change is observed within few days in the ES cell system in vitro, providing unique opportunity to analyze the molecular events in detail. We take this advantage of the ES cell system and perform 3 subjects of the basic research: (1) Analysis of the functions of transcription factor network and epigenetic mechanisms to maintain pluripotency, (2) Analysis of the mechanisms governing the transition of transcription factor network during differentiation, and (3) Analysis of the molecular mechanisms that direct the maintenance of pluripotency.

(1) Analysis of the functions of transcription factor network and epigenetic mechanisms to maintain pluripotency

Pluripotency is determined by the activity of the transcription factor network (Niwa *Development* 2018). The maintenance of the pluripotency-associated transcription factor network depends on the external signals, and the change of the signals triggers its transition for differentiation. It is known that the LIF and Wnt signals involve in the maintenance of self-renewal of mouse ES cells. *Esrrb* involves in the transduction of both signals. Especially, its function is essential to mediate the Wnt signal to maintain pluripotency (Martello et al *Cell Stem Cell* 2012). The LIF signal activates multiple intracellular signal transduction pathways. Among them, the Jak-Stat3 signal mediates the signal to maintain pluripotency whereas the MAPK signal induces differentiation. The quantitative balance of these two signals is varied among the different genetic backgrounds. The Jak-Stat3 signal is predominantly activated in *129* strain whereas the MAPK signal is over-stimulated in *NOD* strain, resulting the different action of LIF to sustain pluripotency (Ohtsuka and Niwa *Development* 2015). In addition, we have revealed that *Trp53, Xpc* and *Zscan10* are dispensable for the maintenance of pluripotency of mouse ES cells although the previous reports suggested their involvement (Shigeta et al *Sci Rep* 2013: Ito et al *FEBS Lett* 2014: Yamane et al *BBRC* 2015).

DNA methylation is one of the major epigenetic

mechanisms to repress gene expression although it is dispensable for self-renewal of ES cells. To address the role of DNA methylation on the regulation of differentiation ability of ES cells, we assessed the response of wild-type and *Dnmt3a/3b*-null ES cells to the artificial activation of Gata4 after induction of differentiation. As the result, we found that the wild-type ES cell-derived cells do not respond to the Gata4 activation, but a subset of the *Dnmt3a/3b*-null differentiated cells respond to it and undergo differentiation toward primitive endoderm lineage as in the case of undifferentiated ES cells. We believe that this is the first demonstration of the functional significance of DNA methylation to determine the restriction of differentiation competency (Oda et al *PLoS Genet* 2013).

(2) Analysis of the mechanisms governing the transition of transcription factor network during differentiation

Mouse ES cells undergo differentiation toward trophoblast stem cells by repression of *Oct3/4*. During this transition, the expression of *Sox2*, one of the essential transcription factors for ES self-renewal, is maintained at functional level. We analyzed the function of *Sox2* in transition of ES cells to TS cells and found that *Sox2* is essential for self-renewal of TS cells by mediating the FGF signal. ChIP-seq analyses of Sox2 during transition revealed that Sox2 targets distinct sets of the genes by cooperating with different transcription factors in ES and TS cells (Adachi et al *Mol Cell* 2013). These findings contribute to the hypothetical transcription factor network model involving in the transition of cell state (Niwa *Development* 2018).

The functional domain of Sox2 is assessed by the complementation assay of inducible *Sox2*-null ES cells. When various Sox family members form different species as well as artificial mutants were examined, we found that few conserved amino acids in the HMG-box are important to define the specific function in ES cells. We confirmed that the *Drosophila SoxB* can replace the endogenous *Sox2* with keeping proper pluripotency that is assessed by chimera formation assay (Niwa et al *BMC Evol Biol* 2016).

(3) Analysis of the molecular mechanisms that direct the maintenance of pluripotency.

To maintain continuous self-renewal, ES cells should keep genome integrity during multiple rounds of replication with maintenance of telomere length. It was reported that ES cells predominantly use atypical telomere elongation system based on the homologous recombination with Zscan4 rather than the canonical telomerase-dependent system. Zscan4 is transiently expressed in 5% of ES cell population, but the molecular mechanism governing this interest regulation pattern is largely unknown.

Nr0b1 encodes nuclear receptor family transcription factor and expresses at high level in ES cells. We addressed the function of *Nr0b1* using inducible knockout system and revealed that it is dispensable for maintenance of pluripotency but essential for rapid proliferation. We found dramatic increase of *Zscan4*-positive population in *Nr0b1*-null ES cells, suggesting the role of Nr0b1 as a transcriptional repressor of *Zscan4* (Fujii et al *Sci Rep* 2015).

For further analysis of the regulatory mechanism of *Zscan4* expression, the fluorescent reporter under the regulation of Zscan4 promoter was introduced into ES cells for time-lapse imaging of *Zscan4* expression. As the result, we found that the cell-cycle length has strong correlation to the activation of *Zscan4*. The cell-cycle length of ES cells is 12 hours in average but varied among the cells in population. During the proliferation, the cell-cycle length tend to elongate, and *Zscan4* is preferentially activated when the length reach to 20 hours. We hypothesize that it happens by the shortening of telomere length and *Zscan4* works to elongate shortened telomere to rejuvenate the cells (Futatsugi-Nakai and Niwa *Stem Cell Rep* 2016).

論文目録　Publications

1. Niwa, H.: The principles that govern transcription factor network functions in stem cells. ***Development,*** 145, 2018. (Review)

2. Ohta, M., Sugano, A., Hatano, N., Sato, H., Shimada, H., Niwa, H., Sakaeda, T., Tei, H., Sakaki, Y., Yamamura, KI. and Takaoka, Y. Co-precipitation molecules hemoplexin and transferrin may be key molecules for fibrillogenesis in TTR V30M amyloidogenesis. ***Transgenic Res,*** 27, 15-23, 2018.

3. Sekikawa, T., Hayashi, Y., Noce, T., Niwa, H. and Matsui, Y. Selective de-repression of germ cell-specific genes in mouse embryonic fibroblasts in a permissive epigenetic environment. ***Sci Rep***, 6, 32932. 2016.

4. Niwa, H., Nakamura, A., Urata, M., Shirae-Kurabayashi, M., Shigehiro, K., Russell, S., and Ohtsuka, S. The evolutionally-conserved function of group B1 Sox family members confers the unique role of Sox2 in mouse ES cells. ***BMC Evolutionary Biology,*** 16, 173, 2016.

5. Nakai-Futatsugi Y. and Niwa H. Zscan4 is activated after telomere shortening in mouse embryonic stem cells. ***Stem Cell Reports***, 6, 1-13, 2016.

6. Hirate, Y., Hirahara, S., Inoue, K., Kiyonari, H., Niwa, H. and Sasaki, H.: Par-aPKC-dependent and –independent mechanisms cooperatively control cell polarity, Hippo signaling, and cell positioning in 16-cell stage mouse embryos. ***Dev Growth Differ,*** 57, 544-56, 2015.

7. Ohtsuka, S, Nakai-Futatsugi, Y. and Niwa, H.: LIF signal in mouse embryonic stem cells. ***JAK-STAT,*** 4, 1-9, 2015. (Review)

8. Yamane, M., Fujii, S., Ohtsuka, S. and Niwa, H. Zscan10 is dispensable for maintenance of pluripotency in mouse embryonic stem cells ***Biochem Biophys Res Commun,*** 468, 826-831, 2015.

9. Kinoshita, M.,Shimosato, D.,Yamane, M. and Niwa, H. Sox7 is dispensable for primitive endoderm differentiation from mouse ES cells ***BMC Dev Biol,*** 15, 37, 2015.

10. Fujii, S., Nishikawa-Torikai, S., Futatsugi, Y., Toyooka, Y., Yamane, M., Ohtsuka, S. and Niwa, H.: Nr0b1 is a negative regulator of Zscan4c in mouse embryonic stem cells. ***Sci Rep***, 5, 9146, 2015.

11. Ohtsuka, S. and Niwa, H.: The differential activation of intracellular signaling pathways confers the permissiveness of embryonic stem cell derivation from different mouse strains. ***Development,*** 142, 431-437, 2015.

12. Frankenberg, S.R., Frank, D., Harland, R., Johnson, A.D., Nichols, J., Niwa, H., Scholer, H.R., Tanaka, E., Wylie, C. and Brickman, J.M.: The POU-er of gene nomenclature. ***Development,*** 141, 2921-2923, 2014.

13. Zhao, J., Li, S., Trilok, S., Tanaka, M., Jokubaitis-Jameson, V., Wang, B., Niwa, H., and Nakayama, N.: Small molecule-directed specification of sclerotome-loke chondroprogeniyotrs and induction of a somitic chondrogenesis program from embryonic stem cells. ***Development,*** 141, 3848-3858, 2014.

14. Niwa, H.: The pluripotency transcription factor network at work in reprogramming. ***Curr Opin Genet Dev,*** 28C, 25-31, 2014. (Review)

15. Ito, S., Yamane, M., Ohtsuka, S. and Niwa, H.: The C-terminal region of Xpc is dispensable for the transcriptional activity of Oct3/4 in mouse embryonic stem cells. ***FEBS Letters,*** 588, 1128-1135, 2014.

16. Storm, M.P., Kumpfmueller, B., Bone, H.K., Buchholz, M., Ripoll, Y.S., Chaudhuri, J.B., Niwa, H., Tosh, D. and Welham, M.J.: Zscan4 is regulated by PI3-linase and DNA-damaging agents and directly interacts with the transcriptional repressors LSD1 and CtBP2 in mouse embryonic stem cells. ***PLoS One,*** 9, e89821, 2014.

17. Le Bin G.C., Munoz-Descalzo, S., Kurowski, A., Leitch, H., Lou, X., Mansfield, W., Etienne-Dumeau, C., Grabole, N., Mulas, C., Niwa, H., Hadjantonakis, A.K. and Nichols, J.:

Oct4 is required for lineage priming in the developing inner cell mass of the mouse blastocyst. ***Development,*** 141, 1001-1010, 2014

18. Leeb, M., Dietmann, S., Paramor, M., Niwa, H. and Smith, A.: Genetic exploration of the exit from self-renewal uisng haploid embryonic stem cells. ***Cell Stem Cell,*** 14, 385-393, 2014.

19. Shigeta, M., Ohtsuka, S., Nishikawa-Torikai, S., Yamane, M., Fujii, S., Murakami, K. and Niwa, H.: Maintenance of pluripotency in mouse ES cells without Trp53. ***Sci Rep,*** 3, 2944, 2013.

20. Adachi, K., Nikaido, I., Ohta, H., Ohtsuka, S., Ura, H., kadota, M., Wakayama, T., Ueda, H.R. and Niwa, H.: Context-dependent wiring of Sox2 regulatory networks for self-renewal of embryonic and trophoblast stem cells. ***Mol Cell,*** 52, 380-392, 2013.

21. Nakatake, Y., Fujii, S., Masui, S., Sugimoto, T., Torikai-Nishikawa, S., Adachi, K. and Niwa, H.: Kinetics of drug selection systems in mouse embryonic stem cells. ***BMC Biotechnol,*** 13, 64, 2013.

22. Oda, M., Kumaki, Y., Shigeta, M., Jakt, L.M., Matsuoka, C., Yamagiwa, A., Niwa, H. and Okano, M.: DNA methylation restricts lineage-specific functions of transcription factor Gata4 during embryonic stem cell differentiation. ***PLoS Genet,*** 9, e1003574, 2013.

23. Nakai-Futatsugi, Y. and Niwa, H.*: Transcription factor network in embryonic stem cells: heterogeneity under the strngency. ***Biol Pharm Bull,*** 36, 166-70, 2013. (Review)

24. Hemmrich, G., Khalturin, K., Boehm, A.M., Puchert, M., Anton-Erxleben, F., Wittlieb, J., Klostermeier, U.C., Rosenstiel, P., Oberg, H.H., Domazet-Loso, T., Sugimoto, T., Niwa, H. and Bosch, T.C.: Molecular signatures of the three germ cell lineages in Hydra and the emergence of stem cell function at the base of multicellularity. ***Mol Biol Evol,*** 29, 3267-3280, 2012.

25. Iwafuchi-Doi, M., Matsuda, K., Murakami, K., Niwa, H., Tesar, P. J., Aruga, J. Matsuo, I. and Kondoh, H.: Transcriptional regulatory networks in epiblast cells and during anterior neural plate development as modeled in epiblast stem cells. ***Development,*** 139, 3926-3937, 2012

26. Ohtsuka, S., Nishikawa-Torikai, S. and Niwa, H.: E-cadherin promotes incorporation of mouse epiblast stem cells into normal development. ***PLoS One,*** 7, e45220, 2012.

27. Martello, G., Sugimoto, T., Diamanti, E., Joshi, A., Hannah, R., Ohtsuka, S., Gottgens, B., Niwa, H. and Smith, A.: Esrrb is a pivotal target of the GSK3/Tcf3 axis regulating embryonic stem cell self-renewal. ***Cell Stem Cell,*** 11, 491-504, 2012.

著書・総説目録　Publications

1. 山根　万里子、丹羽　仁史：着床前後の胚発生の表現型解析、実験医学別冊　「マウス表現型解析スタンダード」（伊川正人、高橋智、若菜茂晴　編集、羊土社）259-266, 2016.

2. 丹羽　仁史：再生医療の基盤となる転写因子ネットワークに関する基礎知識、再生医療、13, 150-154, 2014.

3. 大塚　哲、丹羽　仁史：2i 培養法を用いたマウス ES 細胞樹立法、実験医学別冊「ES・iPS 細胞実験スタンダード」70-78, 2014.

4. 丹羽　仁史：多能性幹細胞という生き物を解剖する、特集「iPS 細胞／ES 細胞の生物学」生物の科学　遺伝、68, 1, 2014.

5. 二木　陽子、丹羽　仁史：多能性幹細胞 – 分化する能力を閉じ込めた「玉手箱」、幹細胞研究と再生医療（中内啓光　編集、南山堂） 13-22, 2013

6. 発生の謎から臨床へ（対談）岡野栄之、丹羽仁史：科学（岩波書店)、83, 1 2013.

7. 丹羽仁史：幹細胞研究の生物学、再生医療叢書１幹細胞（日本再生医療学会　監修：山中伸弥、中内啓光　編集、朝倉書店）1-20, 2012

8. 丹羽　仁史：幹細胞の多能性を規定する分子機構、領域総合レビュー、１、e008, 2012.

9. 丹羽　仁史：成体細胞が多能性に初期化されうることの発見、現代化学（東京化学同人）No. 501、2012.

学会・研究集会 発表目録　Meeting Presentations

1. Niwa, H. Overlapping function of Klf family targets multiple transcription factors to maintain naïve pluripotency of ES cells. KEY FORUM 2018 Stem Cell Traits and Developmental Systems, 11-12 January, 2018, Kumamoto, Japan.

2. Hitoshi Niwa: Molecular mechanism to generate heterogeneous gene expression in mouse ES cell population, 第５５回日本生物物理学会年会　シンポジウム　動的不均一性がもたらす多細胞社会の秩序形成, 9 月 20 日, 2017、熊本.

3. 丹羽仁史. マウス ES 細胞における Klf ファミリー転写因子の機能. モロシヌス研究会、 6 月 23 日 - 24 日, 2017, 阿蘇.

4. Hitoshi Niwa: Function and evolution of pluripotency-associated transcription factors. Joint Meeting of the German and Japanese Societies of Developmental Biologists, 15 March, 2017, Kiel, Germany.

5. Hitoshi Niwa: Overlapped function of Klf family members to maintain pluripotency in mouse mebryonic stem cells. 2nd Kumamoto IRCMS International Symposium and 17th Kumamoto AIDS Seminar, 31 October, 2016, Kumamoto, Japan.

6. Hitoshi Niwa, Yoko Futatsugi: Regulation of 2-cell specific gene expression in mouse ES cells. CSHA/ISSCR Joint Meeting-Stem Cells:from Basic Biology to Disease Therapy, 19-23 October, 2015, Suzhou, China.

7. Hitoshi Niwa, Yoko Ftatsugi: Regulation of 2-cell specific gene expression in mouse ES cells. Cold Spring Harbor Laboratory Meeting: Stem Cell Biology, 7-11 October, 2015, Cold Spring Harbor Laboratory, USA.

8. 丹羽　仁史：マウス ES 細胞の多能性を規定する転写因子ネットワーク, 第３９回阿蘇シンポジウム　幹細胞研究とその応用, 7 月 31 日, 2015, 阿蘇.

9. Hitoshi Niwa: transcription factor network governing pluripotency, ISSCR Regional forum series: Global controls in stem cells, 5-7 November, 2014, Singapore.

10. Hitoshi Niwa: transcription factor network governing pluripotency, The 2014 Till & McCulloch Meetings, 27-29 October, 2014, Ottawa, Canada.

11. Hitoshi Niwa: Input and output of pluripotency-associated transcription factor netowrk, 熊本医学・生物科学国際シンポジウム「幹細胞制御と臓器再建, 9 月 4-5 日, 2014, 熊本.

12. Hitoshi Niwa: Input and output of pluripotency-associated transcription factor network, The 5th conference in the Copenhagen Bioscience Conference: The Stem cell Niche 2014, 18-22 May, 2014, Copenhagen, Denmark.

13. Hitoshi Niwa: restriction of pluripotency in development - modeling in vitro, The 61st NIBB Conference: Cellular Community in

Mammalian Embryogenesis 10-12, July, 2013, 岡崎.

14. Hitoshi Niwa; Molecular mechanism to maintain pluripotency of mouse ES cells, International Symposium on Recent Advances in Stem Cells and Cancer & the 8th Annual Meeting of TSSCR, 14 October, 2012, Kaoshung, Taiwan.

アウトリーチ活動　Outreach activity

1. 丹羽　仁史.　熊本県立八代中学校見学対応. 6 月 16 日、 2017, 熊本.

2. 丹羽　仁史.　熊本県立八代中学校見学対応と講義. 10 月 18 日、 2016, 熊本.

3. 丹羽　仁史.　熊本県立八代中学校見学対応. 6 月 19 日、 2015, 熊本.

© 2018. Published by The Company of Biologists Ltd | Development (2018) 145, dev157420. doi:10.1242/dev.157420

REVIEW

The principles that govern transcription factor network functions in stem cells

Hitoshi Niwa

ABSTRACT

Tissue-specific transcription factors primarily act to define the phenotype of the cell. The power of a single transcription factor to alter cell fate is often minimal, as seen in gain-of-function analyses, but when multiple transcription factors cooperate synergistically it potentiates their ability to induce changes in cell fate. By contrast, transcription factor function is often dispensable in the maintenance of cell phenotype, as is evident in loss-of-function assays. Why does this phenomenon, commonly known as redundancy, occur? Here, I discuss the role that transcription factor networks play in collaboratively regulating stem cell fate and differentiation by providing multiple explanations for their functional redundancy.

KEY WORDS: Embryonic stem cells, Differentiation, Reprogramming, Transcription factor

Introduction

During mouse development, a single, totipotent cell divides repeatedly to give rise to a few billion cells, which differentiate into a few hundred different cell types. Differentiation is the process by which a cell changes phenotype and becomes increasingly specialized. Cell phenotypes are defined by particular combinations of genes expressed in a cell type-dependent manner (Armit et al., 2017). The selection of these combinations is mainly driven by cell type-specific transcription factors (TFs), which in turn are regulated by other TFs that integrate and respond to extracellular signals in order to maintain cell phenotype (Davidson, 1993). Thus, TFs form a network in which each TF is reciprocally regulated to maintain its balanced expression.

