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Ⅳ　研究成果の刊行物・別冊

2．ES 細胞を用いた生殖医療

● ES 細胞の生殖細胞への分化に関する新知見を解説して下さい

■回答■ 阿久津英憲・梅澤明弘

胚性幹細胞（embryonic stem cells 以下 ES 細胞）は胚盤胞の内部細胞塊より樹立される細胞で，幅広い分化能力を保ちつつ体外培養系で無限に増殖できる希有な細胞である．ES 細胞は実験動物のマウスで約 25 年前に樹立され，98 年にヒト ES 細胞が米国ウィスコンシン大学のグループにより報告され，一気に再生医療の分野で注目されるようになった．ES 細胞の分化多能性を証明する方法として，マウスでは ES 細胞を免疫不全マウスへ移植し奇形腫形成を確認する方法と ES 細胞を胚盤胞へ注入し得られた個体で三胚葉組織へ寄与するキメラ個体が得られることと体外培養系で ES 細胞から分化した細胞の集合体（胚様体：embryoid body 以下 EB）を作成し三胚葉組織への分化を確認する方法がある．これまで，ES 細胞から EB へ分化する過程では in vivo で起こる発生の多面性や遺伝子発現と細胞分化のタイミング，in vivo のイベントが同様に起きているとされている．つまり，初期発生の再現が培養ディッシュ上でできていることになる．キメラ個体の産仔は ES 細胞由来の形質を示すことより ES 細胞が in vivo では生殖細胞へ分化することが示されるが，体外培養系で ES 細胞から生殖細胞への分化に関する報告はごくわずかである．生殖細胞の発生を鑑み，最近の ES 細胞由来生殖細胞から産仔に至った報告を検討していきたい．

A．生殖細胞の成り立ち

生殖細胞は種の保存・進化を担う主役の細胞である．胎生期に始原生殖細胞（primordial germ cells；以下 PGC）が形成され，減数分裂を経て染色体が一組の一倍体細胞（精子または卵子）となる．減数分裂期には相同遺伝子の組換え（meiotic recombination）が起こり遺伝情報の多様化に寄与する．通常の発生では，体細胞が生殖細胞になるまでに 3 つの大きなイベントを経ている．① PGC の形成，②減数分裂の完遂，③精子・卵子への形態成熟で，特に①から②の間に親のエピゲノム情報が消去され，新たに付与されるエピゲノムリプログラミングが起こる．

生殖細胞への分化系譜は，発生のごく初期より体細胞の分化成熟とは異なった非常にユニークな道を歩む（図 1）．マウスでは受精後 7 日目頃には生殖細胞の起源となる細胞がエピブラスト上縁部に現れる．将来生殖細胞となるグループの中に，6.25 日頃 Blimp 1 遺伝子を発現する細胞が現れ，体細胞特徴的な Hox 遺伝子群の発現を抑制していく．周囲の細胞からの BMP-Smad シグナリングを介した調和の中，Flagilis，Stella，Oct-3/4 を発現をした細胞は生殖堤へと移動し，真の生殖細胞になるために必要な一大イベントであるエピゲノムのリプログラミングが始まる．これは，親由来のメチル化情報や刷り込み遺伝子の刷り込み因子が消されて自身の新たなエピゲノム情報が刻み込まれていく．それとともに性の分化が始まり，発生の進行に差が出てくる．精原細胞は出生第 2 日目まで減数分裂期へ入らず有糸分裂停止期となるが，卵原細胞は卵母細胞となり第一減数分裂前期で細胞周期はいったん休止するが刷り込みの確立や遺伝子の転写は活発に行われている．

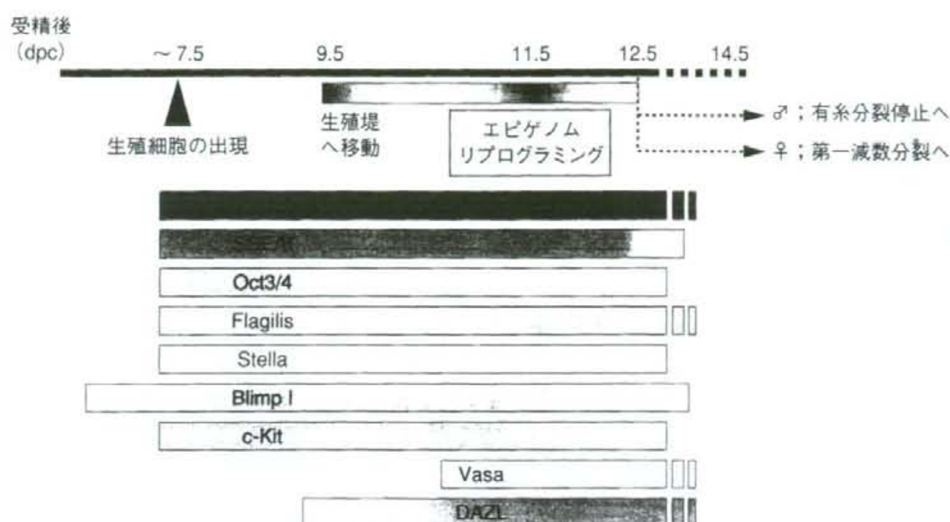

図1　マウス生殖細胞発生

B．ES 細胞から生殖細胞へ

03 年に Hubner らが，ES 細胞から生殖細胞（卵子）への分化をはじめて報告した[1]（表 1）．彼らは，in vivo で起きている PGC の遺伝子発現パターンから ES 細胞由来 PGC を選別し培養を継続していき，卵子特異的遺伝子 ZP-2，ZP-3 の発現が確認できる卵子様細胞を得ることができた．興味深いのは，XY 型の ES 細胞からも卵子が分化できていることである．もう一つは，この ES 細胞分化培養系では，減数分裂期の休止が認められず単為発生胚となり胚盤胞期まで発生している．通常，卵子形成過程では第一減数分裂前期と第二減数分裂中期で休止しているが，ES 細胞分化系では減数分裂停止機構が機能していなかった．筆者らは，出生 1 日目のマウス卵巣器官培養系でも成熟した卵子が単為発生してしまうのを確認しているので，ES 細胞特異的というより全体的な培養システムの中で起こる問題かもしれない．Toyooka らは ES 細胞より PGC 細胞まで分化を進行させた後，胎仔精巣再構成凝集塊との共培養下で精子への発生を初めて報告した[2]．Geijesen らは ES 細胞より精子細胞への分化に成功し，刷り込み遺伝子（Igf2r と H19）の DNA メチル化状態が精子特異的に付与されていると報告した[3]．さらに，ICSI により野生型卵子に注入すると胚盤胞までの発生が認められたが，産仔までは得られていない．ヒト ES 細胞培養系でも生殖細胞分化が Clark らにより初めて報告された[4]．ヒト ES 細胞分化では EB を形成後 3 日目には VASA 発現が認められている．その他，DAZL，STELLAR など生殖細胞特異的遺伝子発現と減数分裂期発現遺伝子である SCP1，SCP3，GDF9 などの発現が認められた．しかし，減数分裂を完了した一倍体細胞は認められなかった．最近，Nayernia らはマウス ES 細胞から PGC そして一倍体精子細胞を分化させ，ICSI により産仔にいたったと報告した[5]．ついに，ES 細胞由来生殖細胞が産仔発生能力のあることを初めて示した．彼らの培養システムでは，ACROSIN や先体膜タンパクの OAM，精子核タンパク PROTAMINE そして精子尾部特異的タンパク PHGPx が認められる細胞が得られている．精子形態形成の途上まで発生したこれらの精子細胞では，3 つの刷り込み遺伝子（H19，Igf2r，Snrpn）の DNA メチル化状態を解析したところ，親のメチル化パターンが消去されまた新

表1 ES細胞の生殖細胞分化に関する報告

	文献	種	ES細胞の性染色体型	分化生殖細胞	コメント
1	Hubner, et al. 2003	マウス	XY, XX	卵子（一倍体）	単為発生胚盤胞
2	Toyooka, et al. 2003	マウス	XY	精子細胞（一倍体）	胎仔精巣再構成凝集塊との共培養下で精子へ発生
3	Geijesen, et al. 2004	マウス		精細胞（一倍体）	ICSIにより胚盤胞
4	Clark, et al. 2004	ヒト	XY, XX	PGC	減数分裂未完了
5	Nayernia, et al. 2006	マウス	XY	精細胞（一倍体）	ICSIで産仔まで発生

