

Systems biology discoveries using non-human primate pluripotent stem and germ cells: novel gene and genomic imprinting interactions as well as unique expression patterns

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REVIEW

Systems biology discoveries using non-human primate pluripotent stem and germ cells: novel gene and genomic imprinting interactions as well as unique expression patterns

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Abstract

The study of pluripotent stem cells has generated much interest in both biology and medicine. Understanding the fundamentals of biological decisions, including what permits a cell to maintain pluripotency, that is, its ability to self-renew and thereby remain immortal, or to differentiate into multiple types of cells, is of profound importance. For clinical applications, pluripotent cells, including both embryonic stem cells and adult stem cells, have been proposed for cell replacement therapy for a number of human diseases and disorders, including Alzheimer's, Parkinson's, spinal cord injury and diabetes. One challenge in their usage for such therapies is understanding the mechanisms that allow the maintenance of pluripotency and controlling the specific differentiation into required functional target cells. Because of regulatory restrictions and biological feasibilities, there are many crucial investigations that are just impossible to perform using pluripotent stem cells (PSCs) from humans (for example, direct comparisons among panels of inbred embryonic stem cells from prime embryos obtained from pedigreed and fertile donors; genomic analysis of parent versus progeny PSCs and their identical differentiated tissues; intraspecific chimera analyses for pluripotency testing; and so on). However, PSCs from nonhuman primates are being investigated to bridge these knowledge gaps between discoveries in mice and vital information necessary for appropriate clinical evaluations. In this review, we consider the mRNAs and novel genes with unique expression and imprinting patterns that were discovered using systems biology approaches with primate pluripotent stem and germ cells.

Introduction

To identify the mechanisms underlying pluripotency, a number of studies have been carried out, and these have been recently summarized [1]. The first two studies that characterized the 'stemness gene' list [2,3] identified about 250 putative genes involved in mouse embryonic stem cell (mESC) pluripotency, and many other genes are being studied today [4-7]. While these experiments identified many genes involved in maintenance of pluripotency, such as *Oct-4*, *Nanog* and *Sox-2*, they also usually showed that human ESCs (hESCs) are quite different from each other [4,8-10]. A more comprehensive

study showed that although closely related, the 59 ESC lines showed heterogeneity in gene expression [11]. Interestingly, variations in gene expression were found not only for genes correlated with the pluripotent state or differentiation, but also for housekeeping genes [12]. Therefore, interactions among many genes likely form an active network that allows the pluripotent state to be maintained [13]. In addition, due to this variation between lines, better models need to be established to understand the true underlying mechanisms of pluripotency.

While gene regulatory networks that enhance our knowledge of pluripotency will help our understanding of stem cell biology, there are additional implications. As described below, ESCs are derived from the inner cell mass of a blastocyst [14,15]. Therefore, the ESCs are closely related to the inner cell mass, from which, via post-implantation development, the embryo and fetus form. Thus, differentiation of ESCs recapitulates the earliest stages of human development, and understanding

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the gene regulatory networks in these cells will enhance our knowledge of the regulation of the earliest stages of development.

Currently, we lack much information on specific stages in development. As described, all tissues of the embryo arise from the same cells; however, they are different from each other, not only in their morphology and function, but also in their total DNA content. While somatic cells are diploid, gametes are only haploid. We have almost no knowledge on how these initial cells are selected and differentiated.

Another area that would benefit from the delineation of pluripotent gene networks is the better understanding of reproductive mechanisms. While the sperm meets the egg to form the zygote, what gene expression in the egg allows the first stages of development to proceed? Since *in vitro* fertilization (IVF) and other assisted reproductive technologies are so commonly used, can better understanding of these first stages increase the efficiency of these technologies?

However, while ESC lines contribute to a chimera - which is when cells injected into a new blastocyst contribute to all tissues in the newborn - epiblast lines do not. We have no understanding of the mechanisms that underline the difference between these two types of cells.