A TF network often forms part of a gene regulatory network (Box 1, Glossary). Gene regulatory networks are divided into functional subcircuits (Davidson, 2010) and consist of multiple layers of regulatory mechanisms at the epigenetic, topological and transcriptional level. The epigenetic regulation of chromatin accessibility is thought to be important for maintaining the irreversibility of a cell's differentiated status under normal physiological conditions (Perino and Veenstra, 2016). The topological regulation of chromatin is also believed to control global gene expression patterns in organisms (Acemel et al., 2017); however, the degree to which these regulatory mechanisms actively determine specific cell types is unclear. By contrast, TF networks have been shown to play a pivotal role in defining cell types, which is reflected in the ability of certain TFs to instruct changes in cell phenotype when ectopically expressed in various contexts (Niwa, 2007; Morris, 2016). The discovery of *MyoD* (*Myod1*), for example, was a key finding in this field as it demonstrated the power of a single TF to define cell phenotype (Davis et al., 1987). This example might be somewhat of a rarity, however, since in general the potential of a single cell-type-specific TF to instruct fate is limited, and it often only regulates differentiation in a particular context. More commonly, specific combinations of TFs are required to instruct cell fate, most famously during the reprogramming of differentiated somatic cells to pluripotent stem cells, which requires a combination of four TFs (Takahashi and Yamanaka, 2006). Other combinations of TFs have also been used to induce direct lineage reprogramming, whereby a cell transitions from one cell type to another without returning to a pluripotent state (Morris, 2016).

These findings raised a key question: why are multiple TFs required to artificially change a cellular phenotype? To answer this question, we need to know how TFs function in a cell to define a phenotype. During differentiation, multiple TFs are known to cooperate with each other to activate transcription of their target genes (Whyte et al., 2013). To stably maintain a certain cell type, multiple TFs form a network that maintains their own expression, as well as that of cell type-specific genes as a downstream subcircuit (Davidson, 2010). While these broad principles have been established for a number of years, specific issues, such as what determines the exact number of TFs required to form a cell type-specific TF network, and how TF networks are sequentially replaced during differentiation, remain unanswered. A simple model system with synchronous differentiation would provide an ideal platform to address these issues. The *in vitro* differentiation system of mouse embryonic stem cells (mESCs) (Box 1, Glossary; Box 2) provides one such model (Niwa, 2010). In this Review, I focus on studies that analyze the role of TFs in regulating mESC self-renewal and differentiation, and summarize the mechanisms involved in the functioning and transitioning of TF networks.

Modes of interaction between TFs and their target sites

To elucidate the structure of the TF network, we first need to know how each TF regulates its target genes. TFs are categorized into two classes: general TFs and tissue-specific TFs (Levine et al., 2014). General TFs bind to a promoter element to recruit RNA polymerase II and initiate transcription. Tissue-specific TFs bind to either a proximal element of a promoter or a distal regulatory element, designated as an enhancer, to promote the recruitment of general TFs to a promoter. Tissue-specific TFs quantitatively regulate the frequency of transcriptional bursting (Box 1, Glossary) by influencing the binding affinity of TFs to each other and of trans-acting co-factors to their target sites, as well as the stability of transcriptional complexes (Bartman et al., 2016; Fukaya et al., 2016). Here, I focus on the mechanisms that mediate the synergistic action of multiple tissue-specific TFs.

Department of Pluripotent Stem Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan.

*Author for correspondence (niwa@kumamoto-u.ac.jp)

H.N., 0000-0002-5280-8031

DEVELOPMENT

The FASEB Journal article fj.201700573R. Published online November 16, 2017.

THE FASEB JOURNAL • RESEARCH • www.fasebj.org

Reduced Dnmt3a increases Gdf5 expression with suppressed satellite cell differentiation and impaired skeletal muscle regeneration

Yukino Hatazawa,[*,†,‡,1] Yusuke Ono,[§,¶,1] Yuma Hirose,[†] Sayaka Kanai,[*] Nobuharu L. Fujii,[‖] Shuichi Machida,[#] Ichizo Nishino,[**] Takahiko Shimizu,[††] Masaki Okano,[‡‡] Yasutomi Kamei,[*,†,2] and Yoshihiro Ogawa[*,§§,¶¶,3]

[*]Department of Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan; [†]Laboratory of Molecular Nutrition, Graduate School of Environmental and Life Science, Kyoto Prefectural University, Kyoto, Japan; [‡]Japan Society for the Promotion of Science, Tokyo, Japan; [§]Musculoskeletal Molecular Biology Research Group, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [¶]Division of Regenerative Medicine Research, Japan Agency for Medical Research and Development (AMED), Tokyo, Japan; [‖]Department of Health Promotion Sciences, Graduate School of Human Health Sciences, Tokyo Metropolitan University, Hachioji, Japan; [#]Graduate School of Health and Sports Science, Juntendo University, Chiba, Japan; [**]National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; [††]Department of Advanced Aging Medicine, Chiba University Graduate School of Medicine, Chiba, Japan; [‡‡]Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan; [§§]Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and [¶¶]Japan Agency for Medical Research and Development (AMED), Core Research for Evolutional Science and Technology (CREST), Tokyo, Japan

ABSTRACT: DNA methylation is an epigenetic mechanism regulating gene expression. In this study, we observed that DNA methyltransferase 3a (Dnmt3a) expression is decreased after muscle atrophy. We made skeletal muscle–specific Dnmt3a knockout mice (Dnmt3a-KO mice). The regeneration capacity after muscle injury was markedly decreased in Dnmt3a-KO mice. Diminished mRNA and protein expression of Dnmt3a were observed in skeletal muscles as well as in satellite cells, which are important for muscle regeneration, in Dnmt3a-KO mice. Dnmt3a-KO satellite cell showed smaller in size (length/area), suggesting suppressed myotube differentiation. Microarray analysis of satellite cells showed that expression of growth differentiation factor 5 (Gdf5) mRNA was markedly increased in Dnmt3a-KO mice. The DNA methylation level of the *Gdf5* promoter was markedly decreased in Dnmt3a-KO satellite cells. In addition, DNA methylation inhibitor azacytidine treatment increased Gdf5 expression in wild-type satellite cells, suggesting *Gdf5* expression is regulated by DNA methylation. Also, we observed increased inhibitor of differentiation (a target of Gdf5) mRNA expression in Dnmt3a-KO satellite cells. Thus, Dnmt3a appears to regulate satellite cell differentiation *via* DNA methylation. This mechanism may play a role in the decreased regeneration capacity during atrophy such as in aged sarcopenia.—Hatazawa, Y., Ono, Y., Hirose, Y., Kanai, S., Fujii, N. L., Machida, S., Nishino, I., Shimizu, T., Okano, M., Kamei, Y., Ogawa, Y. Reduced Dnmt3a increases Gdf5 expression with suppressed satellite cell differentiation and impaired skeletal muscle regeneration. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: DNA methylation · epigenetics · atrophy · knockout mouse

Epigenetic events contribute to skeletal muscle remodeling in a variety of physiologic and pathophysiologic conditions (1). DNA methylation occurs as 5-methylcytosines mainly at cytosine–guanine dinucleotides, so-called CpG sites, and such methylation is a well-studied epigenetic mechanism for transcriptional regulation (2, 3). Generally DNA methylation of the gene promoter is correlated with transcriptional repression (2, 3). Genomic DNA methylation patterns are established by the actions of the *de novo* methyltransferases DNA methyltransferase 3a (Dnmt3a) and Dnmt3b, and are maintained by the methyltransferase Dnmt1 (4). DNA methylation has long been known to be involved in muscle formation. Treatment of fibroblasts with the DNA methylation inhibitor 5-azacytidine caused

ABBREVIATIONS: Bmp, bone morphogenic protein; CTX, cardiotoxin; Dnmt3a, DNA methyltransferase 3a; Dnmt3a-KO mice, skeletal muscle–specific Dnmt3a knockout mice; EDL, extensor digitorum longus; EdU, 5-ethynyl-2′-deoxyuridine; FGFR4, fibroblast growth factor receptor 4; Gdf, growth differentiation factor; HE, hematoxylin and eosin; IAP, intracisternal A particle; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, knockout; MyHC, myosin heavy chain; qPCR, quantitative PCR; WT, wild type; YFP, yellow fluorescent protein

[1] These authors contributed equally to this work.
[2] Correspondence: Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto, 606-8522 Japan. E-mail: kamei@kpu.ac.jp
[3] Correspondence: 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: yogawa@intmed3.med.kyushu-u.ac.jp

doi: 10.1096/fj.201700573R
This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

Transgenic Res (2018) 27:15–23
https://doi.org/10.1007/s11248-017-0054-x

CrossMark

ORIGINAL ARTICLE

Co-precipitation molecules hemopexin and transferrin may be key molecules for fibrillogenesis in TTR V30M amyloidogenesis

Mika Ohta · Aki Sugano · Naoya Hatano · Hirotaka Sato · Hirofumi Shimada · Hitoshi Niwa · Toshiyuki Sakaeda · Hajime Tei · Yoshiyuki Sakaki · Ken-ichi Yamamura · Yutaka Takaoka

Received: 17 August 2017 / Accepted: 14 December 2017 / Published online: 29 December 2017

Abstract The disease model of familial amyloidotic polyneuropathy—7.2-hMet30 mice—manifests amyloid deposition that consists of a human amyloidogenic mutant transthyretin (TTR) (TTR V30M). Our previous study found amyloid deposits in 14 of 27 7.2-hMet30 mice at 21–24 months of age. In addition, non-fibrillar TTR deposits were found in amyloid-negative 7.2hMet30 mice. These results suggested that TTR amyloidogenesis required not only mutant TTR but also an additional factor (or factors) as an etiologic molecule. To determine the differences in serum proteome in amyloid-positive and amyloid-negative mice in the 7.2-hMet30 model, we used proteomic analyses and studied serum samples obtained from these mice. Hemopexin (HPX) and transferrin (Tf) were detected in the serum samples from amyloid-positive mice and were also found in amyloid deposits via immunohistochemistry, but serum samples from amyloid-negative mice did not contain HPX and Tf. These two proteins were also not detected in non-fibrillar TTR deposits. In addition, in silico analyses suggested that HPX and Tf facilitate destabilization of TTR secondary structures and misfolding of TTR. These results suggest that HPX and Tf may be associated with TTR amyloidogenesis after fibrillogenesis in vivo.

Mika Ohta, Aki Sugano and Naoya Hatano have contributed equally to this work.

M. Ohta · A. Sugano · Y. Takaoka (✉)
Division of Medical Informatics and Bioinformatics, Kobe University Hospital, Kobe 650-0017, Japan
e-mail: ytakaoka@ebraille.med.kobe-u.ac.jp

N. Hatano
The Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

H. Sato
Department of Pathology, Division of Anatomical and Cellular Pathology, Iwate Medical University, Morioka 028-3694, Japan

H. Shimada · H. Niwa · K. Yamamura · Y. Takaoka
Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto 860-0811, Japan

T. Sakaeda
Department of Phamacokinetics, Kyoto Pharmaceutical University, Kyoto 607-8414, Japan

H. Tei · Y. Sakaki · Y. Takaoka
Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

K. Yamamura
Yamamura Project Laboratory, Institute of Resource Development and Analysis, Kumamoto University, Kumamoto 860-0811, Japan

SCIENTIFIC REPORTS

OPEN

Selective de-repression of germ cell-specific genes in mouse embryonic fibroblasts in a permissive epigenetic environment

Received: 25 April 2016
Accepted: 17 August 2016
Published: 09 September 2016

Tamotsu Sekinaka[1,2,3], Yohei Hayashi[1,2,3], Toshiaki Noce[4], Hitoshi Niwa[5] & Yasuhisa Matsui[1,2,3]

Epigenetic modifications play crucial roles on establishment of tissue-specific transcription profiles and cellular characteristics. Direct conversions of fibroblasts into differentiated tissue cells by over-expression of critical transcription factors have been reported, but the epigenetic mechanisms underlying these conversions are still not fully understood. In addition, conversion of somatic cells into germ cells has not yet been achieved. To understand epigenetic mechanisms that underlie germ cell characteristics, we attempted to use defined epigenetic factors to directly convert mouse embryonic fibroblasts (MEFs) into germ cells. Here, we successfully induced germ cell-specific genes by inhibiting repressive epigenetic modifications via RNAi or small-molecule compounds. Under these conditions, some tissue-specific genes and stimulus-inducible genes were also induced. Meanwhile, the treatments did not result in genome-wide transcriptional activation. These results suggested that a permissive epigenetic environment resulted in selective de-repression of stimulus- and differentiation-inducible genes including germ cell-specific genes in MEFs.

The early precursors of germ cells, designated primordial germ cells (PGCs), become established at around embryonic day (E)7.25 in the extraembryonic mesoderm[1]. PGCs then migrate into the indifferent embryonic gonads (genital ridges), and subsequently start to differentiate into sperms or eggs. Developing PGCs express several germ cell-specific genes at specific embryonic developmental stages. For example, nascent PGCs express *Blimp1* (also known as *Prdm1*: PR domain containing 1, with ZNF domain), which is necessary for induction of PGCs[2]; *Stella* (also known as *Dppa3*: developmental pluripotency-associated 3), which is important to embryonic development after fertilization[3–5] and *Nanos3* (nanos homolog 3), which is necessary for survival of PGCs[6,7]. Then, during migration into the genital ridges (E10.5-E13.5), PGCs express *Vasa* (also known as *Ddx4*: DEAD box polypeptide 4), which is important for development of male germ cells[8,9]; and PGCs also begin to express meiosis related-genes such as *Dazl* (deleted in azoospermia-like)[10,11] and *Stra8* (stimulated by retinoic acid gene 8) during migration[12,13]. Along with those PGC-specific genes, PGC also express pluripotency-associated gene including *Oct4* (also known as *Pou5f1*: POU domain, class5, transcription factor 1), *Sox2* (SRY-box 2), and *Nanog* (Nanog homeobox); these gene products contribute to survival and/or differentiation of PGCs[14–17].

During their development, PGC undergo characteristic epigenetic reprogramming. During migration, repressive epigenetic modifications, such as histone H3 Lysine 9 di-methylation (H3K9me2) and DNA methylation, are globally reduced[18,19]; simultaneously, histone H3 Lysine 27 tri-methylation (H3K27me3), another repressive histone modification, is elevated[20]. Meanwhile, H3K27 becomes locally hypo-methylated in regulatory regions of germ cell-specific genes prior to their PGC-specific upregulation[21]; these coordinated changes suggest that these epigenetic modifications play important roles in the temporal regulation of germ cell-specific gene expression in

[1]Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (IDAC), Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan. [2]Graduate school of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi, 980-8577, Japan. [3]The Japan Agency for Medical Research and Development-Core Research for Evolutional Science and Technology (AMED-CREST), Tokyo 100-0004, Japan. [4]School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. [5]Department of Pluripotent Stem Cell Biology, Institute of Molecular Embryology and Genetics (IMEG), Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan. Correspondence and requests for materials should be addressed to Y.M. (email: yasuhisa.matsui.d3@tohoku.ac.jp)

Niwa et al. BMC Evolutionary Biology (2016) 16:173
DOI 10.1186/s12862-016-0755-4

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

The evolutionally-conserved function of group B1 Sox family members confers the unique role of Sox2 in mouse ES cells

Hitoshi Niwa[1,2*], Akira Nakamura[3], Makoto Urata[4], Maki Shirae-Kurabayashi[5], Shigehiro Kuraku[6], Steven Russell[7] and Satoshi Ohtsuka[1,8]

Abstract

Background: In mouse ES cells, the function of Sox2 is essential for the maintenance of pluripotency. Since the Sox-family of transcription factors are well conserved in the animal kingdom, addressing the evolutionary origin of *Sox2* function in pluripotent stem cells is intriguing from the perspective of understanding the origin of pluripotency.

Results: Here we approach this question using a functional complementation assay in inducible *Sox2*-null ES cells. Assaying mouse Sox proteins from different Groups, we found that only Group B1 and Group G proteins were able to support pluripotency. Interestingly, invertebrate homologs of mammalian Group B1 Sox proteins were able to replace the pluripotency-associated function of mouse *Sox2*. Moreover, the mouse ES cells rescued by the *Drosophila SoxNeuro* protein are able to contribute to chimeric embryos.

Conclusions: These data indicate that the function of mouse *Sox2* supporting pluripotency is based on an evolutionally conserved activity of the Group B1 Sox family. Since pluripotent stem cell population in developmental process could be regarded as the evolutional novelty in vertebrates, it could be regarded as a co-optional use of their evolutionally conserved function.

Keywords: Pluripotent stem cells, Sox2, Evolution, Co-option

Background

Pluripotency is a unique feature of the cells found in early vertebrate embryos. Pluripotent stem cells give rise to all cell types of the organism, including germ cells, but, unlike zygotes, they do not have the ability to give rise organisms autonomously [1]. The pluripotent phenotype is primarily determined by the expression of a set of pluripotency-associated transcription factors, as demonstrated by the induction of pluripotency in somatic cells transfected with four transcription factors to give rise to induced pluripotent stem (iPS) cells [2]. Of these four transcription factors, Oct3/4 (encoded by *Pou5f1*) and Sox2 are known to be essential for maintaining pluripotency in mouse embryonic stem (ES) cells [3, 4]. In contrast, the functions of Klf4 and Myc are dispensable for pluripotency, but primarily support self-renewal in the absence of the cytokine leukemia inhibitory factor (LIF) [5–8].

Pluripotent stem cell populations have been definitively identified in mammalian embryos, but their presence in other vertebrate embryos remains unclear, with no pluripotent stem cell lines yet isolated from other taxa. Frog animal cap cells behave similarly to pluripotent cells, but have never been shown to yield stem cells capable of propagating in vitro [9]. The absence of pluripotent stem cells is evident in ascidian embryos, since blastomeres exhibit mosaic behavior upon artificial separation [10]. Likewise there is no evidence of pluripotent stem cells in well studied invertebrates,

* Correspondence: niwa@kumamoto-u.ac.jp
[1]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology (CDB), 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
[2]Department of Pluripotent Stem Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan
Full list of author information is available at the end of the article

Stem Cell Reports
Article
ISSCR
OPEN ACCESS

Zscan4 Is Activated after Telomere Shortening in Mouse Embryonic Stem Cells

Yoko Nakai-Futatsugi[1,3,*] and Hitoshi Niwa[1,2,4,*]
[1]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
[2]Japan Science and Technology Agency, CREST, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan
[3]Present address: Center for iPS Cell Research and Application, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[4]Present address: Department of Pluripotent Stem Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan
*Correspondence: y.futatsugi@cira.kyoto-u.ac.jp (Y.N.-F.), niwa@kumamoto-u.ac.jp (H.N.)
http://dx.doi.org/10.1016/j.stemcr.2016.02.010

SUMMARY

ZSCAN4 is a DNA-binding protein that functions for telomere elongation and genomic stability. In vivo, it is specifically expressed at the two-cell stage during mouse development. In vitro, it is transiently expressed in mouse embryonic stem cells (ESCs), only in 5% of the population at one time. Here we attempted to elucidate when, under what circumstances, *Zscan4* is activated in ESCs. Using live cell imaging, we monitored the activity of *Zscan4* together with the pluripotency marker *Rex1*. The lengths of the cell cycles in ESCs were diverse. Longer cell cycles were accompanied by shorter telomeres and higher activation of *Zscan4*. Since activation of *Zscan4* is involved in telomere elongation, we speculate that the extended cell cycles accompanied by *Zscan4* activation reflect the time for telomere recovery. *Rex1* and *Zscan4* did not show any correlation. Taken together, we propose that *Zscan4* is activated to recover shortened telomeres during extended cell cycles, irrespective of the pluripotent status.

INTRODUCTION

Zinc finger and SCAN domain containing 4 (ZSCAN4) is a DNA-binding protein that is specifically expressed in two-cell stage embryos during mouse development (Falco et al., 2007). In vitro, interestingly, *Zscan4* is transiently expressed in a minor population of embryonic stem cells (ESCs) at one time (Carter et al., 2008) but is eventually expressed in all (Zalzman et al., 2010). It functions for telomere elongation and genomic stability (Zalzman et al., 2010) and thus is considered as a rejuvenation factor.