たに付与されるエピジェネティックリプログラミングが行われていた．ICSIを行った結果，得られた65個の2細胞期胚から7匹の産仔が得られた．しかし，7匹全て正常な大きさの個体はなく生後5日から5カ月の間に全て死亡してしまった．さらに，ICSI産仔では，刷り込み遺伝子のメチル化パターンはまちまちであった．ES由来精子細胞では，減数分裂を完了するが，エピジェネティックリプログラミングは不完全でありICSIで得られた産仔も正常な個体は得られなかった．

C．ES細胞由来生殖細胞の課題と展望

これまでの報告よりES細胞からPGCへの分化は可能であり，減数分裂が開始し一倍体の生殖細胞を培養ディッシュ上で産生することができている．ES由来精子細胞からICSIにより産仔獲得まで至ったが，生殖細胞発生系譜で特異的に起こるエピジェネティックリプログラミングは不完全な状態で一倍体細胞となっていた．そのICSI産仔で刷り込み遺伝子領域のDNAメチル化状態は異常であり，5カ月以内に全て死んでしまった．これまでの，ES細胞由来生殖細胞に関する研究より，以下の課題があげられる．

- 体外培養系でPGCのDNAメチル化を含めたエピゲノムのリプログラミングがどの程度行われているのか．数個の刷り込み遺伝子領域だけでなく，ゲノムワイドで解析する必要がある．
- 通常のマウス雄個体では数週間かけて起こる精細胞発生が体外培養系ではPGCの出現から一倍体細胞への分化が数日で起こってしまう．培養方法の検討が必要である．
- 卵細胞分化では，通常起こる減数分裂期での休止がみられず，分化細胞はさらに単為発生を進めてしまう．卵子発生において，体外培養系での詳細な減数分裂進行のメカニズム解析が必要である．
- XX染色体型の個体では卵子，XY個体では精子が発生してくるわけだが，マウスおよびヒトES細胞分化系ではXY型ES細胞からでの卵子発生が引き起こされる．これには，SRYの作用有無または，作用の時期が大きくかかわっているようであるが，体外培養系での性分化決定メカニズム解析は非常に興味深い．

現在の時点ではES細胞由来生殖細胞の臨床応用を考慮する段階ではない．それ以前に，これま

でのわずかな成功例が示唆している点は，発生生物学や基礎医学的にも非常に興味深い．ほ乳類生殖細胞発生の重要な以下の事象が体外培養系で解明できる可能性が出てきた．① PGC の分化発生メカニズム，②クロマチンの再構築および精細胞でのヒストン・プロタミン変換メカニズム，③減数分裂開始・進行機構，④ PGC エピゲノムリプログラミング機構，⑤刷り込み遺伝子樹立メカニズム，⑥性分化メカニズムなどである．最近，Saitou らは，マウス PGC 細胞一つから cDNA ライブラリーを構築し，網羅的遺伝子発現解析を可能にした[6]．この ES 細胞由来生殖細胞分化誘導系と最新の遺伝子発現解析を応用すれば未解明現象に一気に分子レベルで解明できる可能性が大いにある．Nayernia らの結果から，ES 細胞由来生殖細胞分化系で PGC エピゲノムリプログラミングを正常に近づける培養システムの開発が必要である．この体外培養系でのエピゲノム環境への脆弱性改善の研究は，生殖医療における未熟卵子体外培養系の卵細胞質向上に非常に有用と考えられる．

1981 年にマウス ES 細胞樹立が報告されて 25 年になるが，ES 細胞から生殖細胞分化の知見を得たのは最近である．今後，ヒト ES 細胞を用いた生殖細胞分化の知見も得られればヒト初期発生のメカニズムの解明に大きく寄与すると思われる．【注：本邦でヒト ES 細胞を用いた生殖細胞分化研究を行うことは現時点で許可されていない（「ヒト ES 細胞の樹立及び使用に関する指針（平成 13 年文部科学省告示第 155 号）」】．

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Optimal Timing of Inner Cell Mass Isolation Increases the Efficiency of Human Embryonic Stem Cell Derivation and Allows Generation of Sibling Cell Lines

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DOI 10.1016/j.stem.2008.12.001

The capacity of human embryonic stem cells (hESCs) to self-renew indefinitely in culture while retaining their ability to differentiate into all cell types suggests that they have enormous potential both in medical applications and as a research tool (Reubinoff et al., 2000; Thomson et al., 1998). Despite their immortal nature, there is a need for derivation of new hESC lines to meet emerging requirements for their use in cell replacement therapies, disease modeling, and basic research. The need to optimize the use of donated or experimentally generated embryos motivated our attempts to improve methods for the derivation of hESC lines, which have led to practical recommendations and the generation of sibling hESC lines.

Following the derivation of 17 hESC lines (Cowan et al., 2004), we derived an additional 12 lines using the same method and found that the efficiency of these derivations varied greatly from experiment to experiment (see Table S1 available online). To better understand the variables that affect derivation efficiency, we explored methods for ICM isolation and systematically investigated the relationship between preimplantation biology and the timing of ESC derivation. We found that in vitro-cultured human preimplantation embryos undergo major changes in morphology as well as expression of OCT4 and CDX2 from days 5–9 postfertilization. We observed a peak of derivation efficiency using day 6 preimplantation embryos, corresponding to restriction of OCT4 to the ICM and CDX2 to the trophectoderm (TE). These comparative studies have led to the derivation of 45 new hESC lines from 140 blastocysts, of which 22 cell lines are derived from sibling embryos. Global gene expression analysis of hESC lines reveals that lines derived on different days do not significantly differ from one another in transcriptional profile, but lines derived from different genetic backgrounds do significantly differ, suggesting that genetic background, rather than the timing or method of derivation, is a contributing factor in the variability observed among hESC lines.

The most widely used method for hESC derivation involves either chemical or enzymatic removal of the zona pellucida, followed by isolation of the inner cell mass (ICM) of the blastocyst by immunosurgery (Reubinoff et al., 2000; Thomson et al., 1998). In immunosurgery, cells of the TE are destroyed by brief exposure to antibodies directed against human cells in tandem with complement activity (Solter and Knowles, 1975). However, only high-quality embryos with an intact TE can be subjected to this procedure, as only the structural integrity of the blastocyst prevents the ICM from also being destroyed. We reasoned that isolation of the ICM by laser-mediated ablation of the zona pellucida and TE might reduce exposure of the ICM to potentially cytotoxic compounds.

The 584 frozen human embryos used in this study were donated for research following informed consent under protocols reviewed and approved by both the Committee on the Use of Human Subjects (IRB) and the Embryonic Stem Cell Research Oversight Committee (ESCRO) at Harvard University. Human zygotes and cleavage-stage embryos were thawed and cultured to the blastocyst stage (Figure S1A). ICM isolation was carried out by exposing TE cells to cell-lethal laser pulses from a XYClone laser (see also Turetsky et al., 2008) and subsequent removal of dead TE cells either by using piezo drill-assisted micromanipulation or by repeated aspiration into a 50–75 μm glass capillary pipette (Figures S1B and S1C). The isolated ICM (Figure S1D) was then plated onto γ-irradiated mouse embryonic fibroblasts (MEFs) in hESC-conditioned derivation media.

Isolated ICMs attached to the MEF feeder cell layer within 24 hr and 4–13 days later gave rise to an ESC outgrowth (Figures S1E–S1G) composed of cells with typical hESC morphology (Figure S1H) that could be expanded into cell lines (Figures S1I and S1J). HESC lines isolated by laser surgery had a normal karyotype (Figure S1K) and expressed marker antigens found in pluripotent hESCs, including OCT4, SOX2, NANOG, TRA1-81, TRA1-60, and SSEA-4 (Figures S2A–S2F). Upon differentiation in vitro, via embryoid body formation (Figures S2G–S2I), and in vivo, via teratoma formation (Figures S2J–S2O), endoderm, mesoderm, and ectoderm lineages were readily observed, demonstrating that these cell lines are bona fide hESCs (Thomson et al., 1998; The International Stem Cell Initiative, 2007).