ESCs have been studied not only for cell replacement therapy or basic stem cell biology, but also as a tool for development of better and safer drugs. Since the ESC lines resemble, in many aspects, the developing fetus, they can be used as a first and quick tool for drug screening without exposing pregnant mothers and their babies to harmful drugs. Therefore, better understanding of gene regulatory networks that control these cells or allow them to differentiate under specific signals will allow the development of new therapies. These therapies will be based on the finding of new targets and hence the development of treatments specifically for those targets and the processes they control.

Certain cancers have been suggested to have a stem cell origin. Treatment today is usually directed to the amplifying cell rather than to the source of the cancer. Hence, understanding of the gene networks that have changed from those in stem cells and have led to cancer will allow the development of new treatments for the cancer, and as described before, the development of specifically targeted molecules for these pathologies.

To summarize, understanding of the gene regulatory networks that enable a cell to maintain its pluripotent phenotype are of great interest today. Better understanding of these networks will lead to better understanding of basic biology questions, the control of the specific differentiation of stem cells into target cells for cell replacement therapy, and the development of new drugs and treatments for cancer, among other diseases. This

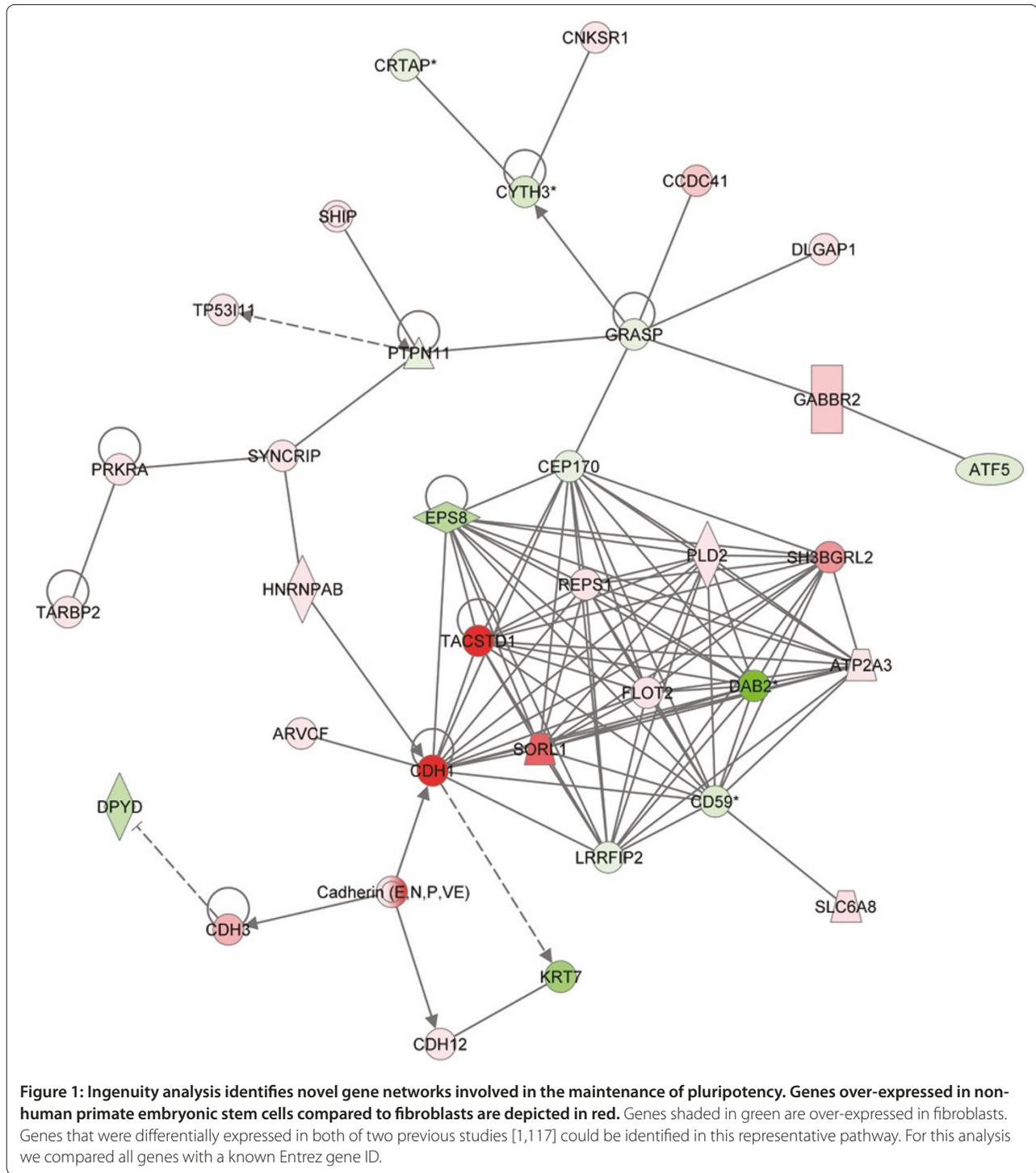
review summarizes our current knowledge of gene expression networks in non-human primates, which resembles the human model and also has greater advantages.