ESCs are a heterogeneous population. If cultured in conventional serum-containing medium supplemented with leukemia inhibitory factor (LIF), they remain undifferentiated but closer studies show they are actually a mixture of cells with higher and lower potential of differentiation (reviewed in Nakai-Futatsugi and Niwa, 2013). Recently even a minor population of two-cell-stage-like ESCs that are not only pluripotent but also capable of differentiating into extra-embryonic lineages was found in the heterogeneous ESC population (Macfarlan et al., 2012). The heterogeneity of ESCs is accompanied by fluctuation of the expression of pluripotency-associated genes such as *Rex1* (also known as *Zfp42*) (Toyooka et al., 2008), *Nanog* (Chambers et al., 2007; Singh et al., 2007), *Klf4* (Niwa et al., 2009), *Tbx3* (Niwa et al., 2009), *Stella* (Hayashi et al., 2008), and so on. However, among the pluripotency-associated genes, *Oct3/4* (also known as *Pou5f1*), whose expression does not fluctuate, is an exception. It is the master gene of pluripotency (Nichols et al., 1998). A constant expression level of *Oct3/4* is crucial for the maintenance of pluripotency, as a slight increase leads to differentiation into primitive endoderm and mesoderm while a slight decrease leads to differentiation into trophoectoderm (Niwa et al., 2000). The expression level of *Oct3/4* is maintained at a constant level downstream of a robust transcription factor network in mouse ESCs (Niwa et al., 2009). *Rex1*, although not essential for ESC self-renewal and pluripotency (Masui et al., 2008), decreases in its mRNA-expression level when the master gene *Oct3/4* is either hyper-expressed or hypo-expressed (Niwa et al., 2000). Thus we consider the promoter activity of *Rex1*, which is high only when the expression of *Oct3/4* is maintained at an optimal range, as a good indicator of pluripotency.

To elucidate whether the expression pattern of *Zscan4* has any correlation with ESC proliferation, we monitored *Zscan4* activity at single cell level. Also to see whether the rejuvenation factor *Zscan4* correlates with the fluctuating wave of ESC pluripotency (Figure S1), we monitored *Zscan4* and the pluripotency indicator *Rex1* simultaneously under live cell imaging. Unexpectedly, we did not see any correlation between the two factors. Instead, we found *Zscan4* is activated when the cell-cycle lengths become long, irrespective of the pluripotent status, presumably sensing shortened telomeres.

RESULTS

Cell-Cycle Length of Mouse ESCs Is Diverse

First we analyzed the proliferation profile of ESCs at the single cell level. ESCs were stably transfected with Fucci vector

Development, Growth & Differentiation
The Japanese Society of Developmental Biologists

Develop. Growth Differ. (2015) **57**, 544–556 doi: 10.1111/dgd.12235

Original Article

Par-aPKC-dependent and -independent mechanisms cooperatively control cell polarity, Hippo signaling, and cell positioning in 16-cell stage mouse embryos

Yoshikazu Hirate,[1†] Shino Hirahara,[2] Ken-ichi Inoue,[3] Hiroshi Kiyonari,[3,4] Hiroshi Niwa[5,6] and Hiroshi Sasaki[1,7*]

[1]*Department of Cell Fate Control, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811,* [2]*Laboratory for Embryonic Induction, RIKEN Center for Developmental Biology,* [3]*Animal Resource Development Unit, Division of Bio-function Dynamics Imaging, RIKEN Center for Life Science Technologies,* [4]*Genetic Engineering Team, Division of Bio-function Dynamics Imaging, RIKEN Center for Life Science Technologies,* [5]*Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047,* [6]*Department of Stem Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, and* [7]*Laboratory for Embryogenesis, Graduate School of Frontier BioSciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan*

In preimplantation mouse embryos, the Hippo signaling pathway plays a central role in regulating the fates of the trophectoderm (TE) and the inner cell mass (ICM). In early blastocysts with more than 32 cells, the Par-aPKC system controls polarization of the outer cells along the apicobasal axis, and cell polarity suppresses Hippo signaling. Inactivation of Hippo signaling promotes nuclear accumulation of a coactivator protein, Yap, leading to induction of TE-specific genes. However, whether similar mechanisms operate at earlier stages is not known. Here, we show that slightly different mechanisms operate in 16-cell stage embryos. Similar to 32-cell stage embryos, disruption of the Par-aPKC system activated Hippo signaling and suppressed nuclear Yap and Cdx2 expression in the outer cells. However, unlike 32-cell stage embryos, 16-cell stage embryos with a disrupted Par-aPKC system maintained apical localization of phosphorylated Ezrin/Radixin/Moesin (p-ERM), and the effects on Yap and Cdx2 were weak. Furthermore, normal 16-cell stage embryos often contained apolar cells in the outer position. In these cells, the Hippo pathway was strongly activated and Yap was excluded from the nuclei, thus resembling inner cells. Dissociated blastomeres of 8-cell stage embryos form polar–apolar couplets, which exhibit different levels of nuclear Yap, and the polar cell engulfed the apolar cell. These results suggest that cell polarization at the 16-cell stage is regulated by both Par-aPKC-dependent and -independent mechanisms. Asymmetric cell division is involved in cell polarity control, and cell polarity regulates cell positioning and most likely controls Hippo signaling.

Key words: asymmetric cell division, cell polarity, Hippo signaling, Par-aPKC, preimplantation embryo.

Introduction

Before implantation in the uterus, mouse embryos undergo several rounds of cell division and form a cyst-like structure called the blastocyst (Yamanaka *et al.* 2006; Sasaki 2010, 2015). The early blastocyst contains two types of cells, the trophectoderm (TE) and the inner cell mass (ICM). The TE is an outer epithelial structure required for implantation that later gives rise to placental tissues, whereas the ICM is a mass of pluripotent cells surrounded by the TE that later forms the embryo proper and some extraembryonic tissues.

Formation of the TE and ICM is the first cell fate specification in mouse development, and it has been

*Author to whom all correspondence should be addressed.
Email: sasaki@fbs.osaka-u.ac.jp
†Present address: Center for Experimental Animals, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
Received 13 May 2015; revised 11 July 2015; accepted 12 July 2015.

JAK-STAT, 4:1–19, 2015
Published with license by Taylor & Francis Group, LLC
ISSN: 2162-3996 online
DOI: 10.1080/21623996.2015.1086520

REVIEW

LIF signal in mouse embryonic stem cells

Satoshi Ohtsuka,[1,*] Yoko Nakai-Futatsugi,[1] and Hitoshi Niwa[1,2,*]

[1]Laboratory for Pluripotent Stem Cell Studies; Center for Developmental Biology (CDB) RIKEN; Kobe, Japan
[2]Department of Pluripotent Stem Cell Biology; Institute of Molecular Embryology and Genetics (IMEG); Kumamoto University; Kumamoto, Japan

Since the establishment of mouse embryonic stem cells (mESCs) in the 1980s, a number of important notions on the self-renewal of pluripotent stem cells *in vitro* have been found. In serum containing conventional culture, an exogenous cytokine, leukemia inhibitory factor (LIF), is absolutely essential for the maintenance of pluripotency. In contrast, in serum-free culture with simultaneous inhibition of Map-kinase and Gsk3 (so called 2i-culture), LIF is no longer required. However, recent findings also suggest that LIF may have a role not covered by the 2i for the maintenance of naïve pluripotency. These suggest that LIF functions for the maintenance of naïve pluripotency in a context dependent manner. We summarize how LIF-signal pathway is converged to maintain the naïve state of pluripotency.

KEYWORDS. Embryonic stem cell (ESC), Lekemia inhibitory factor (LIF) signal, Stat3, MAP kinase, PI3K-Akt, Genetic background, naive state of pluripoetncy, Epigenetics

INTRODUCTION

mESCs are derived from the inner cell mass (ICM) of the blastocyst, and self-renew indefinitely *in vitro*.[1,2] In earlier period, mESCs were maintained in fetal calf serum (FCS)-containing medium with mouse embryonic fibroblast (MEF)-feeder cells. It was technically

*Correspondence to: Satoshi Ohtsuka; Email: sohtsuka@cdb.riken.jp; Hitoshi Niwa; Email: niwa@kumamoto-u.ac.jp
Submitted: 06/23/2015; Revised: 08/14/2015; Accepted: 08/18/2015

Biochemical and Biophysical Research Communications 468 (2015) 826–831

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Zscan10 is dispensable for maintenance of pluripotency in mouse embryonic stem cells

Mariko Yamane [a], Setsuko Fujii [a], Satoshi Ohtsuka [a], Hitoshi Niwa [a, b, c, d, *]

[a] *Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology (CDB), 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[b] *Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe 650-0017, Japan*
[c] *JST, CREST, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan*
[d] *Department of Pluripotent Stem Cell Biology, Institute of Molecular Embryology and Genetics (IMEG), 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan*

ARTICLE INFO

Article history:
Received 28 October 2015
Accepted 7 November 2015
Available online 22 November 2015

Keywords:
Zscan10
Pluripotency
Embryonic stem cells

ABSTRACT

Zinc finger and SCAN domain-containing 10 (*Zscan10*, also known as *Zfp206*) encodes a transcription factor that has been reported to be involved in the maintenance of pluripotency in mouse embryonic stem (ES) cells. Here we generated inducible knockout ES cells for *Zscan10* using the Cre-*loxP* system and analyzed its function. We succeeded in establishing *Zscan10*-null ES cells and confirmed their pluripotency by the generation of chimeric embryos. Our results clearly indicate that *Zscan10* is dispensable for the ability of self-renewal and differentiation in ES cells.

1. Introduction

Embryonic stem (ES) cells are pluripotent stem cells derived from pre-implantation embryos [1,2]. ES cells possess a specific transcriptional factor network downstream of the integrated external signals, and the activity of this network enables to maintain their pluripotency and self-renewability [3]. Zinc finger and SCAN domain-containing 10 (*Zscan10*, also known as *Zfp206*) encodes a transcription factor and is regarded as an important member of the pluripotency-associated transcription factor network. *Zscan10* is highly expressed in ES cells and down-regulated upon differentiation [4,5]. The expression of *Zscan10* is directly regulated by the key pluripotency-associated transcription factors Oct3/4 and Sox2 [6], and Zscan10 binds to the enhancer region of *Oct3/4* with Oct3/4 and Sox2 [7], suggesting its tight integration to the pluripotency-associated transcription factor network. Single cell analysis suggested that *Zscan10* is a component of the feedback regulation of *Oct3/4* [8]. Moreover, the overexpression of *Zscan10* stabilizes self-renewal and prevents differentiation whereas its knock-down by shRNA causes increase in spontaneous differentiation [4,5], suggesting its functional involvement in the maintenance of pluripotency. In addition, it was also reported that Zscan10 might act as a transcriptional activator of the 2-cell-stage specific transcripts, such as *Zscan4* and *Tcstv1*, because their expression levels are decreased in *Zscan10* knocked-down ES cells [5]. In contrast, it was recently reported that *Zscan10* knockout mice can develop to adult organisms although they have some defects such as weight reduce or eye defect [9], suggesting that its function for the maintenance of the pluripotent cell population in early developmental process is either dispensable or masked by the maternal transcripts. In the previous reports that applied shRNA-mediated knock-down strategy for the loss of function experiments, 10–20% of total *Zscan10* transcripts still remained [4,5]. Thus, even though they indicated that *Zscan10* supports pluripotency in ES cells, it is still unclear whether the function of *Zscan10* is necessary for the maintenance of pluripotency in ES cells. To settle the argument, especially whether Zscan10 is the key regulator of 2-cell-stage specific genes and pluripotency-associated genes, we established *Zscan10* inducible-knockout ES cell lines using the Cre-*loxP* system. By this strategy, we found that *Zscan10*-null ES cells continue self-renewal while keeping the expression of pluripotency-associated transcription factors. We also confirmed the pluripotency of the *Zscan10*-null ES cells by the generation of chimeric embryos. These results suggest that the function of *Zscan10* is

* Corresponding author. Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan.
E-mail address: niwa@cdb.riken.jp (H. Niwa).

http://dx.doi.org/10.1016/j.bbrc.2015.11.039
0006-291X/

Kinoshita *et al. BMC Developmental Biology* (2015) 15:37
DOI 10.1186/s12861-015-0079-4

BMC Developmental Biology

RESEARCH ARTICLE Open Access

Sox7 is dispensable for primitive endoderm differentiation from mouse ES cells

Masaki Kinoshita[1], Daisuke Shimosato[1,2], Mariko Yamane[1] and Hitoshi Niwa[1,2,3*]

Abstract

Background: Primitive endoderm is a cell lineage segregated from the epiblast in the blastocyst and gives rise to parietal and visceral endoderm. *Sox7* is a member of the SoxF gene family that is specifically expressed in primitive endoderm in the late blastocyst, although its function in this cell lineage remains unclear.

Results: Here we characterize the function of *Sox7* in primitive endoderm differentiation using mouse embryonic stem (ES) cells as a model system. We show that ectopic expression of *Sox7* in ES cells has a marginal effect on triggering differentiation into primitive endoderm-like cells. We also show that targeted disruption of *Sox7* in ES cells does not affect differentiation into primitive endoderm cells in embryoid body formation as well as by forced expression of *Gata6*.

Conclusions: These data indicate that *Sox7* function is supplementary and not essential for this differentiation from ES cells.

Keywords: ES cells, XEN cells, primitive endoderm, Sox7

Background

Mouse blastocysts at E4.5 consist of three cell types: epiblast, primitive endoderm and trophectoderm. The epiblast is composed of pluripotent cells that give rise to all embryonic lineages in later developmental stages [1]. In contrast, both primitive endoderm and trophectoderm form extra-embryonic parts such as the yolk sac and placenta, respectively. Primitive endoderm differentiates into two types of endoderm after implantation. One is the parietal endoderm (PE) that migrates along the mural trophectoderm and covers its inner surface to form the Reichert membrane. The other is the visceral endoderm (VE) that covers the outer surface of epiblast and extraembryonic ectoderm derived from trophectoderm. PE cells show mesenchymal cell-like characteristics such as stellate morphology, weak cell adhesion and rapid migration ability. In contrast, VE cells show typical epithelial morphology with tight cell adhesion.

Transcription factors (TFs) have pivotal roles in determining cell fates in developmental processes. In pre-implantation embryos, the Gata family zinc-finger transcription factor, Gata6, appears to be the primary TF that determines primitive endoderm fate as it is expressed at the earliest time point (E2.5) [2] among the primitive endoderm-specific TFs, and *Gata6*-null embryos fail to form functional visceral endoderm [3]. In addition, recent reports showed that *Gata6* deficient blastocyst-stage embryos fail to form primitive endoderm before implantation [4, 5]. The family member Gata4 is co-expressed in the primitive endoderm [6] and possibly shares function with Gata6. *Gata4*-null embryos die around E9.5 with both primitive and definitive endoderm and heart defects [7, 8]. However, chimeric complementation of the extraembryonic lineage with wild type cells, allows contribution of *Gata4*-null ES cells to cardiac and definitive endoderm cell lineages without abnormality [9], suggesting that its importance in proper extraembryonic endoderm development is limited. In addition, the involvement of the group F Sox family members, Sox7 and Sox17, is suggested by their expression in the primitive endoderm [10, 11] as well as the inability to derive extraembryonic endoderm (XEN) stem cells from *Sox17*-null blastocysts [11]. *Sox17*-null embryos show a defect in the primitive endoderm lineage

* Correspondence: niwa@kumamoto-u.ac.jp
[1]Laboratory for Pluripotent cell studies, RIKEN, Centre for Developmental Biology, 2-2-3, Minatojima-Minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan
[2]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
Full list of author information is available at the end of the article

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
EMBRYONIC STEM CELLS
CELL GROWTH

Received
24 September 2014

Accepted
9 February 2015

Published
16 March 2015

Correspondence and requests for materials should be addressed to H.N. (niwa@cdb.riken.jp)

Nr0b1 is a negative regulator of *Zscan4c* in mouse embryonic stem cells

Setsuko Fujii[1,4], Satomi Nishikawa-Torikai[1,4], Yoko Futatsugi[1], Yayoi Toyooka[1,2], Mariko Yamane[1], Satoshi Ohtsuka[1] & Hitoshi Niwa[1,3,4]

[1]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan, [2]Division of Embryology, National Institute for Basic Biology (NIBB), Okazaki 444-8787, Japan, [3]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe 650-0017, Japan, [4]JST, CREST, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan.

Nuclear receptor subfamily 0, group B, member 1 **(*Nr0b1*, also known as *Dax1*) is regarded as an important component of the transcription factor network that governs pluripotency in mouse embryonic stem (ES) cells. Here we generated inducible knockout ES cells for *Nr0b1* using the Cre-*loxP* system and analyzed its precise function. We succeeded in establishing the *Nr0b1*-null ES cells and confirmed their pluripotency by showing their contribution to chimeric embryos. However, they proliferated slowly with over-expression of 2-cell stage specific transcripts including *Zscan4c*, which is known to be involved in telomere elongation in ES cells. We revealed that over-expression of *Zscan4c* prevents normal self-renewal by inducing arrest at G2 phase followed by cell death and that Nr0b1 directly represses the *Zscan4c* promoter. These data indicated that *Nr0b1* is not essential to maintain pluripotency but is involved in the proper activation of 2-cell specific transcripts for self-renewal.**

Nr0b1 (also known as *Dosage-sensitive sex reversal-adrenal hypoplasia congenital on the X-chromosome gene-1: Dax1*) is a unique member of the nuclear family because it lacks the DNA binding domain and works to modulate the function of other nuclear receptors[1]. It has been well analyzed that *Nr0b1* is involved in germ cell development[2,3]. *Nr0b1* is expressed at a high level in ES cells but not in epiblast stem cells (EpiSCs), suggesting the role in the naïve state of pluripotency[4]. The expression of *Nr0b1* is regulated by the LIF signal via Jak-Stat3 pathway[5] as well as by Oct3/4[5], Nanog[6], Nr5a2[6] and Esrrb[7]. Conversely, Nr0b1 binds to Oct3/4 to inhibit its transcriptional activity and the over-expression of *Nr0b1* induces differentiation toward trophectoderm as in the case of *Oct3/4* knockout[8]. It was also reported that Nr0b1 interacts with Esrrb to inhibit its transcriptional activity[7]. Interestingly, while the biochemical analyses suggested that Nr0b1 interacts with Nr5a2 to suppress its function[9,10], it was also demonstrated that Nr0b1 cooperates with Nr5a2 and Steroid receptor RNA activator 1 (Sra1) to activate the *Oct3/4* promoter[11]. What happens if *Nr0b1* function is eliminated in ES cells? *Niakan et al* reported that either knock-down of *Nr0b1* by siRNA or knock-out of *Nr0b1* by the Cre-*lox* system induces differentiation of ES cells[12]. This is consistent with the second report by *Khalfallah et al* showing that siRNA-mediated knock-down of *Nr0b1* causes multi-lineage differentiation[13]. However, in both cases, the primary effect of the withdrawal of *Nr0b1* on the pluripotency-associated transcription factor network was not well analyzed. Here we generated an inducible knockout ES cell line of *Nr0b1* with the Cre-*lox* system to examine its precise role to regulate the transcription factor network. Our data indicated that *Nr0b1* is dispensable for maintaining pluripotency but is involved in the transcriptional regulation of 2-cell specific genes in ES cells.