Next, we investigated the consequences of the presence or absence of TE cells in the derivation culture. We compared the efficiency of deriving

Table 1. Derivation Is Most Efficient on Day 6 Postfertilization

Day of ICM Isolation	Number of Blastocysts Used for Derivation	Number of Attachment Sites (Percent of Blastocysts)	Number of ICM Outgrowths	Number of Cell Lines	Percent Derivation Efficiency
5	19	6 (31)	1	1	5
6	27	22 (81)	15	14	52[a]
7	27	22 (81)	9	9	33
8	19	17 (90)	5	5	26
9	11	10 (91)	4	4	36
No isolation	10	10 (100)	1	1	10
Unknown[b]	27	ND	ND	11	40

Derivation efficiency is expressed as percent according to the number of cell lines obtained per total number of blastocysts used for derivation. Results summarize derivations using both laser surgery and immunosurgery. A more detailed presentation of the results is given in Table S2.
[a] $p = 0.00002$, comparison between day 6 and day 5; $p = 0.008$, comparison between day 6 and no isolation (assuming binomial distribution).
[b] HUES 18–28 (see also Table S1).

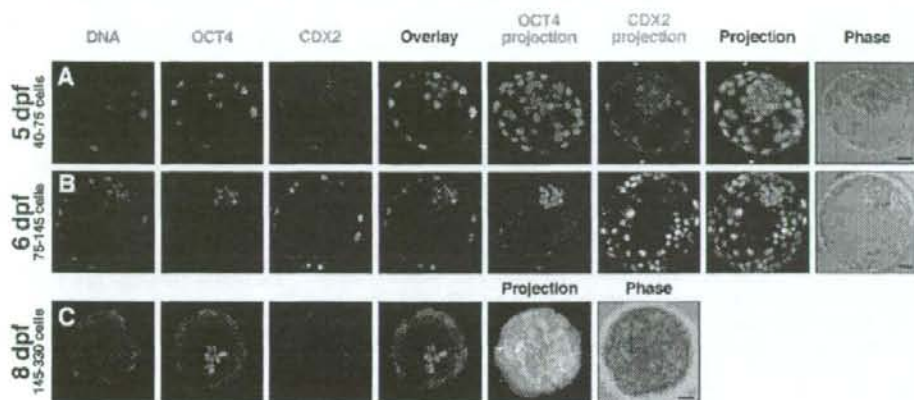
hESCs following plating of intact blastocysts without ICM isolation with the efficiency following ICM isolation on days 5–9 of development (with the day of insemination representing day 0). While derivation from plating intact blastocysts has previously been reported (Baharvand et al., 2004; Bongso et al., 1994; Genbacev et al., 2005; Heins et al., 2004), we found that the efficiency was low (10% of blastocysts used) and required close outgrowth monitoring in order to isolate the ICM before it was lost to rapid differentiation (Table 1 and Figure S3).

To investigate the effects of the timing of ICM isolation on hESC derivation, we systematically tested the derivation efficiencies with ICMs isolated from early blastocysts (day 5) through late blastocysts (day 9). During this prolonged culture period, we observed a number of morphological changes. Between days 5 and 6, ICM cell number increased while cell size decreased, and the TE of high-quality embryos expanded (Figure S4A). Both the ICM and TE of blastocysts continued to grow through day 6 of in vitro culture, but by day 7, the TE frequently collapsed and deteriorated, even in high-quality embryos (Figure S4B). In contrast to the TE, cells in the interior of the embryo continued to grow. For embryo culture beyond day 6, a shift from global medium to hESC-conditioned medium improved development, particularly in poor-quality embryos with a small or indiscernible ICM. In extremely compromised embryos, we switched to conditioned media as early as day 5. Surprisingly, poor-quality embryos without an ICM on day 5 or 6 often developed a distinct ICM after 1–2 days of additional culture (Figures S4C and S4D) in hESC-conditioned media. Extended culture of these embryos occasionally resulted in an atypical morphology in which the interior cells of the blastocysts expanded to form a solid sphere (Figures S4E and S4F), but no disadvantage was observed in subsequent hESC derivation (Table 1 and Table S2).

We found that hESC llines could be derived from embryos at days 5–9 after fertilization, with ICM isolation on day 6 resulting in the most efficient derivation of hESCs (Table 1). HESCs have previously been isolated on various days of development (Hovatta et al., 2003; Mitalipova et al., 2003; Stojkovic et al., 2004; Strom et al., 2007) and even from blastomeres and morula-stage embryos (Chung et al., 2008; Klimanskaya et al., 2006; Strelchenko et al., 2004). The low number of cell lines generated, however, did not allow a conclusion to be drawn regarding the efficiency of derivation. Isolation of the ICM on day 6 resulted in a 10-fold increase over the derivation efficiency on day 5 (52%, $n = 27$ versus 5%, $n = 19$, respectively; $p = 0.00002$). This is also 5-fold higher than derivation without isolation of the ICM (52%, $n = 27$ versus 10%, $n = 10$, respectively; $p = 0.008$). Derivation efficiency correlated with the total number of ICM attachment sites to the feeder layer after ICM plating. The number of attachment sites and resulting cell lines was low on day 5 but increased on day 6 and remained high on days 7–9. Derivation efficiency on days 7–9 was slightly lower but not significantly different from derivation on day 6 ($p > 0.01$). Poor-quality embryos benefited greatly from extended culture in hESC-conditioned media, as only 1–2 days of additional culture promoted ICM growth and allowed hESC derivation (Figures S4C–S4F, Table S2). This approach allowed for derivation of ESCs from embryos that would have otherwise been unlikely to give rise to hESC lines (e.g., grade 2CC embryos, HUES 33, 43, 57; grade 3CC embryos, HUES 32, 54, 59, 64). In summary, by combining extended embryo culture in hESC-conditioned media with laser-assisted isolation of the ICM on day 6 of preimplantation development, a derivation efficiency of 50% can be routinely achieved.

Using these methods, we succeeded in deriving a total of 22 sibling cell lines from seven donor couples (Table S3). To verify their identical maternal origin and demonstrate their karyotypic individuality, we sequenced the hypervariable regions of the mitochondrial genome and a combination of nuclear short tandem repeats (STRs) and compared them to unrelated hESC lines. While their mitochondrial genome was identical, their nuclear genome was different but highly related, sharing more than 50% of STR alleles (Table S4 and Figures S5 and S6).

Next, we explored whether differences in efficiency of hESC derivation correlate with a change in localization and expression of the respective ICM and TE markers, OCT4 and CDX2. It has been shown in mouse preimplantation embryos that by the 65–128 cell stage, OCT4 becomes restricted to the ICM, and CDX2 to the TE, at approximately 3.5 days postfertilization in in vitro-cultured blastocysts (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008). We therefore asked



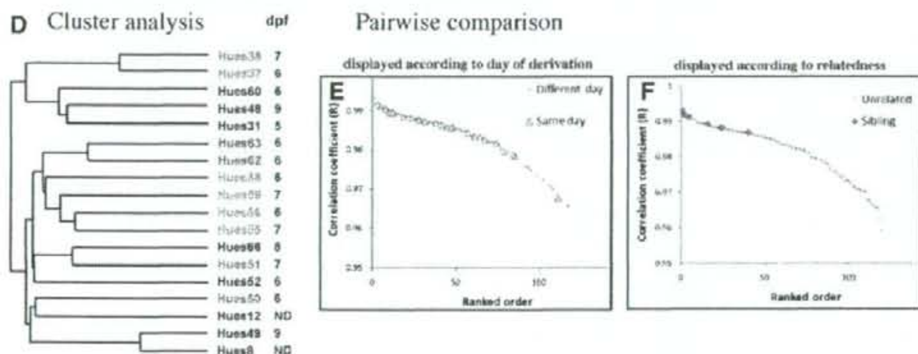


Figure 1. OCT4 and CDX2 Expression during Human Preimplantation Development and Gene Expression Analysis of hESC Lines
(A–C) Representative human preimplantation embryos on day 5 (A), day 6 (B), and day 8 (C) stained for the TE marker, CDX2, and ICM marker, OCT4. Total cell numbers typically found for each day are indicated. Scale bar indicates relative size for comparison.
(D) Cluster analysis of 16 hESC lines derived on different days of development. Sibling cell lines are marked with identical colors.
(E and F) Pairwise comparison of global gene expression profiles between different cell lines. Comparison of 16 cell lines yields 16 × 15/2 = 120 data points for all possible pairwise comparisons. The higher the Pearson correlation coefficient (R), the more similar the gene expression profile between two lines. Pairs were ordered from high to low according to the Pearson correlation coefficient (ranked order). (E) Pairwise comparisons of hESC lines derived on the same versus different days. (F) Pairwise comparisons of sibling hESC lines versus unrelated lines.

whether OCT4 and CDX2 expression and localization in human preimplantation embryos could explain why derivation after day 6 of in vitro culture is more efficient.