Embryonic stem cells

ESCs are defined as a population of cells capable of self-renewing while maintaining their pluripotency. ESCs differentiate and give rise to cells from all three germ layers: ectoderm, mesoderm and endoderm, including the pancreas [16]. These cells are derived from the inner cell mass of mammalian blastocyst stage embryos [17,18]. While mESCs were cloned over three decades ago, we have only recently celebrated our first decade of hESC derivation. Hence, our knowledge on hESCs is more limited than that on mESCs. While the two types of ESC share many features, such as the expression of the pluripotent marker OCT-4, they also differ from each other, such as the dependency of mESCs on leukemia inhibitory factor (LIF) to maintain pluripotency. Presumably, therefore, pathways that contribute to both pluripotency as well as specific differentiation might differ between mESCs and hESCs. Thus, our knowledge on gene expression in mESCs as well as their utilization for therapy must be verified in hESCs.

Nuclear transfer

While ESCs can be differentiated into beta cells, utilizing them in human therapy poses a problem since they do not identically match the patient. Human ESC lines today were derived from 'leftover' blastocysts from IVF clinics. Hence, they might cause an immune reaction when transplanted into human patients. One possibility to overcome this problem is the generation of genetically similar ESCs to the patient. This process would require the use of the patient's genome. While routinely carried out in mice, this process has been found to be more difficult in other species. Nuclear transfer (NT; also termed therapeutic cloning) requires the enucleation of a donor egg and the removal of the spindle DNA. Into this enucleated egg a single differentiated cell, usually a fibroblast, is placed. The two cells fuse together, resulting in a single cell carrying the correct number of chromosomes. This cell then begins its development and will cleave until a blastocyst is formed, which contains an inner cell mass from which ESCs can be derived (termed NT-ESCs). For this process to succeed, a large number of processes are carried out within the cell, including reprogramming of the genome to an undifferentiated state. It is this process that lowers the efficiency of NT-derived blastocysts. Much is unknown about the results of reprogramming, and many theories have been developed, which are summarized by Yang and colleagues [19].



While the best known successful NT was Dolly the Sheep, derived by Sir Ian Wilmut [20], NT has been successful in many species, including dog, cat, mouse, cow, goat and others [21-23]. Recently, primate cells have been cloned, though with low efficiency [24]. These results support the contention that human cells could also be generated by NT, though very significant

bioethical challenges remain. The reasons for the low efficiency of primate cloning are not clear, and better methods for cloning are being investigated.

A recent study showed that it would be difficult to carry out intraspecies cloning [25]. In this method, enucleated animal eggs would be used to house the differentiated cells, thereby overcoming one of the major

hurdles of this process: the lack of sufficient numbers of donor eggs. However, the control experiments in this study were lacking. While the authors were able to demonstrate that there was no development in the cloned intraspecies cells, they did not show their ability to clone human cells, raising questions about the efficiency of cloning in this study [25].