Results

Establishment of inducible knockout ES cell lines for *Nr0b1*. The *Nr0b1* gene consists of 2 exons (Fig. 1a). It has been reported that the deletion of exon 2 results in functional ablation. We made a knockout vector in which two *loxP* sites were inserted in intron 1 and the 3′ of exon 2, with a *PGKpacΔtkpA* cassette flanked by *Frt* sites inserted adjacent to the 5′ end of the 3′ *loxP* site (Fig. 1a). The linearized knockout vector was introduced into male ES cells by electroporation followed by the selection with puromycin. As a result we obtained multiple clones with correct homologous recombination event confirmed by combinations of long-range genomic PCR (Fig. 1b), designated as $Nr0b1^{flFrt/Y}$ ES cells. Then the expression vector of the FLP recombinase (FLPe)[14] was transiently transfected by lipofection followed by the selection with gancyclovir, resulting in the generation of ES cells in which the *PGKpacΔtkpA* cassette was excised, designated as $Nr0b1^{fl-/Y}$ ES cells. Then the piggy-bac vectors for

 | Development (2015) 142, 431-437 doi:10.1242/dev.112375

RESEARCH REPORT STEM CELLS AND REGENERATION

The differential activation of intracellular signaling pathways confers the permissiveness of embryonic stem cell derivation from different mouse strains

Satoshi Ohtsuka[1] and Hitoshi Niwa[1,2,3,*]

ABSTRACT

The requirement of leukemia inhibitory factor (LIF) for the establishment and maintenance of mouse embryonic stem cells (ESCs) depends on the genetic background of the ESC origin. To reveal the molecular basis of the strain-dependent function of LIF, we compared the activation of the intracellular signaling pathways downstream of LIF in ESCs with different genetic backgrounds. We found that the JAK-Stat3 pathway was dominantly activated in ESCs derived from 'permissive' mouse strains (129Sv and C57BL6), whereas the MAP kinase pathway was hyperactivated in ESCs from 'non-permissive' strains (NOD, CBA and FVB). Artificial activation of Stat3 supported stable self-renewal of ESCs from non-permissive strains. These data suggest that the difference in the balance between the two intracellular signaling pathways underlies the differential response to LIF.

KEY WORDS: LIF signaling, MAP kinase, Stat3, Embryonic stem cell, Signal responsiveness

INTRODUCTION

Mouse embryonic stem cells (ESCs) were first established in fetal calf serum (FCS)-containing medium with mouse embryonic fibroblasts (MEF) feeder cells (Evans and Kaufman, 1981; Martin, 1981). The cytokine leukemia inhibitory factor (LIF) was identified as the activator to support self-renewal (Smith, et al., 1988). Supplementation of LIF into FCS-containing medium (FCS/LIF) allowed stable self-renewal of ESCs derived from 129 strains without MEF (Nichols et al., 1990). Combination of MEF with FCS/LIF supported ESCs with other genetic backgrounds than 129, but most of these were unstable in long-term culture (Kawase et al., 1994). Serum-free culture containing inhibitors for glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (MAPK) (2i) provided greatly improved culture conditions for any mouse strain (Ying et al., 2008; Nichols et al., 2009). Combination of 2i with LIF (2iLIF) was more suitable than 2i alone (Kiyonari et al., 2010). The establishment of ESCs from different genetic backgrounds in 2iLIF allowed us to revisit the question why LIF is sufficient to support self-renewal of ESCs derived from limited strains. Here, we demonstrate how ESCs from various genetic backgrounds respond to the LIF signal by assessing the quantitative balance in the activation of the intracellular signaling pathways.

[1]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology (CDB), Minatojima-minamimachi 2-2-3, Chuo-Ku, Kobe 650-0047, Japan. [2]CREST (Core Research for Evolutional Science and Technology), Japan Science Technology Agency, Honcho 4-1-8, Kawaguchi, Saitama 332-0012, Japan. [3]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe 6500017, Japan.

*Author for correspondence (niwa@cdb.riken.jp)

Received 6 May 2014; Accepted 5 December 2014

RESULTS AND DISCUSSION

Comparison of the self-renewing abilities of ESCs derived from different strains

Previous reports indicated that there are two types of mouse strains: strains permissive for the establishment of ESCs in FCS/LIF or FCS/LIF/MEF (129Sv, C57BL6 and BALB/c), and non-permissive strains (NOD, CBA and FVB) (Kawase et al., 1994; Brook et al., 2003; Nagafuchi et al., 1999; Cinelli et al., 2008). We established three male ESCs of each type from these six strains using 2iLIF with MEF. These ESCs continued self-renewal, with maintaining expression of pluripotency-associated genes at comparable levels (Fig. 1A) and compact colony morphologies (Fig. 1C) in 2iLIF. The ability to produce germline chimeras was confirmed in ESCs derived from 129Sv and NOD (supplementary material Fig. S1).

We then tested their characteristics in other culture conditions. ESCs were seeded in 2iLIF, followed by incubation for 24 h. Then, the medium was changed to either 2iLIF or FCS/LIF with or without inhibitors (CHIR for GSK3, PD032 for MAPK or both). After culturing for 6 days, primary colony formation was evaluated by counting colony numbers (Fig. 1B) and by assessing colony morphologies (Fig. 1C-G). The efficiency of primary colony formation was significantly reduced upon removal of one of the inhibitors (Fig. 1B). In the presence of 2i, all ESCs formed stem cell colonies, even in FCS/LIF (Fig. 1C,D). However, in FCS/LIF, ESCs derived from 129Sv and C57BL6 formed stem-cell colonies at a much higher rate than ESCs derived from the other strains (Fig. 1G). Addition of either CHIR or PD032 to FCS/LIF (Fig. 1E,F) was insufficient to support stem cell colony formation of FVB-, CBA- and NOD-ESCs, although BALB/c-ESCs formed small, compact colonies. Addition of a higher dose of LIF (10^4 units/ml) to FCS/LIF also failed to support stem cell colony formation in NOD-ESCs (supplementary material Fig. S2). These data indicate that FCS/LIF is insufficient to support self-renewal of BALB/c-, FVB-, CBA- and NOD-ESCs. BALB/c-ESCs have been previously referred to as permissive for derivation of ESCs in FCS/LIF with MEF (Baharvand and Matthaei, 2004); however, their characteristics were similar to those of non-permissive strains previously categorized (FVB, CBA and NOD) in FCS/LIF without MEF, even though the phenotypes with single inhibitors in FCS/LIF were intermediate (Fig. 1B,E,F). Therefore, hereafter we categorized 129Sv and C57BL6 as permissive, FVB, CBA and NOD as non-permissive strains, and BALB/c as intermediate strain.

Differential activation of intracellular signaling pathways by LIF in strain-dependent manner

We then tested the effect of the LIF signal on activation of the Jak-Stat3 and MAPK pathways, the positive and negative signals to

© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 2921-2923 doi:10.1242/dev.108407

SPOTLIGHT

The POU-er of gene nomenclature

Stephen R. Frankenberg[1,*], Dale Frank[2], Richard Harland[3], Andrew D. Johnson[4], Jennifer Nichols[5], Hitoshi Niwa[6], Hans R. Schöler[7], Elly Tanaka[8], Chris Wylie[9] and Joshua M. Brickman[10,*]

ABSTRACT

The pluripotency factor POU5F1 (OCT4) is well known as a key regulator of stem cell fate. Homologues of POU5F1 exist throughout vertebrates, but the evolutionary and functional relationships between the various family members have been unclear. The level to which function has been conserved within this family provides insight into the evolution of early embryonic potency. Here, we seek to clarify the relationship between POU5F1 homologues in the vertebrate lineage, both phylogenetically and functionally. We resolve the confusion over the identity of the zebrafish gene, which was originally named *pou2*, then changed to *pou5f1* and again, more recently, to *pou5f3*. We argue that the use of correct nomenclature is crucial when discussing the degree to which the networks regulating early embryonic differentiation are conserved.

Class V POU (POUV) transcription factors are important regulators of potency, differentiation and early development in vertebrates. They constitute one of five classes of POU domain-containing proteins defined by similarity within both the homeodomain and the POU-specific domain (Rosenfeld, 1991). Mouse POU5F1 (also called OCT3 or OCT4) was the first class V member identified (Lenardo et al., 1989; Okamoto et al., 1990; Rosner et al., 1990; Schöler et al., 1989, 1990). It is a central regulator of embryonic stem cell (ESC) pluripotency (Nichols et al., 1998; Niwa et al., 2000; Yuan et al., 1995) and the most essential of the four factors originally identified as being able to induce reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Yu et al., 2007). Since the initial characterisation of POU5F1, homologues have been identified in many vertebrates, including frog, chicken, axolotl, teleost fishes and sturgeon.

As more POUV genes were identified, it became apparent that the POUV family has a complex evolutionary history. Some eutherian mammals possess a second, single-exon POUV gene, *POU5F2* (previously called *SPRM-1*), which has a role in spermatogenesis (Andersen et al., 1993; Pearse et al., 1997) and presumably arose in an ancestral eutherian by retroviral insertion of a copy of *POU5F1*. Marsupial and monotreme genomes contain two POUV genes: *POU5F1* and another homologue now called *POU5F3*. Both the marsupial POUV genes (*POU5F1* and *POU5F3*) are expressed in early development in domains similar to those described for *Pou5f1* in the mouse (Frankenberg et al., 2010, 2013). Notably, some vertebrate lineages have orthologues of both *POU5F1* and *POU5F3*, whereas others (including eutherian mammals) have only *POU5F1* and others only *POU5F3* (Fig. 1). The basis for this pattern of evolution is unclear, but is possibly due to distinct roles for each paralogue that became redundant in a taxon-specific manner.

In zebrafish, a POUV gene was identified and originally, albeit confusingly, named *pou2* (Takeda et al., 1994). With the availability of the complete sequence, it became clear that *pou2* encodes a class V POU protein (Burgess et al., 2002) and, based on an assumption of orthology with mammalian *POU5F1* (Lunde et al., 2004), zebrafish *pou2* was subsequently renamed *pou5f1*. However, the zebrafish gene is not a true orthologue of mammalian *POU5F1* but is instead more closely related to mammalian *POU5F3*, as indicated by conservation of both synteny and sequence (Frankenberg et al., 2010; Frankenberg and Renfree, 2013; Niwa et al., 2008; Tapia et al., 2012). Moreover, a recent study (Frankenberg and Renfree, 2013) has now demonstrated unequivocally that the gene duplication event giving rise to *POU5F1* and *POU5F3* occurred before the divergence of extant cartilaginous and bony fishes, showing conclusively that the zebrafish gene is a true *POU5F3* orthologue. On the weight of this evidence, the Zebrafish Nomenclature Committee re-renamed the zebrafish gene *pou5f3*, reflecting its proper place in the POUV family. We now support the application of this nomenclature to all vertebrate orthologues of *POU5F3* (see Table 1).

The degree to which the function of the POUV proteins is conserved in evolution is variable. This has been tested by the capacity of different POUV proteins to rescue the loss of endogenous POU5F1 activity in ESC self-renewal (Hammachi et al., 2012; Morrison and Brickman, 2006) or in the generation of iPSCs (together with three other mammalian factors) (Tapia et al., 2012). Based on these assays, orthologues of POU5F1 and POU5F3 show varying degrees of functional conservation in inducing pluripotency and supporting self-renewal. In particular, in *Xenopus*, which has three *POU5F3* genes (*pou5f3.1*, *pou5f3.2* and *pou5f3.3*) that presumably arose by tandem duplication, the expression pattern and activity of these genes have diversified such that only one of them – *pou5f3.1* – is expressed in primordial germ cells and has 'OCT4-like' activity in both reprogramming and ESC self-renewal (Livigni et al., 2013; Venkatarama et al., 2010; Tapia et al., 2012). Other *POU5F3* genes, including the two other *Xenopus* genes, have varying degrees of OCT4-like activity in such assays, but it is notable that zebrafish *pou5f3* has little activity in either reprogramming or the support of *Pou5f1* mutant ESCs (Lavial et al., 2007; Morrison and Brickman, 2006; Niwa

[1]Department of Zoology, University of Melbourne, Melbourne, VIC 3010, Australia. [2]Department of Biochemistry, The Rappaport Family Institute for Research in the Medical Sciences, Faculty of Medicine, Technion - Israel Institute of Technology, Haifa 31096, Israel. [3]University of California Berkeley, Department of Molecular & Cell Biology, 142 Life Sciences Addition # 3200, Berkeley, CA 94720-3200, USA. [4]School of life Sciences, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK. [5]Wellcome Trust - Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK. [6]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology (CDB), 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 6500047, Japan. [7]Max Planck Institute for Molecular Biomedicine, Röntgenstraße 20, Münster 48149, Germany. [8]DFG Research Center for Regenerative Therapies – CRTD, Technische Universität Dresden, Fetscherstrasse 105, Dresden 01307, Germany. [9]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45227, USA. [10]The Danish Stem Cell Centre (DanStem), University of Copenhagen, Blegdamsvej 3B, Copenhagen N DK-2200, Denmark.

*Authors for correspondence (srfr@unimelb.edu.au; joshua.brickman@sund.ku.dk)

© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 3848-3858 doi:10.1242/dev.105981

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Small molecule-directed specification of sclerotome-like chondroprogenitors and induction of a somitic chondrogenesis program from embryonic stem cells

Jiangang Zhao[1,*], Songhui Li[2,‡], Suprita Trilok[1], Makoto Tanaka[2,¶], Vanta Jokubaitis-Jameson[2,§], Bei Wang[2,‡], Hitoshi Niwa[3] and Naoki Nakayama[1,2,4,]**

ABSTRACT

Pluripotent embryonic stem cells (ESCs) generate rostral paraxial mesoderm-like progeny in 5-6 days of differentiation induced by Wnt3a and Noggin (Nog). We report that canonical Wnt signaling introduced either by forced expression of activated β-catenin, or the small-molecule inhibitor of Gsk3, CHIR99021, satisfied the need for Wnt3a signaling, and that the small-molecule inhibitor of BMP type I receptors, LDN193189, was able to replace Nog. Mesodermal progeny generated using such small molecules were chondrogenic *in vitro*, and expressed trunk paraxial mesoderm markers such as Tcf15 and Meox1, and somite markers such as Uncx, but failed to express sclerotome markers such as Pax1. Induction of the osteochondrogenically committed sclerotome from somite requires sonic hedgehog and Nog. Consistently, Pax1 and Bapx1 expression was induced when the isolated paraxial mesodermal progeny were treated with SAG1 (a hedgehog receptor agonist) and LDN193189, then Sox9 expression was induced, leading to cartilaginous nodules and particles in the presence of BMP, indicative of chondrogenesis via sclerotome specification. By contrast, treatment with TGFβ also supported chondrogenesis and stimulated Sox9 expression, but failed to induce the expression of Pax1 and Bapx1. On ectopic transplantation to immunocompromised mice, the cartilage particles developed under either condition became similarly mineralized and formed pieces of bone with marrow. Thus, the use of small molecules led to the effective generation from ESCs of paraxial mesodermal progeny, and to their further differentiation *in vitro* through sclerotome specification into growth plate-like chondrocytes, a mechanism resembling *in vivo* somitic chondrogenesis that is not recapitulated with TGFβ.

KEY WORDS: Pluripotent stem cells, Paraxial mesoderm, Sclerotome, Chondrogenesis, Mouse

INTRODUCTION

Repair of large bone defects and damaged cartilage remain a significant clinical challenge. The combination of therapy with mesenchymal stromal cells (MSCs) and the use of biodegradable biomimetic scaffolds appears to be a promising therapeutic approach, as MSCs from bone marrow or fat tissue can be both osteogenic and chondrogenic under certain conditions *in vitro* and *in vivo*. However, the difficulty of the approach lies in controlling the clinical outcome. As bone and cartilage are naturally formed during embryogenesis, we hypothesize that one way to alleviate the difficulty is to use embryonic osteochondro-progenitors instead of adult cells.

For human embryonic cells, pluripotent stem cells (PSCs) [i.e. embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)] are thus far the only practical source. PSC culture is scalable, and *in vitro* differentiation of PSCs can mimic early embryogenesis (Murry and Keller, 2008; Nishikawa et al., 2007) such that large numbers of a particular type of embryonic cell can be obtained under the appropriate conditions of differentiation to mimic embryogenesis. Furthermore, the wealth of information emanating from developmental biology studies is expected to aid in the discovery and refinement of such conditions. However, although ESCs have been shown to generate mesenchymal progeny capable of developing cartilage *in vitro* (Nakayama and Umeda, 2011), the resultant chondrogenic activities were generally poor, and with the exception of ours and a few others (Craft et al., 2013; Oldershaw et al., 2010; Tanaka et al., 2009; Umeda et al., 2012), most reports did not define the developmental origin of the mesenchymal cells generated. The osteochondro-progenitors that develop during embryogenesis are limb bud mesenchyme (derived from lateral plate mesoderm) responsible for limb bone and cartilage generation, sclerotome (from somite/rostral paraxial mesoderm) responsible for rib, vertebral joint, intervertebral disc and vertebral body formation, and ectomesenchyme (from cranial neural crest) responsible for craniofacial bone and cartilage generation. These progenitors are differentially specified through the action of protein factors such as Wnt, and members of the transforming growth factor (TGF)β-superfamily: Nodal and bone morphogenetic protein (BMP), and their inhibitors. Therefore, to make use of such knowledge to improve and refine the condition for osteochondro-progenitor development, PSC differentiation should be directed toward a particular lineage.

In the first step toward testing our hypothesis, we have previously demonstrated that somitic/rostral presomitic mesoderm-like progeny (hereafter referred to as 'rostral paraxial mesoderm') is specified from mouse and human ESCs and is enriched in the Flk1(Kdr)$^{-}$Pdgfrα^{+} cell fraction of embryoid bodies (EBs) generated in a chemically defined medium (CDM) (Tanaka et al., 2009; Umeda et al., 2012). The specification process is strictly dependent on the activation of Wnt signaling and suppression of BMP signaling (Craft et al., 2013; Tanaka et al., 2009; Umeda et al., 2012).

Considering future application to humans, it is important to determine whether the PSC progeny developed *in vitro* behave in the

[1]Institute of Molecular Medicine, The University of Texas Health Science Center Medical School at Houston, Houston, TX 77030, USA. [2]Australian Stem Cell Centre, Monash University, Clayton, Victoria 3800, Australia. [3]RIKEN Center for Developmental Biology, Kobe 650-0047, Japan. [4]Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria 3800, Australia. *Present address: The University of Texas Medical Branch at Galveston, Galveston, TX 77555, USA. [‡]Present address: CSIRO, Clayton, Victoria 3168, Australia. [§]Present address: The University of Melbourne, Parkville, Victoria 3052, Australia. [¶]Present address: Department of Orthopaedic Surgery, Osaka Police Hospital, Osaka 543-0035, Japan.

**Author for correspondence (naoki.nakayama@uth.tmc.edu)

Received 13 November 2013; Accepted 16 August 2014

FEBS Letters 588 (2014) 1128–1135

ELSEVIER

FEBS Letters

journal homepage: www.FEBSLetters.org

1964–2014

The C-terminal region of *Xpc* is dispensable for the transcriptional activity of Oct3/4 in mouse embryonic stem cells

Shunsuke Ito [a,b], Mariko Yamane [a], Satoshi Ohtsuka [a], Hitoshi Niwa [a,b,c,*]

[a] *Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan*
[b] *Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe 650-0017, Japan*
[c] *JST, CREST, Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan*

ARTICLE INFO

Article history:
Received 25 November 2013
Revised 8 February 2014
Accepted 14 February 2014
Available online 4 March 2014

Edited by Ivan Sadowski

Keywords:
Embryonic stem cell
Pluripotency
Transcription

ABSTRACT

The transcription factor Oct3/4 is essential to maintain pluripotency in mouse embryonic stem (ES) cells. It was reported that the Xpc DNA repair complex is involved in this process. Here we examined the role of Xpc on the transcriptional activation of the target genes by Oct3/4 using the inducible knockout strategy. We found that the removal of the C-terminal region of Xpc, including the interaction sites with Rad23 and Cetn2, showed faint impact on the gene expression profile of ES cells and the functional *Xpc*-Δ*C* ES cell lines retained proper gene expression profile as well as pluripotency to contribute chimeric embryos. These data indicated that the C-terminal region of Xpc is dispensable for the transcriptional activity of Oct3/4 in mouse ES cells.

1. Introduction

Tissue-specific transcription factors (TFs) bind to the specific sites of the genome in a sequence-specific manner and control the transcription of the target genes positively and negatively. For the activation of transcription, TFs cooperate with coactivators to recruit the general transcription machinery to the promoter, resulting in loading RNA polymerase II (polII) to initiate transcription. As one of the coactivators, the mediator complex (Mediator) has a pivotal role to bridge the TFs binding to their target sites and the general transcriptional machinery [1,2]. Recent finding indicated that Mediator forms a large complex with multiple TFs bound to a large genomic region to direct the specific gene expression, designated as a super enhancer [3]. The system composed of these factors direct tissue-specific expression of the genes from the genome in cooperation with the epigenetic regulation.