Embryos used in stem cell derivation are most commonly staged in terms of days postfertilization, with the day of insemination representing day 0. We observed that the majority of human embryos at day 5 (approximately 40–75 cells, blastocyst grade 3) had high levels of OCT4 expression in both the ICM and TE, while some embryos had begun to express CDX2 in some, but not all, of the TE (Figure 1A). By day 6 (approximately 75–145 cells, blastocyst grades 4 and 5), blastocysts exhibited a clear restriction of high levels of OCT4 expression to the ICM and of CDX2 to the TE (Figure 1B). By day 8, OCT4 expression was confined to a small number of cells in the presumptive ICM (Figure 1C). As most of cells of the day 8 embryo did not express OCT4 or CDX2, this observation suggests that a cell type other than ICM or TE proliferates at this late stage in vitro. While the identity of these cells is unclear, their proliferation and survival are minimal upon ICM explant for hESC derivation.

Together, these observations suggest that the ICM and TE cells of early day 5 blastocysts may not yet be restricted to either fate, and therefore isolated ICM cells only rarely give rise to ESCs. The restriction of OCT4 expression to the ICM and of CDX2 to the TE on day 6, together with the increase in ICM cell number, may explain why derivation on day 6 is most efficient. Once segregation of the ICM and TE

populations has occurred, derivation efficiency remains high on days 7–9 (Table 1) despite a reduction in the number of OCT4-expressing cells.

The morphological and molecular differences we observed between preimplantation embryos at various developmental time points led us to question whether hESCs derived from embryos on days 5–9 differ in their gene expression programs. It was suggested that pluripotent stem cells isolated from the epiblast of mouse peri-implantation embryos are the mouse equivalent to hESCs. These epiblast stem cells differ in their gene expression profile from mouse ESCs isolated from preimplantation stage embryos and share similarities to hESCs (Brons et al., 2007; Tesar et al., 2007). We therefore examined whether hESC lines derived from days 5–9 generate different types of stem cell lines. We found that hESC lines isolated from different days of development were identical in their growth requirements and expressed the same pluripotency-associated antigens (Table S2). We further analyzed the gene expression profile of 16 hESC lines derived on days 5–9 of development and found that these lines did not group into separate clusters based on their day of derivation (Figure 1D). The distribution of pairwise correlation coefficients (R) between lines derived from the same day of development was indistinguishable from lines derived from different days (Student's t test, $p = 0.12$, Figure 1E). In contrast, when all pairwise correlation coefficients were grouped according to genetically related versus unrelated lines, the similarity between sibling lines was significantly higher than between unrelated lines (Student's t test, $p = 1.1 \times 10^{-8}$, Figure 1F). These observations suggest that the gene expression differences among hESC lines are due to genetic parentage rather than the day or method of derivation. Such differences likely contribute to the variation in differentiation propensity reported between hESC lines (The International Stem Cell Initiative, 2007; Osafune et al., 2008).

The 50% derivation efficiency we achieved using day 6 embryos, laser surgery, and modified embryo culture parameters was higher than previously reported from either our or other laboratories (Cowan et al., 2004; Lerou et al., 2008; Thomson et al., 1998) (Table S5). The increased efficiency and reliability of this method has also allowed us to derive cohorts of stem cell lines that would not have previously been obtainable, including 22 sibling cell lines. These sibling cell lines will be a valuable resource for further investigation of the effects of genetic background on the growth characteristics, pluripotency, and differentiation potential of hESCs.

Our findings increase the probability of successful derivation from rare embryos such as those obtained after preimplantation genetic diagnosis or somatic cell nuclear transplantation. A detailed understanding of the naturally occurring variations among hESC lines will also be important for insight into the genetic regulation of human development as well as for evaluating pluripotent stem cells generated by reprogramming.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, six figures, and five tables and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(08)00618-8.

ACKNOWLEDGMENTS

We thank E. Trish for technical assistance, K. Osafune for help with ESC culture, and J. Sprague and G. Hardiman for assistance in microarray analysis. A.E.C. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research and Merck Research Laboratories. H.A. is funded by Health and Labour Sciences Research Grants, Japan. K.E. is a fellow of the John D. and Catherine T. MacArthur Foundation. K.Z. and J.D. are funded by the University of California, San Diego new faculty startup fund. J.D. is a California Institute for Regenerative Medicine postdoctoral fellow. This work was supported by the Harvard Stem Cell Institute as well as the Stowers Medical Institute and Howard Hughes Medical Institute.

Received: July 30, 2008
Revised: November 14, 2008
Accepted: December 2, 2008
Published: February 5, 2009

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RBMOnline - Vol 16 No 1. 2008 41-50 Reproductive BioMedicine Online; www.rbmonline.com/Article/3061 on web 13 November

Symposium: Nuclear reprogramming and the control of differentiation in mammalian embryos

Elucidating nuclear reprogramming mechanisms: taking a synergistic approach

Dr Hidenori Akutsu became interested in nuclear reprogramming in mammalian species when he was a research fellow at University of Hawaii under Dr Ryuzo Yanagimachi. This interest endured and motivated him to undertake further research under Dr Minoru Ko at NIA/NIH (embryo genomics) and Dr Kevin Eggan at Harvard University (epigenetic and nuclear reprogramming). While at Harvard University he also became an important part of Dr Douglas Melton's team, deriving human embryonic stem cell lines which were later offered freely to the scientific community to facilitate the efforts of other scientists. His special interests are egg development, epigenetic and nuclear reprogramming and embryonic stem cells.

Dr Hidenori Akutsu

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Abstract

Nuclear reprogramming is the process by which a differentiated somatic nucleus has developmental potential restored to it. It involves heritable changes in gene expression as well as structural and functional changes to chromatin structure. This process is naturally induced immediately after fertilization, but can also be artificially induced by nuclear transfer, cell fusion and also now by viral transduction with four stem cell genes. However, the frequency of successful reprogramming is low in each system. The highest success rates, those using nuclear transfer, are only of the order of 2–5%. This article briefly reviews these three methods and proposes a synergistic approach where conditions that facilitate reprogramming in one system are transposed to the others. This might increase the incidence of successful reprogramming and identify common steps necessary for the reacquisition of developmental potential.

Keywords: *developmental potential, differentiation, embryonic stem cell, nuclear reprogramming, nuclear transfer, pluripotency*

Cell differentiation and nuclear reprogramming

Cell differentiation is the process by which a cell becomes specialised to perform specific biological functions (Gurdon, 1968). The process is associated with a decline in the range of cell types that the cell is capable of generating (Gurdon, 1968). It had been initially thought that as cells differentiated, hereditary material no longer required was cast off or permanently inactivated (Weismann, 1893). However, this paradigm was shown to be false more than 50 years ago when Briggs and King transferred differentiated nuclei from blastula cells to enucleated eggs of the frog *Rana pipiens* (Briggs and King, 1952). These reconstructed cells went on to generate normal hatched embryos, showing that nuclei of differentiated cells contain the same genetic material as those of undifferentiated cells. The current paradigm for how cell differentiation occurs involves the assembly of condensed chromosomal structures (Kass and Wolffe, 1998). Such structures, formed via interactions between DNA and protein, are thought to compartmentalize chromatin into functional domains and, in some unknown way, stably maintain the differentiated state even when the cell divides.

In terms of mammalian development, differentiation first occurs at the blastocyst stage in the preimplantation embryo. As the embryo develops, the outer layer cells of the embryo (the trophectoderm) become morphologically distinct from the inner cell mass (ICM). Cells of the trophectoderm and ICM have different developmental potentials, e.g. cells of the ICM have the potential to form all the cells of the conceptus, whereas the trophectoderm cells have only the potential to form extraembryonic cells or the placenta.

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The processes responsible for the epigenetic changes that lead to dedifferentiation are referred to as nuclear reprogramming mechanisms (Rideout *et al.*, 2001). Nuclear reprogramming in this sense refers to the process by which a specialized nucleus re-acquires developmental capacity. This definition includes complete reprogramming to a totipotent state (verifiable only by generation of viable offspring) and also partial reprogramming where pluripotency (the capacity to generate cells representative of all three germ layers) is restored. By necessity, it involves heritable changes to gene expression, i.e. changes in gene expression that are passed on to daughter cells. Some have suggested that transient changes to gene expression constitute nuclear reprogramming (Hakelien *et al.*, 2002), but such changes do not persist, nor is there any evidence that they are transferred to progeny cells. Such observations almost certainly result from residual transcription activity rather than the consequence of a reprogrammed genome, and so these examples do not constitute nuclear reprogramming as defined here and elsewhere (Hochedlinger and Jaenisch, 2006).