ESCs and NT-ESCs have been proposed as possible treatments for diseases such as Parkinson's, Alzheimer's, juvenile diabetes and others. Specifically, NT-ESCs as cell replacement therapy - that is, using NT to derive ESCs - have a great advantage when compared to fertilized ESCs. Patients could provide their own fibroblasts for the derivation of NT-ESCs, and the resulting line would be patient-specific. However, since this process uses donated eggs that contain mitochondrial DNA, these cells are not identical to the donor patient [26]. Therefore, the newly derived ESCs are mostly genetically similar to the donor, and hence might be rejected when fully differentiated cells are transplanted. In contrast, many patients could not (for gender reasons) and/or should not (for ethical reasons) [26,27] donate eggs for the derivation of fertilized ESCs, and even then the newly derived cells would only be similar but not identical to the fibroblast from which they were derived, while fertilized ESCs can be closely related to the donor only if the egg donor is the patient herself or a first degree relative.

NT-ESCs and fertilized ESCs have been shown to have many similar properties, including the ability to differentiate into cells from all three germ layers [28]. Since most differentiation procedures used for NT-ESCs will follow the same methods from fertilized ESCs, much emphasis is placed on generating NT-ESCs more efficiently, and other studies are carried out to improve the differentiation procedures. As proof of principle, a number of publications have shown that NT-ESCs can be used for therapy. This was achieved in a Parkinson's model [29] as well as a diabetic model in which NT-ESCs were differentiated into beta cells [30], thereby bridging the gap between the two routes of research. The method of differentiation for these NT-ESCs was based on protocols described previously [30]. These cells were able to maintain normal glucose levels after transplantation into a diabetic mouse [30]; however, an increase in blood glucose levels was seen 8 weeks after transplantation, presumably because the cells were not fully differentiated beta cells.

Induced pluripotent stem cells

One major goal of stem cell research is the generation of patient-specific stem cells. While successful in mice [31], the derivation of genetically matched, patient-specific human ESCs using somatic cell NT (SCNT) has not yet been accomplished. Furthermore, the use of donor oocytes or pre-implantation embryos to derive patient-specific

stem cells using techniques such as SCNT, cell fusion and parthenogenesis elicits ethical concerns. The recent advance in reprogramming adult somatic cells into ES-like cells, termed induced pluripotent stem (iPS) cells, provides another avenue for generating patient-specific stem cells without the ethical concerns of other methodologies. Thus, iPS cell derivation is the latest innovation for generating large pools of patient-specific stem cells that can be used to treat a wide range of human diseases.

In 2006, Takahashi and Yamanaka [32] demonstrated that mouse embryonic fibroblasts and adult tip fibroblasts could be reprogrammed into a pluripotent, ES-like state by transducing these cells with four transcription factors (Oct4, Sox2, Klf4 and c-Myc) along with a knock-in *Fbx15* neomycin-resistant reporter gene. After 2 weeks of culture, these mouse iPS cells exhibited similar characteristics to ESCs, such as alkaline phosphatase activity, expression of SSEA-1 and Nanog (two pluripotency markers), and the ability to differentiate into all three germ layers (endoderm, ectoderm and mesoderm) via *in vitro* differentiation or teratoma formation in immunodeficient mice. While this ground-breaking research provided a proof-of-principle for the reprogramming of adult somatic cells to ES-like cells, these iPS cells differed from ESCs in the genomic expression of several genes and the inability of the iPS cells to fully chimerize with donor mouse embryos. Shortly after the publication of this work, three groups showed that dispensing with the reactivation of the *Fbx15* reporter gene generated iPS cells that yielded fully chimerized pups following blastocyst injection and could contribute to germ cell transmission [33-35].