In mouse ES cells, several key TFs have been identified to determine the specific gene expression pattern coupled with pluripotency [4]. The POU family transcription factor Oct3/4 (encoded by *Pou5f1*) is a pivotal TF since it binds to several pluripotency-associated genes to activate their transcription [5] and its elimination causes the cease of self-renewal and the loss of pluripotency [6]. For the activation of the target genes, Oct3/4 mainly cooperates with the Sry-related HMG-box transcription factor Sox2 [7]. Oct3/4 directly interacts with Sox2 and these TFs in the heterodimer recognize the specific sequences adjoining each other without space or with a space of a few base pairs on the target DNA [8]. Mediator works to transduce the binding of Oct3/4–Sox2 complex to the distal binding site for the recruitment of polII at the promoter [9]. The requirement of Mediator to keep the Oct3/4-dependent gene expression pattern was demonstrated by the knockdown of the component of *Mediator* [9]. It was reported that the DNA repair complex containing Xpc also act as a coactivator of Oct3/4 [10]. The Xpc complex consists of Xpc, Rad23B and Cetn2 and mediates the nucleotide excision repair [11]. In mouse ES cells, the complex directly interacts with Oct3/4 to mediate the transcriptional activation of the target genes such as *Nanog* [10]. It was demonstrated that the simultaneous knock-down of *Xpc*, *Rad23b* and *Cetn2* compromised self-renewal of ES cells with down-regulation of the target genes of Oct3/4 [10]. However, it was also demonstrated that the *Xpc* knockout mice develop to term [12] and the *Xpc*-null ES cells can be established from the *Xpc*-null blastocysts [13], which sounds controversial to the function of the Xpc complex proposed in ES cells.

It has sometimes been observed that the knock-down of a specific gene expression by siRNA or shRNA gives different effects from the knockout of the same gene by the gene-targeting via homologous recombination. For example, several reports demonstrated that the knock-down of *Rex1* (encoded by *Zfp42*)

* Corresponding author at: Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan. Fax: +81 78 306 1929.
E-mail address: niwa@cdb.riken.jp (H. Niwa).

http://dx.doi.org/10.1016/j.febslet.2014.02.033
0014-5793/

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Genetics & Development

The pluripotency transcription factor network at work in reprogramming

Hitoshi Niwa[1,2]

Pluripotency-associated transcription factors possess a pivotal role to maintain pluripotency in pluripotent stem cells as well as to induce pluripotency in somatic cells. They direct specific pattern of gene expression from the genome by co-operating with the genetic and epigenetic mechanisms. Recent findings revealed that these mechanisms possess unique features in pluripotent stem cells, which is different from that in somatic cells either qualitatively and quantitatively. To reprogram somatic cells, pluripotency-associated transcription factors should modulate the co-operating machineries to establish the optimal environment for their function to maintain pluripotency-associated transcription factor network.

Addresses
[1] Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ko, Kobe 650-0047, Japan
[2] CREST, Sanbancho, Chiyodaku, Tokyo 1020075, Japan

Corresponding author: Niwa, Hitoshi (niwa@cdb.riken.jp)

Current Opinion in Genetics & Development 2014, **28**:25–31

This review comes from a themed issue on **Cell reprogramming, regeneration and repair**

Edited by **José CR Silva and Renee A Reijo Pera**

For a complete overview see the Issue and the Editorial

Available online 28th August 2014

http://dx.doi.org/10.1016/j.gde.2014.08.004

0959-437X/

Introduction

Pluripotency is defined as an ability of a cell to differentiate into all somatic cell types. It is observed in limited numbers of cells in pre- and early-post implantation embryos in developmental context. Once cultured *in vitro*, these cells derive pluripotent stem cells with continuous self-renewal. Somatic cells including stem cells of post-natal mice never give rise to pluripotent stem cells without reprogramming. Reprogramming is an event that resets the genetic and epigenetic information in a genome-wide manner. Reprogramming of somatic cell nuclei to totipotent state is achieved by the nuclear transfer technique [1], whereas reprogramming of somatic cells into pluripotent stem cells is induced by the over-expression of pluripotency-associated transcription factors [2]. It has been reported that several molecules are involved in the reprogramming event, but it is still unclear how the pluripotency-associated transcription factor network is finally established to maintain the pluripotent state induced by reprogramming. Here the recent findings were reviewed to understand the reprogramming event from the point of view of the transcription factor network transition.

Transcription factor network in pluripotent stem cells

Mouse embryonic stem (ES) cells continue self-renewal in the presence of leukemia inhibitory factor (LIF). LIF signal integrates into ES cells and activates three different intracellular signal transduction pathways, which target the expressions and activities of multiple pluripotency-associated transcription factors such as *Klf4*, *Tbx3* and *Tfcp2l1* [3,4]. Wnt signal cooperates with LIF signal to promote the maintenance of pluripotency via transcriptional activation of the transcription factor *Esrrb* [5]. These transcription factors form a network in which they regulate each other and process the signal integration to direct self-renewal (or to block differentiation which is a default) by maintaining the expressions of the core transcription factors that consist of *Oct3/4* and *Sox2* (Figure 1). The core transcription factors compose auto-regulatory loops to maintain their own expressions and regulate the components of the pluripotency-associated transcription factor network in a signal integration-dependent manner [6]. Moreover, transcription factor network possesses a positive feedback to amplify LIF signal integration via transcriptional repression of *Socs3*, a negative regulator of Stat3 activation, by Nanog [7]. The essence of the function of the transcription factor network is the maintenance of pluripotency.

Transcriptional activation by transcription factors

Mediating the functions of pluripotency-associated transcription factors involves many parameters that define their abilities to activate or repress the target gene expression. This includes the interactions among the transcription factors, transcriptional co-factors and general transcription factors.

Interaction of the transcription factors

It has been reported that Oct3/4 and Sox2 form a heterodimer to activate the transcription of the target genes including their own. The direct interactions of transcription factors define their specific functions [6]. For example, Sox2 is expressed in a variety of cell types during development including trophoblast stem (TS) cells [8]. In TS cells, Sox2 functions to mediate the mitogen-activated protein kinase (MAPK) signal to maintain self-renewal

OPEN ACCESS Freely available online

PLOS ONE

Zscan4 Is Regulated by PI3-Kinase and DNA-Damaging Agents and Directly Interacts with the Transcriptional Repressors LSD1 and CtBP2 in Mouse Embryonic Stem Cells

Michael P. Storm[1☯], Benjamin Kumpfmueller[1,3☯], Heather K. Bone[1], Michael Buchholz[2], Yolanda Sanchez Ripoll[1], Julian B. Chaudhuri[3], Hitoshi Niwa[4], David Tosh[2], Melanie J. Welham[1*¤]

1 Centre for Regenerative Medicine and Departments of Pharmacy & Pharmacology, University of Bath, Bath, United Kingdom, **2** Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom, **3** Department of Chemical Engineering, University of Bath, Bath, United Kingdom, **4** RIKEN Centre for Developmental Biology, Kobe, Hyogo, Japan

Abstract

The Zscan4 family of genes, encoding SCAN-domain and zinc finger-containing proteins, has been implicated in the control of early mammalian embryogenesis as well as the regulation of pluripotency and maintenance of genome integrity in mouse embryonic stem cells. However, many features of this enigmatic family of genes are poorly understood. Here we show that undifferentiated mouse embryonic stem cell (ESC) lines simultaneously express multiple members of the Zscan4 gene family, with Zscan4c, Zscan4f and Zscan4-ps2 consistently being the most abundant. Despite this, between only 0.1 and 0.7% of undifferentiated mouse pluripotent stem cells express Zscan4 protein at a given time, consistent with a very restricted pattern of Zscan4 transcripts reported previously. Herein we demonstrate that Zscan4 expression is regulated by the p110α catalytic isoform of phosphoinositide 3-kinases and is induced following exposure to a sub-class of DNA-damage-inducing agents, including Zeocin and Cisplatin. Furthermore, we observe that Zscan4 protein expression peaks during the G2 phase of the cell cycle, suggesting that it may play a critical role at this checkpoint. Studies with GAL4-fusion proteins suggest a role for Zscan4 in transcriptional regulation, further supported by the fact that protein interaction analyses demonstrate that Zscan4 interacts with both LSD1 and CtBP2 in ESC nuclei. This study advances and extends our understanding of Zscan4 expression, regulation and mechanism of action. Based on our data we propose that Zscan4 may regulate gene transcription in mouse ES cells through interaction with LSD1 and CtBP2.

Citation: Storm MP, Kumpfmueller B, Bone HK, Buchholz M, Sanchez Ripoll Y, et al. (2014) Zscan4 Is Regulated by PI3-Kinase and DNA-Damaging Agents and Directly Interacts with the Transcriptional Repressors LSD1 and CtBP2 in Mouse Embryonic Stem Cells. PLoS ONE 9(3): e89821. doi:10.1371/journal.pone.0089821

Editor: Michael Kyba, University of Minnesota, United States of America

Received September 1, 2013; **Accepted** January 23, 2014; **Published** March 3, 2014

Funding: Funded by grant G0801108 from The Medical Research Council (http://www.mrc.ac.uk/index.htm). BK and MB were supported by the Marie Curie Early Stage Training programme MEST-CT-2005-019822. YSR was the recipient of an MRC Capacity Building Studentship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: M.J.Welham@bath.ac.uk

☯ These authors contributed equally to this work.

¤ Current address: Biotechnology and Biological Sciences Research Council, Polaris House, Swindon, United Kingdom

Introduction

Embryonic stem cells (ESCs) self-renew and are pluripotent, meaning they can differentiate into all cells comprising an adult organism [1]. These properties have made ESCs an attractive source of differentiated cell types for use in both drug discovery and regenerative medicine. While the potential of ESCs has been widely recognized, it is imperative that the mechanisms regulating their self-renewal, pluripotency and stability are better understood, to ensure their efficacy and safety.

The extrinsic factors, signaling pathways and transcription factor networks that contribute to maintenance of mouse ESC self-renewal and pluripotency, referred to as the 'ESC state', have been extensively studied [1,2,3,4]. Leukaemia inhibitory factor (LIF) and Bone morphogenetic protein 4 (BMP4) are the key cytokines required for maintenance of ESC self-renewal in culture, acting via the Jak-Stat3 and Smad-Id pathways respectively [5,6,7]. Inhibition of glycogen synthase kinase 3 (Gsk-3), which mimics both activation of the Wnt pathway and growth factor-induced PI3K signaling, can enhance mouse ESC self-renewal [8,9] and assist in maintaining the 'ground state' of mouse ESC pluripotency [10,11,12]. Inhibition of MAPK signaling, in addition to Gsk-3 inhibition (referred to as 2i conditions) is sufficient to maintain self-renewal of mouse ESCs in the absence of additional exogenous factors [10]. Phosphoinoside 3-kinase (PI3K) signaling has also been implicated in the maintenance of both mouse [13,14,15] and human ESC [16] pluripotency.

Oct4, Sox2 and Nanog are amongst the most important transcription factors that contribute to regulation of ESC pluripotency, often referred to as the 'core transcription factors' or 'master regulators' [1,17]. Other transcription factors work in concert with these core factors and include Zfx [18], Klf 2 & 4

THE COMPANY OF Biologists

RESEARCH ARTICLE STEM CELLS AND REGENERATION

Oct4 is required for lineage priming in the developing inner cell mass of the mouse blastocyst

Gloryn Chia Le Bin[1,2,‡], Silvia Muñoz-Descalzo[3,4,*,‡], Agata Kurowski[1,5,‡], Harry Leitch[1,2], Xinghua Lou[3], William Mansfield[1], Charles Etienne-Dumeau[1], Nils Grabole[5,6], Carla Mulas[1,6], Hitoshi Niwa[7], Anna-Katerina Hadjantonakis[3] and Jennifer Nichols[1,2,§]

ABSTRACT

The transcription factor Oct4 is required *in vitro* for establishment and maintenance of embryonic stem cells and for reprogramming somatic cells to pluripotency. *In vivo*, it prevents the ectopic differentiation of early embryos into trophoblast. Here, we further explore the role of Oct4 in blastocyst formation and specification of epiblast versus primitive endoderm lineages using conditional genetic deletion. Experiments involving mouse embryos deficient for both maternal and zygotic Oct4 suggest that it is dispensable for zygote formation, early cleavage and activation of Nanog expression. Nanog protein is significantly elevated in the presumptive inner cell mass of *Oct4* null embryos, suggesting an unexpected role for Oct4 in attenuating the level of Nanog, which might be significant for priming differentiation during epiblast maturation. Induced deletion of *Oct4* during the morula to blastocyst transition disrupts the ability of inner cell mass cells to adopt lineage-specific identity and acquire the molecular profile characteristic of either epiblast or primitive endoderm. Sox17, a marker of primitive endoderm, is not detected following prolonged culture of such embryos, but can be rescued by provision of exogenous FGF4. Interestingly, functional primitive endoderm can be rescued in Oct4-deficient embryos in embryonic stem cell complementation assays, but only if the host embryos are at the pre-blastocyst stage. We conclude that cell fate decisions within the inner cell mass are dependent upon Oct4 and that Oct4 is not cell-autonomously required for the differentiation of primitive endoderm derivatives, as long as an appropriate developmental environment is established.

KEY WORDS: Blastocyst, Chimaera, Nanog, Oct4 (Pou5f1), Primitive endoderm, Sox17

[1]Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK. [2]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3DY, UK. [3]Developmental Biology Program, Sloan-Kettering Institute, New York, NY 10065, USA. [4]Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK. [5]Wellcome Trust – Cancer Research UK Gurdon Institute, The Henry Wellcome Building of Cancer and Developmental Biology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK. [6]Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, UK. [7]Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 6500047, Japan.
*Present address: Biology and Biochemistry Department, University of Bath, Bath BA2 7AY, UK.
‡These authors contributed equally to this work

§Author for correspondence (jn270@cam.ac.uk)

Received 26 March 2013; Accepted 17 December 2013

INTRODUCTION

A prerequisite for the viviparous development of the mouse embryo is the capacity to generate enveloping tissues to facilitate implantation in the mother, while preserving the potential to produce a foetus. The first extra-embryonic lineage, the trophectoderm, forms the outer layer of the blastocyst. The internal population of cells, termed the inner cell mass (ICM), is protected from differentiation by expression of the POU domain transcription factor Oct4 (also known as Oct3, Oct3/4 and Pou5f1). Following zygotic deletion of *Oct4*, the blastocyst eventually differentiates into trophectoderm (Nichols et al., 1998). However, expression of trophectoderm markers such as Troma1 (keratin 8) and the homeobox transcription factor *Cdx2* is not apparent in the inside cells until after blastocyst formation, suggesting that the initial allocation of these cells to the ICM lineage occurs normally (Nichols et al., 1998; Ralston et al., 2010). The presence of Oct4 protein has been reported in developing oocytes (Schöler et al., 1989) and unfertilised eggs (Palmieri et al., 1994). To eliminate the possibility that lingering maternal Oct4 might facilitate normal gene expression during cleavage, both maternal and zygotic deletion has been performed. Interestingly, early ICM markers such as Nanog and Gata6 still localise to the inside cells in maternal-zygotic *Oct4* mutants (Frum et al., 2013; Wu et al., 2013).

After segregation of the trophectoderm, the ICM becomes partitioned into epiblast, which is the founder of the foetus, and primitive endoderm (PrE), or hypoblast, which is the source of the extra-embryonic endoderm lineage. By means of immunohistochemistry, Oct4 protein has been detected in the PrE following its segregation from the epiblast prior to implantation (Palmieri et al., 1994). Intriguingly, the fluorescence appeared to be more intense in the PrE compared with the epiblast. This led to speculation that elevation of Oct4 might be a prerequisite for PrE differentiation. This hypothesis was further endorsed by the observation that transgenic enhancement of *Oct4* expression in embryonic stem cells (ESCs) resulted in differentiation accompanied by the expression of markers of extra-embryonic endoderm (Niwa et al., 2000). A requirement for Oct4 in PrE specification *in vivo* was inferred using maternal and/or zygotic deletion (Frum et al., 2013). However, the conversion of the majority of presumptive ICM into trophectoderm before implantation in embryos lacking Oct4 (Frum et al., 2013; Nichols et al., 1998; Ralston et al., 2010) somewhat compromises the investigation of a role for Oct4 specifically in subsequent PrE differentiation and function.

Embryos lacking fibroblast growth factor (FGF) 4, a target of Oct4 (Nichols et al., 1998; Yuan et al., 1995), fail to generate PrE unless supplemented with excess FGF4 or FGF2 (Feldman et al., 1995; Kang et al., 2013). A role for FGF4 in directing differentiation of PrE has also been elegantly demonstrated by addition of high concentrations of FGF4 to embryos before blastocyst expansion

Genetic Exploration of the Exit from Self-Renewal Using Haploid Embryonic Stem Cells

Martin Leeb,[1,*] Sabine Dietmann,[1] Maike Paramor,[1] Hitoshi Niwa,[3] and Austin Smith[1,2,*]
[1]Wellcome Trust – Medical Research Council Stem Cell Institute, University of Cambridge, Tennis Court Road, CB2 1QR, Cambridge, UK
[2]Department of Biochemistry, University of Cambridge, Tennis Court Road, CB2 1GA, Cambridge, UK
[3]RIKEN Center for Developmental Biology, 650-0047 Kobe, Japan
*Correspondence: ml553@cam.ac.uk (M.L.), austin.smith@cscr.cam.ac.uk (A.S.)
http://dx.doi.org/10.1016/j.stem.2013.12.008

SUMMARY

Self-renewal circuitry in embryonic stem cells (ESCs) is increasingly defined. How the robust pluripotency program is dissolved to enable fate transition is less appreciated. Here we develop a forward genetic approach using haploid ESCs. We created libraries of transposon integrations and screened for persistent self-renewal in differentiation-permissive culture. This yielded multiple mutants in the Fgf/Erk and GSK3/Tcf3 modules known to drive differentiation and in epigenetic modifiers implicated in lineage commitment. We also identified and validated factors not previously considered. These include the conserved small zinc finger protein Zfp706 and the RNA binding protein Pum1. Pum1 targets several mRNAs for naive pluripotency transcription factors and accelerates their downregulation at the onset of differentiation. These findings indicate that the dismantling of pluripotent circuitry proceeds at multiple levels. More broadly they exemplify the power of haploid ESCs for genetic interrogation of developmental processes.

INTRODUCTION

Rodent ESCs exhibit the identity and pluripotency of naive preimplantation epiblast cells with the additional attribute of extended self-renewal (Nichols and Smith, 2012). The molecular machinery and underlying genetic circuitry that sustain ESC character during self-renewal have been extensively characterized (Young, 2011). Less studied is the process by which ESCs exit the naive state to embark upon differentiation. In contrast to the ordered program of germ layer segregation that unfolds deterministically in the embryo and is obeyed by ESCs in chimeras, differentiation in vitro is asynchronous and disorganized (Lowell et al., 2006). Identifying factors and pathways that direct developmental progression from self-renewal to lineage commitment is a challenge. A timely opportunity for application of forward genetics to dissect this complex developmental transition arises from the recent derivation of haploid mouse ESCs (Elling et al., 2011; Leeb and Wutz, 2011).

Haploid ESCs can be derived from parthenogenetic embryos generated following chemical activation of unfertilized MII oocytes. Based on molecular marker analysis and gene expression profiles, haploid ESCs cannot be distinguished from their diploid counterparts. Notably, they retain full developmental potential and give rise to germline-competent chimeras (Leeb et al., 2012). Haploid ESCs are prone to diploidization in culture but can be maintained by periodic flow cytometric purification. Mutagenesis of the haploid genome allows recessive phenotypes to be directly unmasked. Proof of principle has been shown by screens to identify mutations that confer resistance to toxic compounds (Elling et al., 2011; Leeb and Wutz, 2011). Therefore haploid ESCs could provide a powerful system for elucidating the genetic circuitry of mammalian developmental processes.