Naturally induced nuclear reprogramming

The differentiated state of cells is found to be extremely stable (Kato and Gurdon, 1993). The only stage during which normal mammalian cells seem to naturally dedifferentiate immediately follows fertilization (Schultz *et al.*, 1999). The sperm and oocyte, both highly differentiated cells with condensed chromatin structure, fuse to produce a zygote. Within the zygote, changes lead to the reversion to a less specialised totipotent cellular state (Kelly, 1977). Although the mechanism responsible is unknown, two events are associated with this dedifferentiation: chromatin structure becomes less dense: protamines are removed from sperm-derived chromatin and replaced by oocyte-derived histones (Perreault, 1992); and methylated haploid parental genomes are demethylated (Barton *et al.*, 2001).

Additionally, it has been speculated that inappropriate or incomplete nuclear reprogramming may occur in a pathological context, i.e. during the generation of teratomas. Teratomas are benign tumours associated with chaotic cell-lineage formation. The 'dedifferentiation' theory of cancer states that such lineages may arise from cells that have undergone dedifferentiation to a multipotent state (Ribbert, 1911). Teratomas can also be produced experimentally by injection of pluripotent stem cells into ectopic sites of a syngeneic animal (Evans and Kaufman, 1981; Matsui *et al.*, 1992; Rensnick *et al.*, 1992), so it is conceivable that inappropriately reprogrammed somatic cells could be the origin of such cancers.

Artificially induced nuclear reprogramming

In non-transformed somatic cells, once the differentiation programme of a cell has started, the process is normally irreversible. However, this programme may be reversed artificially. Using nuclear transfer (NT) (Wilmut *et al.*, 1997), cell fusion (Tada *et al.*, 1997), or even viral transduction of four specific stem cell genes (Takahashi and Yamanaka, 2006), it is possible to artificially and heritably alter a cell's gene expression and its functional identity. These techniques are collectively termed 'artificial induction of nuclear reprogramming' (**Figure 1**). The conversion of differentiated cells to pluripotent cells illustrates that cells do not permanently lose the ability to be pluripotent during differentiation.

Frustratingly, the mechanism by which a somatic nucleus may be reprogrammed remains unknown, aside from the fact that such a mechanism almost certainly involves both structural (Kikyo *et al.*, 2000) and chemical (Monk *et al.*, 1987) changes to chromatin. It may be possible for human somatic cells to be reprogrammed to a pluripotent state. If successful, this strategy would provide a potentially endless source of cells for biological research, as well as medical applications (Stojkovic *et al.*, 2005; Verlinsky *et al.*, 2005), toxicity assessment, drug testing and possibly even gene therapy (Wobus and Boheler, 2005). **Figure 2** illustrates how identification of reprogramming molecules and mechanisms could facilitate cell replacement therapy in humans. Over the past century, organ transplantation has evolved rapidly to the current widespread use of donated organs for the treatment of end-stage kidney, heart, and liver failure. However, with limited supplies of organs and an increasing demand for them, many patients who need transplants do not receive them (Gridelli and Remuzzi, 2000). The increasing gap between supply and demand for tissue and organ transplants means that harnessing nuclear reprogramming mechanisms is important (Sullivan and Eggan, 2007).

Nuclear transfer: the oldest and still the most reliable reprogramming technique

Spemann (1938) originally suggested transplantation of nuclei between cells as a technique to study the role of genetic material in cellular differentiation. In nuclear transfer, the nucleus from a differentiated donor cell is transplanted into an enucleated oocyte. The oocyte can reprogramme even an adult differentiated nucleus and the new cell can develop as an embryo. Artificially induced nuclear reprogramming by NT was first demonstrated by Briggs and King in 1952, when they showed that transfer of somatic nuclei to enucleated eggs can direct development so that tadpoles are generated (Briggs and King, 1952). Gurdon later refined the technique so that adult and fertile frogs could be generated (Gurdon *et al.* 1958). Decades later, the production of 'Dolly', the first viable mammal derived by reprogramming a fully differentiated adult somatic cell, illustrated that the mammalian nuclear genome can be completely reprogrammed and totipotency of the nucleus restored (Wilmut *et al.*, 1997). The nuclei of these offspring contain genomes of identical sequence to that of the nuclear donor. At present, nuclear transfer is the only technique in which one can accomplish total nuclear reprogramming in an unequivocal manner; by deriving viable offspring from a reconstituted embryo. More recently, embryonic stem (ES) cells have been derived from cloned mice (Wakayama *et al.*, 2001). The ES cells produced by somatic cell nuclear transfer retained self-renewal and pluripotent features, contributing to all germ layers, including the germline. In addition, gene expression profiling experiments showed the ES cell lines derived from cloned and fertilized mouse blastocysts are indistinguishable (Brambrink *et al.*, 2006). The NT–ES cells are developmentally and functionally equivalent to the fertilization-derived ES cells.

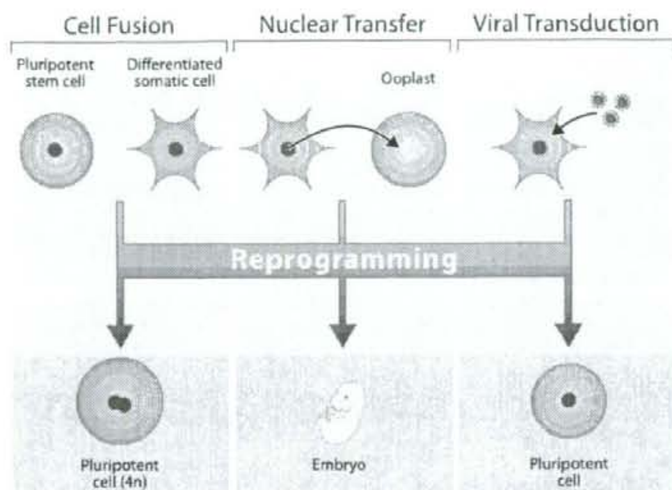

Figure 1. Artificially induced nuclear reprogramming. Cell fusion: a somatic cell fused with a pluripotent stem cell can be reprogrammed in the hybrid cell. These fused hybrid cells show similar features as embryonic stem (ES) cells; however the hybrid cell has atetraploid karyotype and is unable to contribute to chimeras. Nuclear transfer: an adult somatic cell is transferred into an enucleated oocyte followed by artificial activation. These nuclear-transferred embryos can produce ES cells which are pluripotent, contributing to all germ layers including the germ cell lineage. Viral transduction: a somatic cell transduced by retroviruses expressing four key genes, *Oct3/4*, *Sox2*, *Klf4* and *c-Myc*, can be reprogrammed into iPS cells resembling ES cells in a cell-autonomous fashion. Only the nuclear transfer method can produce viable animals as it can return an adult nucleus to a totipotent, embryonic state.

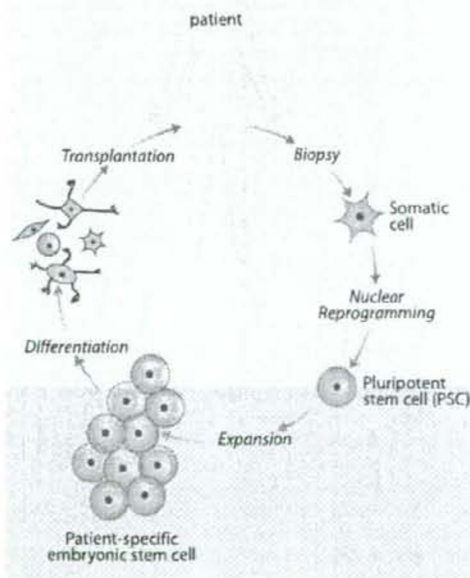

Figure 2. The ultimate goal of nuclear reprogramming research: controlled restoration of developmental potential. Once the mechanism by which nuclear reprogramming is understood, human somatic cells could be induced to dedifferentiate into pluripotent stem cells (PSC). PSC could then be expanded in culture and induced to redifferentiate into the cell type(s) required by the patient. These non-allogenic differentiated cells could then be transplanted into the patient with a decreased risk of immunorejection. It is also important to point out that patient matched pluripotent stem cells can also serve as in-vitro models for studying human disease and development at a cellular and molecular level. Such reprogramming will also allow the generation of genetically matched ES cells will, in themselves, provide scientists and clinicians an important new tool to recapitulate onset of specific diseases *in vitro* (Di Giorgio *et al.*, 2007).