Recently, this work has been extended to human cells, as three groups originally demonstrated that human iPS cells could be generated from embryonic, neonatal and adult fibroblasts [36-38]. Like ESCs, these human iPS cells exhibited alkaline phosphatase activity, expressed SSEA-3, TRA-1-60, Oct4 and Nanog (human pluripotency markers) from endogenous loci, and exhibited a genomic profile more similar to ESCs than the originating fibroblasts. Interestingly, two different combinations of retrovirally introduced transcription factors were used: Oct4, Sox2, Klf4 and c-Myc were utilized by two groups [36,37], while Yu and colleagues [38] generated human iPS cells using Oct4, Sox2, Nanog and Lin28, an RNA binding protein that regulates synthesis of the let-7 family of microRNAs (miRNAs). Currently, this work has been repeated in several other labs and has included reprogramming of more terminally differentiated cells, such as pancreatic beta cells [39-42].

The potential for iPS cells to be utilized therapeutically has recently been examined [43]. In this study, researchers were able to ameliorate a mouse sickle-cell

anemia model by differentiating iPS cells into hematopoietic stem cells and re-introducing these cells back into the animal model [43]. Similarly, human iPS cells have been directly differentiated into motor neurons and insulin-secreting islet-like clusters *in vitro* [44,45]. While these results highlight the great promise for iPS cells in a clinical setting, transplanting iPS cells or differentiated iPS cells into humans carries a high risk. Around 20% of chimeric mice generated from iPS cells developed tumors within a 2- to 10-month time frame [34]. This finding is most likely due to the reactivation of *c-Myc* in iPS cells. While dispensing with *c-Myc* in iPS cell formation reduces the overall efficiency of obtaining iPS cell colonies, chimeric mice derived from these cells appear to be free of tumors [46,47]. However, aberrant expression of *Oct4*, *Sox2*, *Klf4* and/or *Nanog* has been observed in a number of human malignancies [48-50]. Likewise, the random integration of the retroviruses could induce tumorigenesis by activating other oncogenic factors [51]. Because of these concerns, current research has been targeted at deriving iPS cells in a more clinically-friendly manner. Such protocols include using adenoviruses instead of retroviruses, and using miRNAs or a combination of chemical and genetic modifications to induce reprogramming [52-54]. While iPS cell derivation is years away from being utilized in a clinical setting, the proof-of-principal results shown thus far indicate that iPS cells have the promise of treating a wide range of human disorders without the concern of immuno-rejection. Also, without requiring donor oocytes or pre-implantation embryos, iPS cell technology reduces the ethical concerns about generating patient-specific, pluripotent stem cells (PSCs).

Germ cells

Another potential source of stem cells is derived from precursor germ cells. In early embryonic development, a subset of pluripotent cells differentiate into primordial germ cells (PGCs) [55,56]. These cells migrate, proliferate and colonize the genital ridge and represent a population of cells that will eventually further differentiate to form gametes. Initially discovered in mice, failure of PGCs to mitotically arrest following colonization leads to the formation of teratomas, tumors that contain cells representing all three germ layers: ectoderm, mesoderm, and endoderm [57]. The first isolations and cultures of these proliferating PGCs yielded a multipotent cell line termed embryonal carcinoma cells. These cells are capable of being differentiated in culture into various cell types, including neurons and cardiomyocytes [58,59]. It was also shown that culture of isolated PGCs prior to genital ridge colonization resulted in germ cell colonies that express numerous pluripotency markers akin to those of ESCs, such as *OCT-4* [60,61]. These unique cells, termed

embryonic germ cells (EGCs) were shown to be highly pluripotent. EGCs have previously been an interesting cell source for studying gametogenesis *in vitro* because mouse EGCs appear to follow similar differentiation patterns as observed in *in vivo* gametogenesis [62]. However, ethical concerns about obtaining human EGCs have tamed interest in this field.