Suppression of differentiation is sufficient to allow ESC self-renewal. This can be achieved by application of two small molecules (2i) that block the inductive stimulus of fibroblast growth factor 4 (Ffg4)/mitogen activated protein kinase (MAPK) signaling and partially inhibit glycogen synthase kinase-3 (GSK3) (Ying et al., 2008). 2i may capture ESCs in a "ground state" of self-renewal by insulating the core pluripotency transcription factor circuit (Nichols and Smith, 2012). Consistent with this idea, deficiency in components that promote collapse of the pluripotency network liberates self-renewal from a requirement for 2i (Betschinger et al., 2013; Wray et al., 2011).

Importantly, capacity for proliferation in 2i is rather specific for undifferentiated ESCs and is lost early in differentiation (Betschinger et al., 2013). Thus, the ability to self-renew in 2i after a period of permissive culture provides a powerful means to identify and quantify delayed exit from the ground state. Here we combine this functional assay together with haploid ESC mutagenesis in a genetic screen for differentiation inducers.

RESULTS

A Haploid ESC Screen to Identify Genes that Promote Exit from Ground State Self-Renewal

To isolate and analyze mutant ESCs impeded in progression from self-renewal, we used a haploid reporter cell line (HRex1GFPd2) in which a destabilized version of GFP is expressed from the endogenous Rex1 (*Zfp42*) locus (Wray et al., 2011). Rex1 expression is tightly linked to naive pluripotency and is rapidly lost at the onset of differentiation. Importantly, lack of Rex1 is inconsequential for ESCs (Masui et al., 2008) and HRex1GFPd2 reporter ESCs lacking Rex1 protein contribute extensively to chimeras (Leeb et al., 2012). After withdrawal of 2i/LIF, GFP is substantially downregulated by 48 hr and <1% of cells remain positive by

Developmental Epigenetic Modification Regulates Stochastic Expression of Clustered *Protocadherin* Genes, Generating Single Neuron Diversity

Shunsuke Toyoda,[1,2] Masahumi Kawaguchi,[1] Toshihiro Kobayashi,[3,4] Etsuko Tarusawa,[2,5] Tomoko Toyama,[1] Masaki Okano,[6] Masaaki Oda,[6] Hiromitsu Nakauchi,[3,4] Yumiko Yoshimura,[5,7] Makoto Sanbo,[2,8] Masumi Hirabayashi,[2,7,8] Teruyoshi Hirayama,[1,2] Takahiro Hirabayashi,[1,2] and Takeshi Yagi[1,2,*]
[1]KOKORO-Biology Group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences
[2]Japan Science and Technology Agency-Core Research for Evolutional Science and Technology, CREST
Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan
[3]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[4]Japan Science Technology Agency, ERATO, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[5]Division of Developmental Neurophysiology, National Institute for Physiological Sciences, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan
[6]Laboratory for Mammalian Epigenetic Studies, RIKEN Center for Developmental Biology, Minatojima-minamimachi 2-2-3, Chuo-ku, Kobe, Hyogo 650-0047, Japan
[7]Department of Physiological Sciences, The Graduate University for Advanced Studies, Okazaki, Aichi 444-8585, Japan
[8]Section of Mammalian Transgenesis, Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Okazaki, Aichi 444-8585, Japan
*Correspondence: yagi@fbs.osaka-u.ac.jp
http://dx.doi.org/10.1016/j.neuron.2014.02.005

SUMMARY

In the brain, enormous numbers of neurons have functional individuality and distinct circuit specificities. Clustered Protocadherins (Pcdhs), diversified cell-surface proteins, are stochastically expressed by alternative promoter choice and affect dendritic arborization in individual neurons. Here we found that the *Pcdh* promoters are differentially methylated by the de novo DNA methyltransferase Dnmt3b during early embryogenesis. To determine this methylation's role in neurons, we produced chimeric mice from Dnmt3b-deficient induced pluripotent stem cells (iPSCs). Single-cell expression analysis revealed that individual Dnmt3b-deficient Purkinje cells expressed increased numbers of *Pcdh* isoforms; in vivo, they exhibited abnormal dendritic arborization. These results indicate that DNA methylation by Dnmt3b at early embryonic stages regulates the probability of expression for the stochastically expressed *Pcdh* isoforms. They also suggest a mechanism for a rare human recessive disease, the ICF (Immunodeficiency, Centromere instability, and Facial anomalies) syndrome, which is caused by *Dnmt3b* mutations.

INTRODUCTION

The mammalian brain contains enormous numbers of neurons that have distinct circuit specificities, and the stochastic expression of diversified cell-surface proteins is important for specifying individual neuronal identity (Yagi, 2013). In mice, each olfactory sensory neuron expresses only one of more than 1,000 odorant receptor genes, which is selected by common enhancer elements and allelic exclusion and permits the precise recognition of their axonal targets (Buck and Axel, 1991; Chess et al., 1994; Serizawa et al., 2003). In *Drosophila*, a subset of more than 19,000 isoforms of Down syndrome cell adhesion molecule (Dscam) 1 is alternatively spliced and expressed stochastically and combinatorially in each neuron; this diversity is required for self- and nonself recognition in neurite arborizations (Schmucker et al., 2000; Hattori et al., 2007; Matthews et al., 2007).

Clustered protocadherins (Pcdhs) comprise a large subfamily of the cadherin superfamily (Zipursky and Sanes, 2010; Yagi, 2013; Chen and Maniatis, 2013). In mammals, more than 50 *Pcdh* genes are organized into three gene clusters, *Pcdh-α*, *Pcdh-β*, and *Pcdh-γ*, which encode diversified transmembrane proteins that are predominantly expressed in the nervous system (Kohmura et al., 1998; Wu and Maniatis, 1999) (Figure 1A). In mice, the *Pcdh-α* and *Pcdh-γ* clusters contain, respectively, 14 and 22 variable exons encoding the extracellular, transmembrane, and membrane-proximal part of the cytoplasmic domain, and three constant region (CR) exons encode the common portion of the cytoplasmic domain. In contrast, the *Pcdh-β* cluster consists of 22 single-exon genes. Each variable exon of the clustered *Pcdhs* is transcribed from its own promoter (Tasic et al., 2002; Wang et al., 2002a).

Gene ablation experiments showed that Pcdh-α and Pcdh-γ are required for neuronal survival, synapse formation, axonal targeting, dendritic arborization, and the self-avoidance of dendrites (Wang et al., 2002b; Weiner et al., 2005; Hasegawa

© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 269-280 doi:10.1242/dev.099622

PRDM14 promotes active DNA demethylation through the Ten-eleven translocation (TET)-mediated base excision repair pathway in embryonic stem cells

Naoki Okashita[1,6,*], Yuichi Kumaki[2,*], Kuniaki Ebi[1,*], Miyuki Nishi[1], Yoshinori Okamoto[3], Megumi Nakayama[1], Shota Hashimoto[1], Tomohumi Nakamura[4], Kaoru Sugasawa[4], Nakao Kojima[3], Tatsuyuki Takada[5], Masaki Okano[2] and Yoshiyuki Seki[1,6,‡]

ABSTRACT

Ten-eleven translocation (TET) proteins oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5fC and 5caC can be excised and repaired by the base excision repair (BER) pathway, implicating 5mC oxidation in active DNA demethylation. Genome-wide DNA methylation is erased in the transition from metastable states to the ground state of embryonic stem cells (ESCs) and in migrating primordial germ cells (PGCs), although some resistant regions become demethylated only in gonadal PGCs. Understanding the mechanisms underlying global hypomethylation in naive ESCs and developing PGCs will be useful for realizing cellular pluripotency and totipotency. In this study, we found that PRDM14, the PR domain-containing transcriptional regulator, accelerates the TET-BER cycle, resulting in the promotion of active DNA demethylation in ESCs. Induction of *Prdm14* expression transiently elevated 5hmC, followed by the reduction of 5mC at pluripotency-associated genes, germline-specific genes and imprinted loci, but not across the entire genome, which resembles the second wave of DNA demethylation observed in gonadal PGCs. PRDM14 physically interacts with TET1 and TET2 and enhances the recruitment of TET1 and TET2 at target loci. Knockdown of TET1 and TET2 impaired transcriptional regulation and DNA demethylation by PRDM14. The repression of the BER pathway by administration of pharmacological inhibitors of APE1 and PARP1 and the knockdown of thymine DNA glycosylase (TDG) also impaired DNA demethylation by PRDM14. Furthermore, DNA demethylation induced by PRDM14 takes place normally in the presence of aphidicolin, which is an inhibitor of G1/S progression. Together, our analysis provides mechanistic insight into DNA demethylation in naive pluripotent stem cells and developing PGCs.

KEY WORDS: DNA demethylation, Ten-eleven translocation (TET), Embryonic stem cells, Base excision repair (BER), Mouse

[1]Department of Bioscience, School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan. [2]Laboratory for Mammalian Epigenetic Studies, RIKEN Centre for Developmental Biology, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan. [3]Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan. [4]Biosignal Research Center, and Graduate School of Science, Kobe University 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan. [5]Laboratory of Cell Engineering, Department of Pharmaceutical Sciences Ritsumeikan, University Nojihigashi 1-1-1, Kusatsu, Shiga, Japan. [6]Research Center for Environmental Bioscience, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan.
*These authors contributed equally to this work

‡Author for correspondence (yseki@kwansei.ac.jp)

Received 30 May 2013; Accepted 14 October 2013

INTRODUCTION

DNA methylation at position 5 of cytosine (5mC) is a central epigenetic modification that plays crucial roles in embryonic development, genomic imprinting and X chromosome inactivation (Suzuki and Bird, 2008). In mammals, three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) coordinately establish and maintain the DNA methylation patterns of the genome (Li et al., 1992; Okano et al., 1999). DNMT3A and DNMT3B are developmentally regulated enzymes required for *de novo* DNA methylation during postembryonic development of mice. DNA methylation patterns established by DNMT3A and DNMT3B are accurately maintained by the DNMT1-UHRF1 complex, which recognizes hemimethylated CpG in newly synthesized DNA during cell division (Okano et al., 1999; Sharif et al., 2007).

DNA demethylation can occur through passive or active mechanisms. Passive DNA demethylation is triggered by inhibition of the maintenance activity of DNA methyltransferases during *de novo* DNA synthesis in DNA replication (Wu and Zhang, 2010). By contrast, hydroxylation of 5mC to 5-hydroxymethylcytosine (5hmC), catalyzed by the ten-eleven translocation (TET) proteins is the first step of active DNA demethylation through the base excision repair (BER) pathway in mammals (Hackett et al., 2012b). Deamination of 5hmC by activation-induced cytidine deaminase (AID; AICDA – Mouse Genome Informatics) produces 5-hydroxymethyluridine (5hmU), which can serve as a substrate for BER in cytosine regeneration (Guo et al., 2011). By contrast, a recent study has shown that C and 5mC, but not 5hmC, are substrates for AID, suggesting that the function of deaminases in active DNA demethylation is limited (Nabel et al., 2012). Alternatively, 5hmC is further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are both repaired by thymine DNA glycosylase (TDG) to produce unmodified cytosine (He et al., 2011; Ito et al., 2011). Although both oxidation from 5mC to 5hmC and from 5hmC to 5fC/5caC are catalyzed by the same TET proteins, the genomic content of 5hmC is much higher than that of 5fC/5caC in embryonic stem cells (ESCs), which suggests that the conversion of 5hmC to 5fC/5caC is tightly controlled through the regulation of TET protein activity. However, the molecular mechanisms underlying the regulation of TET protein activity in active DNA demethylation remain largely unknown.

In a previous study, we found that PRDM14 is a crucial regulator for specification of germ cell fate and proper genome-wide epigenetic reprogramming (Yamaji et al., 2008). In mice, *Prdm14* is expressed not only in primordial germ cells (PGCs), but also in the inner cell mass (ICM) and ESCs (Yamaji et al., 2008). *Prdm14*-deficient mice are infertile, but viable, providing evidence that *Prdm14* expression in the ICM is not required for pluripotency in

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
DIFFERENTIATION
SELF-RENEWAL
EMBRYONIC STEM CELLS
PLURIPOTENCY

Received
3 April 2013

Accepted
27 September 2013

Published
15 October 2013

Correspondence and requests for materials should be addressed to H.N. (niwa@cdb.riken.jp)

Maintenance of pluripotency in mouse ES cells without *Trp53*

Masaki Shigeta[1], Satoshi Ohtsuka[1], Satomi Nishikawa-Torikai[1], Mariko Yamane[1], Setsuko Fujii[1], Kazuhiro Murakami[1] & Hitoshi Niwa[1,2,3]

[1]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology (CDB), 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 6500047, Japan, [2]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe, Hyogo 6500017, Japan, [3]CREST, Sanbancho, Chiyoda-ku, Tokyo, 1020075, Japan.

Tumor suppressor Trp53 works as a guardian of the genome in somatic cells. In mouse embryonic stem (ES) cells, it was reported that Trp53 represses pluripotency-associated transcription factor *Nanog* to induce differentiation. However, since *Trp53*-null mice develop to term, *Trp53* is dispensable for both the maintenance and differentiation of the pluripotent stem cell population *in vivo*, suggesting the differential functions of Trp53 in ES cells and embryos. To reveal the basis of this discrepancy, here we established a new line of *Trp53*-null ES cells by sequential gene targeting and evaluated their ability to differentiate *in vitro* and *in vivo*. We found that *Trp53*-null ES cells had defects in differentiation *in vitro* as reported previously, whereas they were able to contribute to normal development in chimeric embryos. These data indicated that the requirement of *Trp53* for maintaining and executing the ES pluripotency is not absolute.

Maintenance of the genome integrity in cells is important for keeping homeostasis of multi-cellular organisms. Tumor suppressor Trp53 is one of the most important components to protect the genome from the oncogenic mutations. It controls cell-cycle arrest, apoptosis and stem cell differentiation by activating and repressing its downstream targets[1,2]. Trp53 mainly acts as a transcription factor to activate and repress the target gene expressions. It is expressed ubiquitously in somatic cells and normally its protein product Trp53 is in rapid turnover by active degradation mediated by the E3 ubiquitin ligase Mdm2 or Mdmx. Induction of the DNA damage induces inactivation of Mdm2 that results in accumulation of Trp53 and its nuclear localization. Nuclear localized Trp53 causes arrest of cell-cycle progression and apoptosis to eliminate the cells with damaged genome from the organisms[3].

Mouse embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass of the blastocyst-stagte embryos[4,5]. They continue self-renewal in the optimal culture condition *in vitro*, which commonly contain the cytokine leukemia inhibitory factor (LIF) as a repressor of differentiation[6]. Even after a prolonged culture, their pluripotency is maintained as confirmed by injection of these ES cells into blastocyst that give rise to chimeric embryos in which ES cell-derived cells contribute to all germ layers including germ cells[7]. It was reported that Trp53 functions in a unique mode in mouse ES cells[2]. Trp53 is expressed in mouse ES cells, localized in cytoplasm and degradated in a Mdm2/Mdmx-dependent manner as found in other somatic cell types[8,9]. Induction of differentiation activates Trp53, which represses the pluripotency-associated transcription factor *Nanog*, suggesting its function to drive differentiation program properly[10]. This process could be regulated by a Trp53 deacetylase Sirt1 by controlling Trp53 subcellular localization[11] as well as by the expression of a specific isoform of Trp53, delta40p53, in ES cells[12]. Recently, Aurora kinase A was identified as a repressor of Trp53 by phospholyrating it directly, which could also be one of the mechanisms to maintain self-renewal by repressing the differentiation program induced by Trp53[13].

In contrast to these suggested functions of Trp53 in mouse ES cells, it was known that although Trp53 is activated by DNA damage in mouse ES cells, it has no ability to activate Trp53-mediated DNA damage response such as cell-cycle arrest, apoptosis or senescense as found in somatic cells[14]. This might be due to the unique cell-cycle regulation in mouse ES cells lacking the check point in transition from G1 to S phase[15]. Moreover, *Trp53*-null mice develop normally although they showed high incidence of tumor formation, indicating that the function of *Trp53* is dispensable for self-renewal and differentiation of pluripotent stem cells transiently appeared in the developmental process[16]. Why does the requirement of *Trp53* in differentiation of pluripotent stem cells look different between embryos and ES cells? The distinct role of the LIF signaling in ES cells and embryo has been well

Please cite this article in press as: Adachi et al., Context-Dependent Wiring of Sox2 Regulatory Networks for Self-Renewal of Embryonic and Trophoblast Stem Cells, Molecular Cell (2013), http://dx.doi.org/10.1016/j.molcel.2013.09.002

Molecular Cell
Article

Cell PRESS

Context-Dependent Wiring of Sox2 Regulatory Networks for Self-Renewal of Embryonic and Trophoblast Stem Cells

Kenjiro Adachi,[1,9,10,*] Itoshi Nikaido,[2,9,11] Hiroshi Ohta,[3,12] Satoshi Ohtsuka,[1] Hiroki Ura,[1] Mitsutaka Kadota,[5] Teruhiko Wakayama,[3,6] Hiroki R. Ueda,[2,4] and Hitoshi Niwa[1,7,8,*]
[1]Laboratory for Pluripotent Stem Cell Studies
[2]Functional Genomics Unit
[3]Laboratory for Genome Reprogramming
[4]Laboratory for Systems Biology
[5]Genome Resource and Analysis Unit
RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 6500047, Japan
[6]Faculty of Life and Environmental Sciences, University of Yamanashi, Yamanashi 4008510, Japan
[7]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe 6500017, Japan
[8]JST, CREST, Sanbancho, Chiyoda-ku, Tokyo, 1020075, Japan
[9]These authors contributed equally to this work
[10]Present address: Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, 48149 Münster, Germany
[11]Present address: Bioinformatics Research Unit, Advanced Center for Computing and Communication, RIKEN, Wako, Saitama 3510198, Japan
[12]Present address: Department of Anatomy and Cell Biology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 6068501, Japan
*Correspondence: kenjiro.adachi@mpi-muenster.mpg.de (K.A.), niwa@cdb.riken.jp (H.N.)
http://dx.doi.org/10.1016/j.molcel.2013.09.002

SUMMARY

Sox2 is a transcription factor required for the maintenance of pluripotency. It also plays an essential role in different types of multipotent stem cells, raising the possibility that Sox2 governs the common stemness phenotype. Here we show that Sox2 is a critical downstream target of fibroblast growth factor (FGF) signaling, which mediates self-renewal of trophoblast stem cells (TSCs). Sustained expression of *Sox2* together with *Esrrb* or *Tfap2c* can replace FGF dependency. By comparing genome-wide binding sites of Sox2 in embryonic stem cells (ESCs) and TSCs combined with inducible knockout systems, we found that, despite the common role in safeguarding the stem cell state, Sox2 regulates distinct sets of genes with unique functions in these two different yet developmentally related types of stem cells. Our findings provide insights into the functional versatility of transcription factors during embryogenesis, during which they can be recursively utilized in a variable manner within discrete network structures.

INTRODUCTION

The transcriptional output of a given cell type is controlled by unique combinations of transcription factors under the control of extrinsic signals that can modulate the expression and activity of transcription factors, forming a gene regulatory network that dictates a specific cellular phenotype. Tissue-specific transcription factors play deterministic roles in cell-type specification, which is manifested as lineage reprogramming by forced expression of such transcription factors (Graf and Enver, 2009; Zhou and Melton, 2008). Sox2 is one such transcription factor required for the maintenance of pluripotent stem cells in vivo (Avilion et al., 2003) and in vitro (Masui et al., 2007) and for the induction of pluripotency (Takahashi and Yamanaka, 2006). However, it is also preferentially expressed in neural, retinal, and trophoblast stem cells (TSCs) (Avilion et al., 2003; Pevny and Nicolis, 2010), suggesting a possible role for Sox2 in governing a common stemness phenotype.