Successful reprogramming of somatic nuclei by placing them in enucleated oocytes should perhaps not have been completely unexpected. There are compelling reasons why a system should exist for the removal of epigenetic modifications (excluding gametic imprints) in the oocytes and sperm. They are both highly specialized differentiated cells, and removal of their epigenetic patterns is essential to allow development of pluripotent cells from the inner cell mass (ICM). The same mechanism may be causing reprogramming of a somatic nucleus when exposed to the cytoplasm of an oocyte (Surani, 1999).

Many variables affect reprogramming success with NT. Some of these have been identified, i.e. structural integrity of the nuclear membrane (Willadsen 1986), quality and copy number of donor genetic material, chromatin conformation, histone composition, methylation and acetylation patterns (Campbell, 1999). Also important is the level of maturation or mitosis promoting factor (MPF) (Fulka *et al.*, 1996) and synchronization of donor and recipient cell cycles prior to embryo reconstruction (Campbell, 1996). High MPF concentrations in the oocyte and appropriate synchronization of donor and nuclear cell cycle using serum starvation are thought to minimize chromosomal damage and promote generation of reconstructed embryos that divide to produce normal diploid daughter cells.

Campbell suggests that the frequency of live offspring generation from reconstructed mammalian embryos made by NT is improved when the donor nuclei are in a quiescent state (Campbell *et al.*, 1996; Campbell, 1999). The successful production of Dolly, the first viable animal to be generated by nuclear transfer, used a nucleus from a cultured adult-differentiated somatic cell that had been serum starved into quiescence (Wilmut *et al.*, 1997). Kato *et al.* (1998) reported cloning of calves at 80% success ratio based on the number of transferred embryos using quiescent cumulus cells and oviduct epithelial cells that were cultured for several passages followed by serum starvation. Alternatively, using non-cultured cells also succeeded in producing cloned animals. Wakayama *et al.* (1998) used mouse cumulus cells, a naturally quiescent cell population, as nuclear donating cells in successful nuclear transfer experiments with mouse ooplasts. Ogura *et al.* (2000) made cloned mice by transferring Sertoli cells into enucleated mature oocytes. In both of these experiments, the cell cycle stage of the nuclear donors was controlled but the possibility that animals can be generated using non-quiescent cells as nuclear donors cannot be dismissed. Other researchers claim successful generation of mammalian offspring from nuclei not intentionally induced into a quiescent state (Cibelli *et al.*, 1998). Also, the possibility that transferred nuclei in Wakayama's and Ogura's experiments were non-quiescent cannot be eliminated.

Presumably, the importance of the state of the donor nucleus cell cycle is directly linked to compatibility with the recipient oocyte cytoplasm. Metaphase of second meiotic division (MII) oocytes has typically become the state of choice of recipient cytoplasts for NT procedures (Campbell *et al.*, 1996). MII oocytes contain active MPF to induce nuclear envelope breakdown (NEBD), premature chromosome condensation (PCC), and dispersion of nucleoli in the transferred nucleus, which may be essential for nuclear reprogramming. The donor nucleus in S phase of the cell cycle is likely to be incompatible with a high MPF state, leading to DNA damage and arrest at an early cleavage stage. However, inter-species NT experiments suggest that the occurrence and extent of NEBD and PCC in the donor nucleus are variable between different species, donor cell types and different procedures (Meissner and Jaenisch, 2006).

It had been thought previously that only the cytoplasm of the MII oocyte can support reprogramming after NT, so numerous species have been cloned by NT into MII oocyte (Meissner and Jaenisch, 2006). It seemed necessary for initiating reprogramming that the donor nucleus had elevated MPF concentrations, since NT embryos fail to develop, transforming into interphase zygotes (McGrath and Solter, 1984; Wakayama, 2000). However, more recently, a new insight disproving a myth of MII necessity for NT has been reported (Egli *et al.*, 2007). Unlike interphase zygotes, fertilized zygotes arrested in mitosis can fully support the reprogramming of somatic cells to the totipotent state. This indicates that factors sufficient for reprogramming are not limited to oocytes, and suggests that a continuum of activity extends beyond the unfertilized egg (Egli *et al.*, 2007). Why is the metaphase cell useful for reprogramming? A possible explanation is that condensed chromatin expels transcription factors like Oct-3/4 and Sox2 (Martinez-Balbas *et al.*, 1995), and without a nuclear membrane to enclose them, they are free to interact with any foreign chromatin introduced. Also, as the cell is poised to divide in M phase, it has synthesized many components of the cell to elevated levels, so presumably factors necessary for reprogramming are present in a greater abundance than at other stages of the cell cycle.

Experiments by Eggan *et al.* (2001) show that the number of live mice generated from cells reprogrammed via nuclear transfer is dependent on the genetics of the mouse from which the nuclear donor cell is taken. ES cells taken from inbred 129/SvJae mice fail to produce any post-natal surviving offspring, whereas cloned pups derived from ES cells of C57BL/6 and 129/SvJae matings can survive to adulthood. It may be that the use of inbred animals as nuclear donors introduces a reprogramming barrier not present in hybrids strains. Investigating why this occurs might elucidate more about mechanisms involved in nuclear reprogramming.

Much remains to be learnt about how somatic nuclei are reprogrammed after being transferred into ooplasts. For example, what factors and signalling pathways are involved in altering the chromatin structure, methylation patterns, and gene expression during reprogramming? Is there a master trigger that induces a cascade of downstream events or does it take several factors working in parallel pathways to initiate reprogramming? This might be the case as the frequency of successful reprogramming is so low. How do subtle epigenetic differences from normal animals, such as methylation/acetylation patterns, contribute to the abnormalities that cloned animals often exhibit?

In summary, successful production of cloned animals by NT proved that somatic nuclei could reverse their developmental clock to recreate totipotency in the oocyte. The transferred nuclei must be reprogrammed in resetting of an embryonic transcriptional programme. Although NT remains the tool

of choice for studying reprogramming at a functional level, less technically demanding approaches may be helpful for dissecting reprogramming at the cellular, molecular and biological levels (Hochedlinger and Jaenisch, 2006).

Cell fusion: a reprogramming system with the challenge of tetraploidy

Cell fusion is the mechanism by which reprogramming occurs naturally; a haploid oocyte fuses with a haploid spermatozoan. Artificially induced cell fusion generates tetraploid cells which, due to their lack of contribution to chimeras and their perceived susceptibility to turn aneuploid and abnormal, are of limited therapeutic use (Tada *et al.*, 1997; Sullivan and Eggan, 2007). However, cell fusion is the only system yet to show reprogramming in humans (Cowan *et al.*, 2005), and if it was possible to harness cell enucleation strategies either by naturally occurring (erythrocyte enucleation or selective genome ejection systems seen in insects species such as fire-ants) or artificial means (cytoplast/whole cell fusions, or manual chromatin removal), this problem could be surmounted (Sullivan and Eggan, 2007).

Cell fusion, apart from being a potential therapy, has provided a model system where aspects of how cell-specific phenotypes are initiated and maintained can be examined in fusion products of different cell types (intertypic synkaryons). Monoclonal antibodies and polymorphisms between fusion partners can be used to study gene expression at the single cell level or in mass cultures at a biochemical and molecular level. Regulatory mechanisms governing cell fate and differentiation have been partially elucidated by studying differences among cell types in the frequency, kinetics, and patterns of gene expression. The results of both strategies applied to heterokaryons and cell hybrids show that the expression of genes in the nuclei of differentiated cells is remarkably plastic and susceptible to modulation by the cytoplasm (Boshart *et al.*, 1993). Isolation of genetically stable cell hybrids can be achieved using selection for transgenes integrated in, or against mutations occurring in, only one of the parental cell types. Generation of cell hybrids has elucidated three principles of cell differentiation (Boshart *et al.*, 1993): (i) trans-acting gene regulators are involved in cell differentiation; (ii) such regulators repress as well as activate cell-specific gene expression; and (iii) maintenance of the differentiated state is dependent on such factors.

In intertypic somatic hybrids, genes associated with specialized function are often shut down. Such repression is termed 'extinction'. Extinction is a commonly observed feature of intertypic hybridization (Davidson, 1974). One interesting example of hybridization provided the first direct evidence that telomere length determines proliferative capacity in human cells (Wright *et al.*, 1996). In immortal cell lines, the ends of the chromosomes (telomeres) are constitutively replenished by the ribonucleoprotein enzyme telomerase (Counter *et al.*, 1992), while in somatic cell types, telomere length is found to shorten with age (Lindsey *et al.*, 1991; Vaziri, 1997). Hybrids of immortal and somatic cells are found to have limited life span, and this is due to the extinction of the telomerase gene (Wright, 1996). Treating these cell hybrids with specific oligonucleotides results in telomere elongation. It is thought that telomere elongation reduces the probability of DNases cutting into essential regulatory and expressed sequences in chromosomal DNA and so extends the life span of the hybrids (Wright *et al.*, 1996).

Gene repression is far more commonly observed than activation (Baron *et al.*, 1996). However, it has been observed that activation of cell-type specific gene expression can also occur when different cell types are fused (Baron *et al.*, 1996). An interesting example of activation involves fusing erythroid cells at different developmental stages (Broyles, 1999). The phenotype of hybrid cells involves the retention of specific chromosomes (Weiss and Chaplain, 1971), and is dependent on the number of copies of the individual chromosomes retained. For example in hepatoma × fibroblast hybrids possessing only one copy of hepatic chromosomes, the hepatic phenotype is not observed; if, however, the hybrid contains two sets of hepatic chromosomes, the hepatic phenotype is present. Clearly a delicate equilibrium between positive and negative trans-acting factors mediates hybrid phenotype (Peterson and Wess, 1972). It is interesting to juxtapose these data with similar findings from imprinting experiments injecting transgenes containing differentially methylated regions (Reik *et al.*, 1999). Introduction of such genes alters the methylation status of the chromosomal DNA, also indicating a trans-acting mechanism with a delicate equilibrium (Reik *et al.*, 1999).

In summary, cell hybridization experiments have shown that trans-acting gene regulators control the differentiated state of a cell. Somatic cells may be reprogrammed by fusion with pluripotent stem cells; however, in this case, the persistence of ES cell-derived chromatin causes applicative and interpretive complications, i.e. the resulting tetraploid cells are of limited therapeutic use and it is still unknown whether the ES cell chromatin remaining in the fusion product is playing an active role in the perpetuation of the resultant phenotype.

iPS cell transduction: a technique to study reprogramming at the molecular level

There is currently much interest in the reprogramming community surrounding 'induced pluripotent stem (iPS) cell transduction' (Takahashi and Yamanaka, 2006) (**Figure 1**), a novel approach that uses four transcription factors to restore an ES cell-like phenotype to murine fibroblasts (Rodolfa and Eggan, 2006). By simply transducing murine fibroblast cultures with Moloney virus coding for four stem cell factors (Oct3/4, Sox2, Klf4 and c-Myc), it appears that a pluripotent stem cell-like state can be restored. This is particularly exciting when one considers that the techniques involved (cell culture and viral transduction) are commonly used in many laboratories worldwide already. New work on iPS cells has recently been published from three different laboratories (Rodolfa *et al.* 2007). They showed iPS cells selected for Nanog expression can contribute to all tissue types including germ cells. Amazingly, the Nanog–iPS cells closely resemble ES cells in their epigenetic state as well as genetic activity (Okita *et al.*, 2007; Wernig *et al.*, 2007; Maherali *et al.*, 2007). Many laboratories worldwide can now use this method to elucidate

reprogramming mechanisms. Further published work with this technique is eagerly anticipated, as several questions have still to be answered: for example what cells are being transduced to generate these iPS cells? Can this be done with human cells? What is the molecular basis of reprogramming induced by the four factors? Is it the same process that happens during NT and cell fusion reprogramming? Can the implicated genes be activated and induce reprogramming without use of oncogenic virus (Surani, 2007)?

Screening for reprogramming factors

Reprogramming remains largely phenomenological, and efforts should now aim to dissect the mechanism at the molecular level (Hochedlinger and Jaenisch, 2006). Oocytes, preimplantation embryos, and pluripotent stem cells contain factors sufficient for reprogramming, and so constitute good material for identifying reprogramming factors (Hamatani *et al.*, 2004; Ko, 2006). Beyhan *et al.* (2007) reported global gene expression analysis of bovine NT, IVF embryos and donor somatic cells to characterize differences in their transcription profiles. They have found a small set of genes differentially expressed as well as genes of donor cells persistently expressed in NT embryos. Investigating gene expression changes that occur during or soon after reprogramming should elucidate the molecular mechanisms involved.

Another approach includes the use of mass spectrometry to identify reprogramming factors in cells and cell-derived extracts (Koziol *et al.*, 2007). Cell extracts have been shown to induce transient changes in gene expression and chromatin structure in differentiated cells (Dimitrov and Wolffe, 1996), which, if maintained, could possibly result in reprogramming. However, a caveat to these approaches is that the initial induction of reprogramming may only involve subtle changes in gene expression that then cumulatively elicit a pronounced effect. A more forceful approach would be to individually overexpress the four factors shown by Yamanaka and colleagues to reprogram differentiated cells (Takahashi and Yamanaka, 2006) and analyse the resulting genome-wide changes in gene expression. Alternatively, small molecule or RNAi screens could be performed to identify the important factors (Edwards, 2006).

Induction and maintenance of nuclear programmes has, for many years, been considered to be directed solely by proteins involved in gene regulation and morphogenic signalling. Many researchers have carried out reprogramming screens for proteins only to pull out generic chromatin remodeling factors. Additional candidates now need to be considered, including non-proteinaceous macromolecules. RNA, for example, has now emerged as a key player in a surprisingly large number of gene regulation studies. For example, the activity of X chromosomes in female mammals is controlled by non-coding RNAs such as *Xist* and *Tsix*. Furthermore, microRNAs (miRNAs), a large family of short non-coding RNAs (17–25 nucleotides) that mainly function to repress expression of their target genes, regulate blood development (Yekta *et al.* 2004). Tang *et al.* (2007) have recently showed a large proportion of the maternal genes are directly or indirectly under the control of miRNAs, which demonstrates that the maternal miRNAs are essential for the earliest stages of mouse embryonic development. It would not be surprising if non-coding RNA has further roles in specific and stable regulation of developmental programmes. miRNA may have an important role in nuclear reprogramming.

An alternative approach to studying artificial reprogramming, which could be expanded further, has been to study naturally induced reprogramming in lower vertebrates where it occurs successfully and more frequently and to look for common elements in more complex organisms. Unlike mammals, many fish and amphibia have the capacity to regenerate complex structures such as limbs after injury. Even mammals have this capacity in Msx1 expressing regions at the digit termini and more widely during early embryonic phases (Han *et al.*, 2003). This process involves cell migration and a change in cell phenotype in response to the injury. There are certain caveats here, however. It is hard to dissect process important for reprogramming from other processes such as the innate immune response, cell migration, and other consequences of injury. It is also unknown to what extent these processes are conserved in mammals. Still, dedifferentiation of cells to form proliferating progenitor cells is interesting, and systems such as skeletal muscle, limb and tail regeneration or dorsal iris epithelium during lens regeneration should be studied further with screens designed to find the key players involved.

The main challenge facing elucidation of nuclear reprogramming mechanisms using the conventional approaches, and potential solutions

The main problem with current studies investigating nuclear reprogramming mechanisms is the lack of material due to the low frequencies of reprogramming using artificial methods. Conventional approaches entail isolating and expanding reprogrammed cells in strongly selective culture conditions [e.g. in cell fusion experiments (Tada *et al.*, 1997; Cowan *et al.*, 2005) hybrid clones were isolated by antibiotic resistance and expanded]. Analysing such material, however, does not allow discrimination between the epigenetic changes necessary for the induction of reprogramming versus those that happen independently of such induction; i.e. it does not allow the study of reprogramming as it is happening.

How can the study of this process be facilitated? One strategy is to use easily reprogrammable cells, such as cells differentiated from ES cells in culture (Blelloch *et al.*, 2006; Silva *et al.*, 2006). Perhaps the initial focus should be on cultured cells instead of later primary cells, as these will still have strong epigenetic regulation, and thus would be harder to reprogram. Experiments with cultured cells should yield more reprogrammed material.

Additionally, it would be possible to use chromatin modifying drugs such as trichostatin A and 5-aza-2'-deoxycytidine to make the chromatin less condensed and more accessible. Factors required for activating the *Oct-3/4* gene are unknown, but recently it has been shown that two chromatin modifying drugs can activate the *Oct-3/4* gene in cells (Hattori *et al.*, 2004). These two drugs, trichostatin A (TSA) and 5-aza-2'-deoxycytidine

(5-aza-dC), which inhibit histone deacetylation and DNA methylation respectively, are thought to make the chromatin structure more open and consequently the *Oct-3/4* gene easier to activate. However, such drug treatment is quite toxic to the cells as well as being non-specific (these drugs reactivate many genes including those not associated with an ES cell phenotype (S Sullivan, unpublished data). Tsuji-Takayama *et al.* (2004) have recently shown that treatment of differentiated ES cells with a similar chemical to 5-aza-dC, called 5-azacytidine, causes the up-regulation of stem cell marker genes *Oct-3/4*, *Nanog* and *Sox2*. As with Hattori's work, the expression of genes associated with differentiated cells were not studied, and it is expected that these too will be up-regulated. It will be very interesting to screen for more specific drugs that increase the frequency of reprogramming.