In embryonic stem cells (ESCs), Sox2 forms a heterodimer with Oct3/4 (also known as Pou5f1) on DNA with the OCT-SOX composite motifs, and these factors cooperatively activate pluripotency-related target genes such as *Nanog*, *Fgf4*, *Utf1*, *Lefty1*, and *Fbxo15*, as well as their own expression (Nakatake et al., 2006, and references therein). *Oct3/4*-knockout ESCs are differentiated along the trophoblast lineage in a highly homogeneous manner (Niwa et al., 2000). In contrast, the loss of *Sox2* causes differentiation of ESCs accompanied by upregulation of markers for trophoblast and embryonic germ layers, although artificial maintenance of *Oct3/4* from the transgene can sustain self-renewal and pluripotency of *Sox2*-null ESCs (Masui et al., 2007), suggesting that the unique function of Sox2 may be to maintain *Oct3/4* expression. These two core transcription factors, along with *Nanog*, form an interconnected and hierarchical network downstream of the leukemia inhibitory factor (LIF)-Stat3 and LIF-phosphatidylinositol 3-kinase (PI3K) signaling

Nakatake *et al. BMC Biotechnology* 2013, **13**:64
http://www.biomedcentral.com/1472-6750/13/64

BMC Biotechnology

METHODOLOGY ARTICLE Open Access

Kinetics of drug selection systems in mouse embryonic stem cells

Yuhki Nakatake[1,2], Setsuko Fujii[1,5], Shinji Masui[1,3], Toshimi Sugimoto[1,4], Satomi Torikai-Nishikawa[1], Kenjiro Adachi[1] and Hitoshi Niwa[1,4,5*]

Abstract

Background: Stable expression of transgenes is an important technique to analyze gene function. Various drug resistance genes, such as *neo, pac, hph, zeo, bsd,* and *hisD,* have been equally used as selection markers to isolate a transfectant without considering their dose-dependent characters.

Results: We quantitatively measured the variation of transgene expression levels in mouse embryonic stem (mES) cells, using a series of bi-cistronic expression vectors that contain *Egfp* expression cassette linked to each drug resistant gene via *IRES* with titration of the selective drugs, and found that the transgene expression levels achieved in each system with this vector design are in order, in which *pac* and *zeo* show sharp selection of transfectants with homogenously high expression levels. We also showed the importance of the choice of the drug selection system in gene-trap or gene targeting according to this order.

Conclusions: The results of the present study clearly demonstrated that an appropriate choice of the drug resistance gene(s) is critical for a proper design of the experimental strategy.

Keywords: Transgene, Expression, Marker, Gene targeting, Vector

Background

The introduction of exogenous transgene cassettes into culture cells to direct their expressions is an important strategy in molecular biology to analyze the functions of the genes. However, a simple introduction of the DNA fragment into cells by either electroporation or lipofection results in its stable integration into the genome of the host cells only at a low frequency. Therefore, it is always required to select the cells carrying the integrated copies of the transgenes by using dominant selection markers. The combinations of the antibiotics that kill the mammalian cells and the genes that establish the resistance against them have been preferentially applied for this purpose: such as *neomycin phosphotransferase II* from transposon *Tn5* (designated as *neo* in this paper) against the neomycin derivative G418, *puromycin N-acetyltransferase* from *Streptomyces alboniger* (*pac*) against puromycin, *hygromycin B phosphotransferase* from *Escherichia coli* (*hph*) aginst hygromycin B, *Streptoalloteichus hindustanus ble* (*Sh ble*: designated as *zeo* in this paper) against the bleomycin derivative zeocin, *blasticidin S deaminase* from *Aspergillus terreus* (*bsd*) against blasticidin S, and *histidinol dehydrogenase* from *Salmonella typhimurium* (*hisD*) against histidinol [1-6]. These drugs and the resistance genes have equally been regarded as dominant selection markers that reflect the introduction of the transgenes into mammalian cells. Transfection of drug resistance genes together with transgenes, each in separate expression cassette, to obtain stable transfectants has been a commonly used method. However, in this strategy, the drug resistance does not always appropriately reflect the expression level of the transgene because generally the stable expression levels of exogenous expression cassettes are highly sensitive to thier sites of integration, as a result of the local chromatin environment when the transgenes are randomly integrated into the host genome [7], which affect the expression levels of the drug resistance gene cassette and the transgene cassette separately.

* Correspondence: niwa@cdb.riken.jp
[1]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
[4]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe 650-0017, Japan
Full list of author information is available at the end of the article

PLOS | GENETICS

DNA Methylation Restricts Lineage-specific Functions of Transcription Factor Gata4 during Embryonic Stem Cell Differentiation

Masaaki Oda[1☯¤a], Yuichi Kumaki[1☯¤b], Masaki Shigeta[2☯], Lars Martin Jakt[3¤c], Chisa Matsuoka[1], Akiko Yamagiwa[1], Hitoshi Niwa[2], Masaki Okano[1]*

1 Laboratory for Mammalian Epigenetic Studies, Center for Developmental Biology, RIKEN, Kobe, Japan, **2** Laboratory for Pluripotent Cell Studies, Center for Developmental Biology, RIKEN, Kobe, Japan, **3** Laboratory for Stem Cell Biology, Center for Developmental Biology, RIKEN, Kobe, Japan

Abstract

DNA methylation changes dynamically during development and is essential for embryogenesis in mammals. However, how DNA methylation affects developmental gene expression and cell differentiation remains elusive. During embryogenesis, many key transcription factors are used repeatedly, triggering different outcomes depending on the cell type and developmental stage. Here, we report that DNA methylation modulates transcription-factor output in the context of cell differentiation. Using a drug-inducible Gata4 system and a mouse embryonic stem (ES) cell model of mesoderm differentiation, we examined the cellular response to Gata4 in ES and mesoderm cells. The activation of Gata4 in ES cells is known to drive their differentiation to endoderm. We show that the differentiation of wild-type ES cells into mesoderm blocks their Gata4-induced endoderm differentiation, while mesoderm cells derived from ES cells that are deficient in the DNA methyltransferases Dnmt3a and Dnmt3b can retain their response to Gata4, allowing lineage conversion from mesoderm cells to endoderm. Transcriptome analysis of the cells' response to Gata4 over time revealed groups of endoderm and mesoderm developmental genes whose expression was induced by Gata4 only when DNA methylation was lost, suggesting that DNA methylation restricts the ability of these genes to respond to Gata4, rather than controlling their transcription *per se*. Gata4-binding-site profiles and DNA methylation analyses suggested that DNA methylation modulates the Gata4 response through diverse mechanisms. Our data indicate that epigenetic regulation by DNA methylation functions as a heritable safeguard to prevent transcription factors from activating inappropriate downstream genes, thereby contributing to the restriction of the differentiation potential of somatic cells.

Citation: Oda M, Kumaki Y, Shigeta M, Jakt LM, Matsuoka C, et al. (2013) DNA Methylation Restricts Lineage-specific Functions of Transcription Factor Gata4 during Embryonic Stem Cell Differentiation. PLoS Genet 9(6): e1003574. doi:10.1371/journal.pgen.1003574

Editor: John M. Greally, Albert Einstein College of Medicine, United States of America

Received November 4, 2012; **Accepted** May 2, 2013; **Published** June 27, 2013

Funding: This work was supported by the RIKEN Center for Developmental Biology intramural fund and by the Strategic Programs for R&D of RIKEN (Japan) to M. Okano and H. Niwa. This work was also supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to Y. Kumaki (Grant No. 22510220). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: okano@cdb.riken.jp

☯ These authors contributed equally to this work.

¤a Current address: Department of Pathology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan.
¤b Current address: Department of Anatomy and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
¤c Current address: Department of Systems Medicine, Mitsunada Sakaguchi Laboratory, Keio University School of Medicine, Tokyo, Japan.

Introduction

Development is based on a series of cell-fate decisions and commitments. Transcription factors and epigenetic mechanisms coordinately regulate these processes [1,2]. Transcription factors play dominant roles in instructing lineage determination and cell reprogramming [3,4]. Transcription factor and co-factor networks regulate cell-specific gene programs, allowing a given transcription factor to be used repeatedly in different cellular and developmental contexts [5]. In addition, epigenetic mechanisms, which establish and maintain cell-specific chromatin states (or epigenomes) during differentiation and development [6], modulate the functions of transcription factors in cell-type-dependent manners [7,8]. Alterations of chromatin states can increase the efficiency of transcription factor-induced cell reprogramming [9,10] and lineage conversion *in vivo* [11,12]. However, how epigenetic mechanisms and transcription factor networks coordinately regulate cell differentiation remains elusive.

DNA methylation at cytosine-guanine (CpG) sites is a heritable genome-marking mechanism for epigenetic regulation, modulating gene expression through chromatin regulation [13]. Genome-wide DNA methylation profiles have revealed that the methylated CpG in the mammalian genome is specifically distributed in a cell-type-dependent manner [14–16], and the methylated CpG sites are dynamically reprogrammed during embryogenesis and gametogenesis [17–19]. The DNA methylation profile is established and maintained by three DNA methyltransferases (DNMTs), Dnmt1, Dnmt3a, and Dnmt3b [20], together with DNA demethylation mechanisms [21]. Dnmt1 is required for the maintenance of DNA methylation profiles, whereas Dnmt3a and Dnmt3b are required to establish them. The inactivation of Dnmt1 or both Dnmt3a and Dnmt3b in mice leads to early embryonic lethality, showing that

Current Topics

Stem Cell Research for Regenerative Medicine/Personalized Medicine

Transcription Factor Network in Embryonic Stem Cells: Heterogeneity under the Stringency

Yoko Nakai-Futatsugi and Hitoshi Niwa*

Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, 2–2–3 Minatojima-minamimachi, Chuo-ku, Kobe 650–0047, Japan.

Received October 30, 2012

Leukemia inhibitory factor (LIF) signaling regulates transcription factors to maintain the self-renewability and pluripotency of embryonic stem (ES) cells. Recently, we have proposed a network model that consists of transcription factors such as, Klf4, Sox2, Tbx3, Nanog, and Oct3/4, which form a parallel pathway downstream from LIF signaling (*Nature*, 460, 2009, Niwa *et al.*). In this parallel pathway, the transcription factors maintain the pluripotency of ES cells through mutual balance with some degree of redundancy and compensation. While self-renewability and pluripotency are maintained well under such seemingly stringent regulation, studies of single cells revealed heterogeneity among individual ES cells. This heterogeneity may underlie the mechanism that allows ES cells to exit self-renewal and enter into differentiation to exert pluripotency. Here we focus on recent studies on the heterogeneity of ES cells and discuss their inherent metastability.

Key words embryonic stem cell; transcription factor network; metastability

1. INTRODUCTION

During mouse development, pluripotency is established in the inner cell mass (ICM) of the blastocyst around embryonic day 3.0 (E3.0) (Fig. 1). Pluripotency is the capacity of an individual cell to give rise to all other cell types of the body and the germ line, which is an emergent property realized in the ICM. Pluripotency persists until the ICM develops into the primitive ectoderm (PrEc) by E5.5 (Fig. 1). Embryonic stem (ES) cells are the cells captured from the ICM during this short time period (24 h *in vivo*), which continue to multiply while remaining pluripotent indefinitely under certain culture conditions.[1,2] The factor that maintains the cells in this "forever-young" state was identified[3] and determined to be identical to the cytokine, leukemia inhibitory factor (LIF).[4,5] LIF signaling is mediated *via* the signaling molecule Stat3[6] and regulates the pluripotency transcription factor network.[7] This regulation maintains the core transcription factor Oct3/4 at a certain level, which apparently makes the population 'homogeneous' with indefinite self-renewability and pluripotency. Then

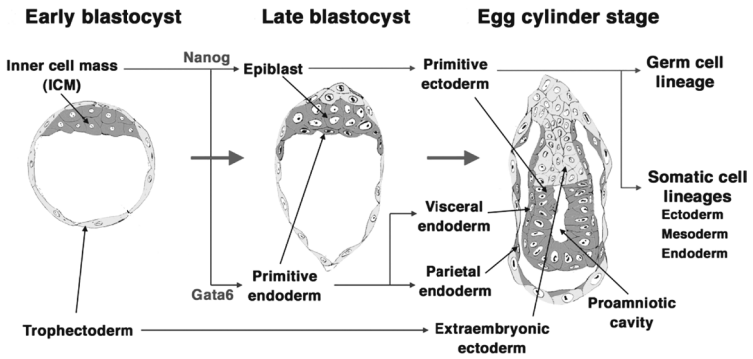


Fig. 1. Pluripotent Lineages in the Mouse Embryo
Modified from Niwa H, *Development*, **134**, 635–646 (2007).

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: niwa@cdb.riken.jp

Molecular Signatures of the Three Stem Cell Lineages in *Hydra* and the Emergence of Stem Cell Function at the Base of Multicellularity

Georg Hemmrich,[†,1] Konstantin Khalturin,[†,1] Anna-Marei Boehm,[†,1] Malte Puchert,[1,2] Friederike Anton-Erxleben,[1] Jörg Wittlieb,[1] Ulrich C. Klostermeier,[3] Philip Rosenstiel,[3] Hans-Heinrich Oberg,[2] Tomislav Domazet-Lošo,[4,5] Toshimi Sugimoto,[6] Hitoshi Niwa,[6] and Thomas C.G. Bosch[*,1]

[1]Zoological Institute, Christian-Albrechts University, Kiel, Germany

[2]Institute of Immunology, University Hospital Schleswig-Holstein, Kiel, Germany

[3]IKMB, University Hospital Schleswig-Holstein, Kiel, Germany

[4]Laboratory of Evolutionary Genetics, Max-Planck-Institute for Evolutionary Biology, Plön, Germany

[5]Laboratory of Evolutionary Genetics, Division of Molecular Biology, Ruđer Bošković Institute, Zagreb, Croatia

[6]Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology, Kobe, Japan

[†]These authors contributed equally to this work.

***Corresponding author:** E-mail: tbosch@zoologie.uni-kiel.de.

Associate editor: Billie Swalla

Abstract

How distinct stem cell populations originate and whether there is a clear stem cell "genetic signature" remain poorly understood. Understanding the evolution of stem cells requires molecular profiling of stem cells in an animal at a basal phylogenetic position. In this study, using transgenic *Hydra* polyps, we reveal for each of the three stem cell populations a specific signature set of transcriptions factors and of genes playing key roles in cell type-specific function and interlineage communication. Our data show that principal functions of stem cell genes, such as maintenance of stemness and control of stem cell self-renewal and differentiation, arose very early in metazoan evolution. They are corroborating the view that stem cell types shared common, multifunctional ancestors, which achieved complexity through a stepwise segregation of function in daughter cells.

Key words: aging, adult stem cell, self-renewal, differentiation, evolution.

Introduction

The evolution of multicellular animals most likely started from a number of multifunctional cell types that existed in the metazoan ancestor (Arendt 2008; Arendt et al. 2009). To maintain tissue homeostasis and repair function, these ancestral cell types, which simultaneously carry out a number of different functions, must have had properties known from adult multipotent stem cells. The evolutionary origin of stem cells is unknown. Stem cells might have originated from flagellated cells at the surface of the body of the ur-metazoan (King 2004). Proliferative cells in sponges also have features that justify referring them as unipotent stem cells (Funayama 2010). Yet, the origins and mechanisms for establishing stem cell populations, however, remain obscure and the search for a clear stem cell "genetic signature" continues. Cnidarians are not only among the earliest-known phyletic lineages known to contain stem cells (fig. 1A) (Hemmrich et al. 2007) but also possess most of the gene families found in bilaterians (Putnam et al. 2007; Dunn et al. 2008; Philippe et al. 2009; Schierwater et al. 2009). They have retained many ancestral genes that have been lost in *Drosophila* and *Caenorhabditis elegans* (Kortschak et al. 2003; Kusserow et al. 2005; Miller et al. 2005; Technau et al. 2005; Chapman et al. 2010). The genome of *Hydra magnipapillata* has been sequenced and analyzed and is a major resource for understanding the molecular "toolbox" of the earliest common ancestors of metazoans (Chapman et al. 2010). Several cnidarians are used as model organisms in developmental biology, including *Nematostella* (Anthozoa), *Clytia* (Hydrozoa), *Hydractinia* (Hydrozoa), *Acropora* (Anthozoa), and *Hydra* (Hydrozoa). All of them have their own benefits and are informative for an understanding of bilaterian evolution and development (Technau and Steele 2011). *Hydra* is the only cnidarian where the cell lineages and the differentiation pathways have been completely understood and a transgene technology is developed to a level that fluorescence-activated cell sorting (FACS) of individually labeled cell lineages is possible. There is some evidence that cnidarians differ in their stem cell differentiation pathways because a interstitial cell lineage seems to be present only in hydrozoans (Technau and Steele 2011). Tissue function, behavioral traits, and sexual reproduction in *Hydra* are

Development 139, 3926-3937 (2012) doi:10.1242/dev.085936

Transcriptional regulatory networks in epiblast cells and during anterior neural plate development as modeled in epiblast stem cells

Makiko Iwafuchi-Doi[1,*], Kazunari Matsuda[1], Kazuhiro Murakami[2,‡], Hitoshi Niwa[2], Paul J. Tesar[3], Jun Aruga[4], Isao Matsuo[5] and Hisato Kondoh[1,§]

SUMMARY

Somatic development initiates from the epiblast in post-implantation mammalian embryos. Recent establishment of epiblast stem cell (EpiSC) lines has opened up new avenues of investigation of the mechanisms that regulate the epiblast state and initiate lineage-specific somatic development. Here, we investigated the role of cell-intrinsic core transcriptional regulation in the epiblast and during derivation of the anterior neural plate (ANP) using a mouse EpiSC model. Cells that developed from EpiSCs in one day in the absence of extrinsic signals were found to represent the ANP of ~E7.5 embryos. We focused on transcription factors that are uniformly expressed in the E6.5 epiblast but in a localized fashion within or external to the ANP at E7.5, as these are likely to regulate the epiblast state and ANP development depending on their balance. Analyses of the effects of knockdown and overexpression of these factors in EpiSCs on the levels of downstream transcription factors identified the following regulatory functions: cross-regulation among Zic, Otx2, Sox2 and Pou factors stabilizes the epiblastic state; Zic, Otx2 and Pou factors in combination repress mesodermal development; Zic and Sox2 factors repress endodermal development; and Otx2 represses posterior neural plate development. All of these factors variably activate genes responsible for neural plate development. The direct interaction of these factors with enhancers of *Otx2*, *Hesx1* and *Sox2* genes was demonstrated. Thus, a combination of regulatory processes that suppresses non-ANP lineages and promotes neural plate development determines the ANP.

KEY WORDS: Epiblast stem cells, Anterior neural plate, Zic factors, Pou factors, Otx2, Sox2

INTRODUCTION

The epiblast serves as the primordium of all somatic lineages in amniotes, where the earliest derivative is the anterior neural plate (ANP). Despite profound interest in the process by which somatic lineages are generated, cell-intrinsic and transcription factor-dependent regulatory mechanisms remain poorly understood, primarily because of the difficulty in accessing the epiblast in post-implantation mammalian embryos. The cell-extrinsic mechanisms that regulate the fate of the epiblast are better understood. It has been shown, for example, that Nodal antagonists secreted from the anterior visceral endoderm disrupt Nodal signaling, which otherwise stabilizes the epiblast state and elicits ANP development (Camus et al., 2006; Perea-Gomez et al., 2002).

The ANP and the posterior neural plate (PNP) are generated via different mechanisms. The ANP is derived directly from the epiblast (Iwafuchi-Doi et al., 2011), whereas the development of the PNP from the epiblast passes through an intermediate state of axial stem cells, which are common precursors for the PNP and paraxial mesoderm (Kondoh and Takemoto, 2012; Takemoto et al., 2011; Tzouanacou et al., 2009).

Recent success in establishing cell lines directly from the egg cylinder epiblast [epiblast stem cells (EpiSCs)] (Brons et al., 2007; Tesar et al., 2007) has opened up new avenues to investigate the cell-intrinsic mechanisms in the epiblast and its derivatives. The epiblast state of EpiSCs is maintained by activin (Nodal substitute) and Fgf2 signaling, and the interruption of these pathways elicits the development of neural plate cells (NPCs), partly mimicking the action of Nodal antagonists during the derivation of the ANP in embryos.

In our present study, we first characterized EpiSCs and their immediately derived NPCs under culture conditions without supply of extrinsic signals. The expression profiles of transcription factor genes under these conditions were compared with those in embryonic tissues. Our findings indicate that the immediate derivatives of EpiSCs under the NPC culture conditions represent the ANP cell state in ~E7.5 embryos. Taking advantage of this observation, we then systematically analyzed the effects on downstream genes of the knockdown and overexpression in EpiSCs of several transcription factors.

These analyses of the transcriptional regulatory networks indicated intricate cross-regulation among the factors to stabilize the epiblast state and to derive the ANP. During the derivation of ANP, inhibitory mechanisms were found to play key roles to suppress the development of mesodermal, endodermal and PNP cells, confining the developmental pathway of epiblast derivatives to ANP. This analysis also highlights the crucial involvement of Zic2/3, Otx2 and Sox2 in these processes. This study thus highlights the advantages of using EpiSCs to examine in detail the regulatory mechanisms that underlie the derivation of somatic lineages.

[1]Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan. [2]RIKEN Center for Developmental Biology (CDB), Chuo-ku, Kobe, Hyogo 650-0047, Japan. [3]Department of Genetics and Center for Stem Cell and Regenerative Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA. [4]RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan. [5]Osaka Medical Center and Research Institute for Maternal and Child Health, Murodo-cho, Izumi, Osaka 594-1101, Japan.

*Present address: Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
‡Present address: The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK
§Author for correspondence (kondohh@fbs.osaka-u.ac.jp)

Accepted 3 August 2012

OPEN ACCESS Freely available online

PLOS ONE

E-Cadherin Promotes Incorporation of Mouse Epiblast Stem Cells into Normal Development

Satoshi Ohtsuka[1], **Satomi Nishikawa-Torikai**[1,2], **Hitoshi Niwa**[1,2]*

1 Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology (CDB), Kobe, Japan, **2** CREST (Core Research for Evolutional Science and Technology), Japan Science Technology Agency, Kawaguchi, Japan

Abstract

Mouse epiblast stem cells (mEpiSCs) are pluripotent stem cells derived from epiblasts of postimplantation mouse embryos. Their pluripotency is distinct from that of mouse embryonic stem cells (mESCs) in several cell biological criteria. One of the distinctions is that mEpiSCs contribute either not at all or at much lower efficiency to chimeric embryos after blastocyst injection compared to mESCs. However, here we showed that mEpiSCs can be incorporated into normal development after blastocyst injection by forced expression of the E-cadherin transgene for 2 days in culture. Using this strategy, mEpiSCs gave rise to live-born chimeras from 5% of the manipulated blastocysts. There were no obvious signs of reprogramming of mEpiSCs toward the mESC-like state during the 2 days after induction of the E-cadherin transgene, suggesting that mEpiSCs possess latent ability to integrate into the normal developmental process as its origin, epiblasts.

Citation: Ohtsuka S, Nishikawa-Torikai S, Niwa H (2012) E-Cadherin Promotes Incorporation of Mouse Epiblast Stem Cells into Normal Development. PLoS ONE 7(9): e45220. doi:10.1371/journal.pone.0045220

Editor: Qiang Wu, National University of Singapore, Singapore

Received June 25, 2012; **Accepted** August 14, 2012; **Published** September 18, 2012

Funding: This work was supported by the Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and RIKEN (Center for Developmental Biology (CDB)) grant to H.N. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: niwa@cdb.riken.jp

Introduction

Pluripotent stem cells (PSCs) are defined by their ability to differentiate into the cell types of all three germ layers, *i.e.*, the ectoderm, mesoderm, and endoderm. To date, various types of PSCs from different origin with distinct characters have been reported. It has been proposed that PSCs can be categorized into two major types, naïve and primed PSCs [1]. The former category includes mouse embryonic stem cells (mESCs) [2,3] and mouse embryonic germ cells, whereas the latter includes mouse epiblast stem cells (mEpiSCs) [4,5] and human embryonic stem cells [6].

There are several criteria that distinguish naïve and primed PSCs. From the cell biological viewpoint, the most remarkable difference is the ability to contribute to chimeric embryos after blastocyst injection, which is only observed in naïve PSCs. mESCs can contribute to chimeric embryos and form embryos consisting of ESCs when injected into tetraploid blastocysts [7]. In contrast, mEpiSCs barely contribute to chimeric embryos when injected into blastocysts, as Brons *et al.* reported that only 2 chimeras were obtained from 385 injected blastocysts and no germline transmission was observed [5]. However, as mEpiSCs are derived from epiblasts of the postimplantation embryos, they may retain the latent ability to contribute to embryonic development as they originally do *in vivo*. The blastocyst is the orthotopic location for mESCs but an ectopic location for mEpiSCs, which may explain why mEpiSCs were unable to contribute to normal development by blastocyst injection. Indeed, Tesar *et al.* showed that mEpiSCs formed a segregated clump in the blastocyst cavity after injection although mESCs attached to the inner cell mass (ICM) under the same conditions [4]. Therefore, the differences in ability of mESCs and mEpiSCs to contribute to chimeras may be due to the different affinities for ICM attachment rather than their cell biological potential. Orthotopic transplantation of mEpiSCs would be an ideal way to evaluate their ability to contribute to normal development, but it is technically difficult due to the small size of the embryos *in utero* that makes them inaccessible for manipulation.

An alternative is artificial enhancement of mEpiSCs integration into the ICM after blastocyst injection. E-cadherin encoded by *Cadherin 1 (Cdh1)* is responsible for mediating homophilic adhesion of mESCs [8] and its level of expression is higher in mESCs than in mEpiSCs [4,5]. Therefore, artificial upregulation of E-cadherin in mEpiSCs may accelerate their attachment to the ICM after blastocyst injection and result in efficient generation of chimeric mice. Here, we tested this possibility and succeeded in generating mEpiSC-derived chimeras in a reproducible manner.

Results

Establishment of mEpiSCs with Inducible E-cadherin Transgene

To establish EpiSCs in which E-cadherin can be overexpressed in an inducible manner, we introduced a tetracycline-inducible *E-cadherin* expression cassette and tetracycline-dependent activator expression vector into two different EpiSC lines using the piggyBac transposon system [8]. We chose two parental EpiSC lines–female mEpiSCs reported by Tesar *et al.* [4], which is a standard and was designated as PTmEpiSCs in this manuscript, and female mEpiSCs established in our laboratory from 129+ter/SvJcl, designated hereafter as SOmEpiSCs. Use of female EpiSCs allowed us to monitor the X-inactivation status, which is one of the markers for distinguishing between naïve and primed states [4,5].

Cell Stem Cell
Article

Cell PRESS

Esrrb Is a Pivotal Target of the Gsk3/Tcf3 Axis Regulating Embryonic Stem Cell Self-Renewal

Graziano Martello,[1,2,6] Toshimi Sugimoto,[4,5,6] Evangelia Diamanti,[1,3] Anagha Joshi,[1,3] Rebecca Hannah,[1,3] Satoshi Ohtsuka,[4] Berthold Göttgens,[1,3,*] Hitoshi Niwa,[4,5,*] and Austin Smith[1,2,*]

[1]Wellcome Trust - Medical Research Council Stem Cell Institute
[2]Department of Biochemistry
[3]Cambridge Institute for Medical Research and Department of Haematology
University of Cambridge, Cambridge UK
[4]RIKEN Center for Developmental Biology, Kobe 650-0047, Japan
[5]Laboratory for Development and Regenerative Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuo-ku, Kobe, Hyogo 6500017, Japan
[6]These authors contributed equally to this work
*Correspondence: bg200@cam.ac.uk (B.G.), niwa@cdb.riken.jp (H.N.), austin.smith@cscr.cam.ac.uk (A.S.)
http://dx.doi.org/10.1016/j.stem.2012.06.008

SUMMARY

Inhibition of glycogen synthase kinase-3 (Gsk3) supports mouse embryonic stem cells (ESCs) by modulating Tcf3, but the critical targets downstream of Tcf3 are unclear. We analyzed the intersection between genome localization and transcriptome data sets to identify genes repressed by Tcf3. Among these, manipulations of Esrrb gave distinctive phenotypes in functional assays. Knockdown and knockout eliminated response to Gsk3 inhibition, causing extinction of pluripotency markers and loss of colony forming capability. Conversely, forced expression phenocopied Gsk3 inhibition or Tcf3 deletion by suppressing differentiation and sustaining self-renewal. Thus the nuclear receptor Esrrb is necessary and sufficient to mediate self-renewal downstream of Gsk3 inhibition. Leukaemia inhibitory factor (LIF) regulates ESCs through Stat3, independently of Gsk3 inhibition. Consistent with parallel operation, ESCs in LIF accommodated Esrrb deletion and remained pluripotent. These findings highlight a key role for Esrrb in regulating the naive pluripotent state and illustrate compensation among the core pluripotency factors.

INTRODUCTION

Since the original derivation of mouse embryonic stem cells (ESCs) in 1981 (Evans and Kaufman, 1981; Martin, 1981), culture conditions for sustaining pluripotency ex vivo have been progressively refined. Following the demonstration that the cytokine leukemia inhibitory factor (LIF) could replace feeder cells (Smith et al., 1988; Williams et al., 1988) and that ESC differentiation is suppressed by inhibition of mitogen activated protein kinase (Erk) signaling (Burdon et al., 1999; Kunath et al., 2007), further addition of an inhibitor of glycogen synthase kinase-3 (Gsk3) has enabled robust ESC propagation in well-defined conditions (Ying et al., 2008). When cultured using the two inhibitors (2i), ESCs display rather uniform marker expression (Wray et al., 2010) and exhibit distinctive gene expression and epigenetic features (Marks et al., 2012). A practical consequence is that it has become facile to establish ESCs from different strains of mice and also rats (Blair et al., 2011). It is noteworthy that, while the triple combination of 2i/LIF appears optimal, mouse ESCs can be propagated by providing any two of these three components (Wray et al., 2011; Wray et al., 2010), implying complementary inputs to a flexible gene regulatory circuit. However, understanding how intracellular signaling pathways engage with the core transcription factor circuitry to maintain or extinguish pluripotency remains fragmentary (Chen et al., 2008; Jaenisch and Young, 2008; Nichols and Smith, 2012; Niwa et al., 2009).

Gsk3 is a negative regulator of many different proteins (Doble and Woodgett, 2003), including transcription factors such as cMyc (Singh and Dalton, 2009). Nonetheless, the effect of Gsk3 inhibition ESC self-renewal is mediated primarily via β-catenin because ESCs lacking β-catenin do not respond productively to Gsk3 inhibitors (Lyashenko et al., 2011; Wray et al., 2011). Consistent with action through intracellular β-catenin, mutation of *Apc* or expression of stabilized β-catenin variants can reduce ESC differentiation (Kielman et al., 2002; Sato et al., 2004). Furthermore, Wnt3a can partially substitute for Gsk3 inhibition and support ESC propagation in conjunction with LIF (ten Berge et al., 2011; Hao et al., 2006; Ogawa et al., 2006; Yi et al., 2011).

It has been suggested that β-catenin might interact directly with Oct4 to promote pluripotent gene expression (Kelly et al., 2011). On the other hand, genetic evidence is incontrovertible that a definitive β-catenin partner, Tcf3 (also known as Tcf7l1), is a major negative regulator of ESC self-renewal (Guo et al., 2011; Pereira et al., 2006). Indeed, ablation of Tcf3 phenocopies deletion or inhibition of Gsk3 (Wray et al., 2011). Genome location analyses indicate that Tcf3 binds in proximity to many core pluripotency genes (Cole et al., 2008; Marson et al., 2008; Tam et al., 2008). Although it has been proposed that β-catenin is recruited to stimulate transcription at these sites (Cole et al., 2008), this model seems inconsistent with the *Tcf3* loss of function phenotype. Furthermore, available evidence indicates that Tcf3 functions in a repressor complex (Pereira et al., 2006; Sokol, 2011; Yi et al., 2011), activity of which can be abrogated by

Chromosome Res (2012) 20:837–848
DOI 10.1007/s10577-012-9317-9

Chromosome-wide regulation of euchromatin-specific 5mC to 5hmC conversion in mouse ES cells and female human somatic cells

Musashi Kubiura · Masaki Okano · Hiroshi Kimura · Fumihiko Kawamura · Masako Tada

Received: 10 August 2012 / Revised: 9 October 2012 / Accepted: 10 October 2012 / Published online: 31 October 2012

Abstract DNA cytosine methylation (5mC) is indispensable for a number of cellular processes, including retrotransposon silencing, genomic imprinting, and X chromosome inactivation in mammalian development. Recent studies have focused on 5-hydroxymethylcytosine (5hmC), a new epigenetic mark or intermediate in the DNA demethylation pathway. However, 5hmC itself has no role in pluripotency maintenance in mouse embryonic stem cells (ESCs) lacking *Dnmt1*, *3a*, and *3b*. Here, we demonstrated that 5hmC accumulated on euchromatic chromosomal bands that were marked with di- and trimethylated histone H3 at lysine 4 (H3K4me2/3) in mouse ESCs. By contrast, heterochromatin enriched with H3K9me3, including mouse chromosomal G-bands, pericentric repeats, human satellite 2 and 3, and inactive X chromosomes, was not enriched with 5hmC. Therefore, enzymes that hydroxylate the methyl group of 5mC belonging to the Tet family might be excluded from inactive chromatin, which may restrict 5mC to 5hmC conversion in euchromatin to prevent nonselective de novo DNA methylation.

Keywords DNA methylation · Embryonic stem cells · Tet enzymes · 5-Hydroxymethylation

Abbreviations

5hmC	5-Hydroxymethylcytosine
5mC	DNA cytosine methylation
Dnmt	DNA methyltransferase
ESCs	Embryonic stem cells
iPSCs	Induced pluripotential stem cells
MBD	Methyl-binding domain
PGCs	Primordial germ cells
PN	Pronucleus
Tet	Tet (Ten-eleven translocation) methylcytosine dioxygenase

Responsible Editor: Tatsuo Fukagawa.

Electronic supplementary material The online version of this article (doi:10.1007/s10577-012-9317-9) contains supplementary material, which is available to authorized users.

M. Kubiura · F. Kawamura
Division of Molecular and Cell Genetics,
Department of Molecular and Cellular Biology,
School of Life Sciences, Faculty of Medicine, Tottori University,
Yonago 683-8503, Japan

M. Okano
Laboratory for Mammalian Epigenetic Studies,
Center for Developmental Biology, RIKEN,
Kobe 650-0047, Japan

H. Kimura
Graduate School of Frontier Biosciences, Osaka University,
Suita 565-0871, Japan

M. Tada (✉)
Chromosome Engineering Research Center,
Tottori University,
Yonago, Tottori 683-8503, Japan
e-mail: matada@med.tottori-u.ac.jp

ORIGINAL ARTICLE

Role of DNA Methylation in the Regulation of Lipogenic Glycerol-3-Phosphate Acyltransferase 1 Gene Expression in the Mouse Neonatal Liver

Tatsuya Ehara,[1,2] Yasutomi Kamei,[1,3] Mayumi Takahashi,[1] Xunmei Yuan,[1] Sayaka Kanai,[1] Erina Tamura,[1] Miyako Tanaka,[1,3] Tomomi Yamazaki,[4] Shinji Miura,[4] Osamu Ezaki,[4] Takayoshi Suganami,[1] Masaki Okano,[5] and Yoshihiro Ogawa[1,6,7]

The liver is a major organ of lipid metabolism, which is markedly changed in response to physiological nutritional demand; however, the regulation of hepatic lipogenic gene expression in early life is largely unknown. In this study, we show that expression of glycerol-3-phosphate acyltransferase 1 (GPAT1; *Gpam*), a rate-limiting enzyme of triglyceride biosynthesis, is regulated in the mouse liver by DNA methylation, an epigenetic modification involved in the regulation of a diverse range of biological processes in mammals. In the neonatal liver, DNA methylation of the *Gpam* promoter, which is likely to be induced by Dnmt3b, inhibited recruitment of the lipogenic transcription factor sterol regulatory element–binding protein-1c (SREBP-1c), whereas in the adult, decreased DNA methylation resulted in active chromatin conformation, allowing recruitment of SREBP-1c. Maternal overnutrition causes decreased *Gpam* promoter methylation with increased GPAT1 expression and triglyceride content in the pup liver, suggesting that environmental factors such as nutritional conditions can affect DNA methylation in the liver. This study is the first detailed analysis of the DNA-methylation–dependent regulation of the triglyceride biosynthesis gene *Gpam*, thereby providing new insight into the molecular mechanism underlying the epigenetic regulation of metabolic genes and thus metabolic diseases. ***Diabetes* 61:2442–2450, 2012**

The liver is a major organ of lipid metabolism, which is physiologically changed during organ maturation (1,2). The rate of hepatic de novo lipogenesis (i.e., triglyceride [TG] biosynthesis) is very low during the suckling period, when fat intake is high from milk, but it rises with the onset of weaning, when glucose is used as a source of energy (1). Thus, hepatic gene expression may change markedly before and after weaning, which could be regulated in response to nutritional demand.

TG is the major storage form of energy in animals. TG biosynthesis begins with the acylation of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase 1 (GPAT1; *Gpam*) to form lysophosphatidic acid; this is the rate-limiting step in the hepatic TG biosynthesis pathway (3). In the acylation process, fatty acids produced by stearoyl CoA desaturase 1 (SCD1; *Scd1*) and fatty acid synthase (FAS; *Fasn*) are used as acyl donors. Among the lipogenic enzymes, GPAT1 plays an important role in the regulation of hepatic TG biosynthesis (4,5). The lipogenic genes such as *Gpam*, *Scd1*, and *Fasn* are activated by sterol regulatory element–binding protein-1c (SREBP-1c), which is a transcription factor and master regulator of lipogenesis. Indeed, their promoter regions contain the SREBP-responsive elements (SREs) (6–8). Aberrant lipogenic gene regulation can contribute to fatty liver, which is associated with obesity, type 2 diabetes, and insulin resistance (9). However, the molecular mechanism involved in the regulation of lipogenic genes during early life remains largely unclear.

The methylation of cytosine residues in DNA is a major epigenetic modification, and its role is well studied in organ development and cell differentiation (10–12). In most instances, DNA methylation of the promoter regions causes suppression of gene expression (13). In mammals, three CpG DNA methyltransferases (Dnmt)—Dnmt1, Dnmt3a, and Dnmt3b—coordinately regulate DNA methylation in the genome. Dnmt1 promotes DNA methylation after DNA replication and plays a major role in the maintenance of methylation (14). Dnmt3a and Dnmt3b are required for the initiation of de novo DNA methylation (10).

DNA methylation may be affected by environmental factors, thereby regulating a variety of metabolic processes and diseases (15–18). Although the fetal and neonatal periods, which are highly plastic to environmental changes, should be under the epigenetic control, the role of DNA methylation in early life has not fully been addressed. This study is the first demonstration that the DNA methylation status of the *Gpam* promoter and its mRNA expression are inversely correlated during mouse liver maturation. This study highlights the role of DNA methylation in the regulation of lipogenic genes, thereby providing new insight into the molecular mechanism underlying epigenetic regulation of metabolic diseases.

RESEARCH DESIGN AND METHODS

Animals and the experiment with high-fat/high-sucrose diet–fed dams. Pregnant female C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were fed ad libitum a standard rodent chow, CRF1 (Charles River Japan, Tokyo, Japan). Offspring at the indicated ages were used for tissue

From the [1]Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; the [2]Functional Food Research Department, Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd., Kanagawa, Japan; the [3]Department of Organ Network and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; the [4]Nutritional Science Program, National Institute of Health and Nutrition, Tokyo, Japan; the [5]Laboratory for Mammalian Epigenetic Studies, Center for Developmental Biology, RIKEN, Kobe, Japan; the [6]Department of Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan; and the [7]Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.
Corresponding author: Yoshihiro Ogawa, ogawa.mem@tmd.ac.jp.
Received 4 January 2012 and accepted 19 April 2012.
DOI: 10.2337/db11-1834
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-1834/-/DC1.
T.E. and Y.K. contributed equally to this work.