Thirdly, although the reason is unknown, cell cycle synchronization by serum starvation makes murine embryonic fibroblasts (MEF) more easily reprogrammed both by NT (Campbell, 1996) or cell fusion (Sullivan *et al.*, 2006) This strategy could also facilitate reprogramming studies.

Can one learn about reprogramming and improve its efficiency by transposing conditions between the three reprogramming methods?

In order to learn from experiments using the three different methods to deduce the reprogramming mechanism(s) and improve their efficiencies, it is necessary to compare and contrast observations from them. At present, it is difficult to dissect the important events such as changes in gene regulation and chromatin structure during the reprogramming processes due to the inefficiency of all three methods, but some hints can be gathered from existing kinetic, gene expression, and cell cycle data. The kinetics of reprogramming appears to be very similar between NT and cell fusion. Somatic cell-derived transgenic Oct-3/4 is expressed within 24 h after NT and cell fusion (Sullivan and Egli, unpublished data). In contrast, reprogramming experiments using viral transduction have shown that stem cell genes *Alkaline Phosphatase*, *SSEA-1*, and *Nanog* are not highly expressed until 2–3 weeks post-infection (Blelloch *et al.*, 2007; Maherali *et al.* 2007; Meissner *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007), indicating that reprogramming proceeds at a slower pace with this method. The need to synthesize the four reprogramming genes *de novo* can only partially explain the slower kinetics of reprogramming using the viral transduction method. It is likely that other proteins that facilitate the induction of reprogramming during NT and cell fusion are missing, or that the entire transcriptional programme required for reprogramming, which is more completely expressed by the oocyte during NT or the ES cell during cell fusion, is vast and requires a substantial amount of time to execute. For example, demethylation of promoters of endogenous genes such as Oct-3/4 may occur very slowly during reprogramming by viral transduction if factors required for active demethylation are not produced as they are thought to be during NT (Yamazaki *et al.* 2006).

The two pluripotency genes used in the iPS cell viral transduction approach, *Oct-3/4* and *Sox2*, are expressed in oocytes (Avilion *et al.*, 2003; Monti *et al.*, 2006) and mouse ES cells (Yamanaka, 2007), suggesting that their roles in establishing and/or maintaining pluripotency are conserved in all three reprogramming approaches. Yamanaka posits that c-Myc may make the chromatin more accessible to transcription factors by binding to many sites in the genome and inducing histone deacetylation in addition to promoting self-renewal, as it does in murine ES cells (Cartwright *et al.* 2005; Yamanaka, 2007). c-Myc is expressed in oocytes (Naz *et al.* 1994) but is not highly expressed in mouse ES cells (Blelloch *et al.*, 2007). However, a functionally equivalent family member, n-Myc, is expressed and can substitute for c-Myc in iPS cell transduction (Blelloch *et al.*, 2007). Thus, Myc proteins may stimulate self-renewal in iPS cell transduction, cell fusion and NT. *Klf-4* is highly expressed in mouse ES cells (Yamanaka, 2007) and thus may play a role in reprogramming during cell fusion.

Cell cycle synchronization of the somatic cells into G_0/G_1 or G_2/M prior to NT or cell fusion increases the efficiency of reprogramming (Campbell *et al.*, 1996; Sullivan *et al.*, 2006). This effect is attributable to avoiding the aneuploidy or chromosomal damage risked by nuclear transfer or cell fusion during S phase. Yamanaka used unsynchronized cells in the iPS cell transduction experiments because active cell division is a requirement for infection by Moloney retrovirus. Egli and coworkers determined that a zygote arrested in mitosis can reprogram a somatic nucleus while an interphase zygote cannot (Egli *et al.*, 2007). A major difference between a mitotic zygote and an interphase zygote is that the nuclear membrane has broken down in the mitotic zygote. Therefore, it is possible that factors required for reprogramming are sequestered in the nucleus during interphase and released during mitosis. In cell fusion in mice, ES cells in G_2/M phase were the most effective at reprogramming, suggesting that key reprogramming activities at that stage of the cell cycle (Sullivan *et al.*, 2006).

Now there is the opportunity to use observations made in one method of reprogramming to try to improve the other methods. For example, will overexpressing some or all of the four Yamanaka factors in ES cells make reprogramming by cell fusion more efficient? The best evidence that this might be the case is given by Silva and coworkers. They reported elevated frequencies of reprogramming in a cell fusion system where *Nanog*, a pluripotency gene not necessary for iPS cell formation by viral transduction, was overexpressed in the ES cell fusion partner (Silva *et al.*, 2006). High Nanog levels may assist the induction of reprogramming indirectly as positive feedback circuits involving Nanog elevate Oct-3/4 and Sox2 levels (Loh *et al.*, 2006).

It will also be interesting to introduce c-*Myc* and *Klf-4* transgenically into cells to be reprogrammed by NT or cell fusion, to see if this increases the frequency of reprogramming; however, as these gene are both oncogenes, the resultant cells should be tested for epigenetic and genetic abnormalities. There is an additional caveat with this approach; what is learned from reprogramming genetically manipulated, cultured cells may not immediately inform the process of reprogramming normal primary somatic cells, which still have all epigenetic regulatory processes intact. It is, however, a first step towards reprogramming primary cells and should give enough material to untangle the various mechanisms.

Slow demethylation or chromatin re-structuring may be why Yamanaka's viral transduction method proceeds more slowly than NT or cell fusion. This seems likely, given that the other two methods have other factors that could potentially speed up these processes. For example, Yamazaki and coworkers found that even in NT, demethylation of the *Oct-4* promoter proceeds gradually and is probably a result of both active and passive mechanisms for demethylation (Yamazaki *et al.*, 2006). Yamanaka's four factors may not be sufficient to induce active demethylation, and may be dependent on the passive mechanism alone, causing slower reprogramming. Overexpression of de-novo methyl-transferase genes such as *Dnmt-1* or *Dnmt-3* might facilitate the process. Alternatively, if chromatin remodelling is the rate-limiting step, small molecule HDAC inhibitors could expedite reprogramming.

In the future, determining the list of genes that are up-regulated in ES cells during G_2/M phase or proteins that are localized in the nucleus during interphase in zygotes will significantly concentrate the search for genes necessary for reprogramming. Additionally, Yamanaka's work suggests that transcription factor libraries may be the most fruitful source of reprogramming factors.

Currently, it seems reasonable that all three reprogramming methods share a general mechanism involving chromatin remodelling to allow changes in gene expression as the first step, followed by changes to prevent cell death. The last step would be the induction of pluripotency. It also seems likely that the genes used to induce pluripotency are the same in all three methods, while there could be different molecular pathways to cell immortalization and altering DNA accessibility.

Conclusion

NT is the only reprogramming technique known not to require addition of foreign genes to induce restoration of developmental potential. Furthermore, it is still the only method can restore pluripotency without a high risk of oncogenesis. Thus, NT remains a very important system for studying reprogramming. Efficiency by this and the other two methods discussed is, however, still very low and the lack of material limits efforts to identify important factors for reprogramming induction. All three methods (NT, cell fusion, and iPS cell transduction) should be perused so that conditions optimal in one system can be implemented in the others to try to improve reprogramming frequencies. The four iPS cell factors can be introduced into cells that are to be used in NT and cell fusion experiments with the hope of increasing the frequency of reprogramming. It is hoped this will provide more material to study mechanisms and so help understanding of reprogramming. The scarcity of tissues and organs for transplantation, as well as the need for pluripotent stem cells to develop in-vitro models of human disease and development, compel further study of reprogramming mechanisms.

Acknowledgements

SS is funded by a fellowship from the Harvard Stem Cell Research Foundation. JI is funded by a fellowship from the New York Stem Cell Foundation. AU and HA are funded by Health and Labour Sciences Research Grants, Japan. The authors thank Esther Son, Kit Rodolfa, Katelyn Foley, and Gabriella Boulting for proofreading the manuscript.

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Received 27 July 2007; refereed 12 September 2007; accepted 17 October 2007.