Several groups have shown the ability of mouse, non-human primate and human ESCs to differentiate into germ cell lineages, specifically *in vitro*-derived PGCs (invPGCs) [63-79]. However, three groups in particular have demonstrated three different methodologies for faithfully deriving invPGCs from ESCs at higher efficiencies [63-65]. Yamauchi and colleagues [64] successfully differentiated cynomolgus monkey ESCs into germ cells by forming embryoid bodies (EBs) with retinoic acid and culturing these EBs for 28 days. At day 28, germ cells could be identified by positive immunostaining for *SSEA1*, *VASA* or *DAZL*. Furthermore, these researchers showed up-regulation of germ cell gene expression for *CXCR4*, *NANOS1*, *NANOS2*, *NANOS3*, *VASA*, *PIWILI* and *TEKT1* upon EB formation with retinoic acid for 28 days. Likewise, this group was able to demonstrate that day 28 EBs grown in retinoic acid or bone morphogenetic protein (BMP)-4 elevated expression of the meiotic marker *SCP1* but not *SCP3*. Kee and colleagues [63] showed that adherent differentiation with a BMP cocktail (*BMP4*, *BMP7* and *BMP8b*) induced differentiation of hESCs into invPGCs in 7 to 14 days. Using a green fluorescent protein (GFP) transgene driven by the *VASA* promoter, these researchers showed that differentiation medium supplemented with BMPs resulted in increased expression of two PGC markers in differentiating hESCs: *VASA* and *DAZL*. Kee and colleagues also demonstrated that *VASA*-GFP⁺ cells could be isolated and cultured on mouse embryonic fibroblasts for 7 days to form invPGC colonies. These cultured cells also exhibited hypomethylation of the *H19* locus, suggesting that these cells, like *in vivo* PGCs, undergo de-methylation prior to gametogenic progression [63]. More importantly, Kee and colleagues demonstrated that overexpression of *DAZ* family members (*DAZ*, *DAZL* and *BOULE*) in cultured invPGCs induces meiotic progression as determined by immunofluorescence staining for *SCP3* and γ H2AX [63]. Even more striking, they demonstrated haploid formation by overexpression of the *DAZL* family members by the appearance of a small 1N peak in their propidium iodide FACS analysis and the expression of acrosin in a small fraction of cells. This remarkable discovery highlights the potential of driving gametogenesis *in vitro* from PSCs [63].

More recently, Amander Clark's group demonstrated a novel approach for rapidly and more efficiently differentiating hESCs into PGCs. Park and colleagues [65]

showed that differentiation of hESCs on human fetal gonadal stromal cells significantly improved germ cell differentiation. Strikingly, these researchers showed that c-kit+/SSEA1+/VASA+ invPGCs (5% of the total population of cells) could be isolated from differentiated hESCs as early as 3 days of culture on human fetal gonadal stromal cells. Similarly to Kee and colleagues, Park and colleagues demonstrated that invPGCs exhibit imprint erasures and show expression of a wide range of germ cell markers [63,65]. The work of Park and colleagues demonstrates progress towards a highly efficient methodology for generating PGCs from ESCs *in vitro* [65]. Furthermore, Park and colleagues are the first group to differentiate human iPS cells into early germ cell lineages. These exciting results combined with the work of Kee and colleagues [63] and Yamauchi and colleagues [64] highlight the similarities between *in vivo* PGCs and invPGCs illustrate the possibility of treating infertility by differentiating patient-matched ESCs into gametes or male germline stem cells for transplantation.

The ability to generate transplantable male germline stem cells or haploid gametes in culture has significant therapeutic implications for couples with infertility [80,81]. The appeal of these approaches is enhanced by iPS cell and NT technologies, which would theoretically enable men to derive germline stem cells or sperm from their own skin cells *in vitro*. Thus, it is hypothetically possible for a man who is rendered infertile by toxic treatment for cancer (chemotherapy or radiation), and who did not cryopreserve semen prior to treatment, to father his own genetic children from germ cells derived from NT-ESCs or iPS cells. This potential can only be realized after extensive feasibility and safety studies are conducted, ideally in nonhuman primate models that are relevant to human physiology. There is a lack of consensus among species regarding the potential of PGCs to undergo spermatogenesis when introduced into seminiferous tubules (mouse PGCs can [82] and rat PGCs cannot (K Orwig, unpublished)). However, there is consensus in rodents and several large animal species that gonocytes and spermatogonia from neonate, pup and adult testes undergo spermatogenesis when transplanted into the testes of infertile recipients [82-87]. Human PSCs can be differentiated into PGCs in the context of EBs [70,88] or adherent differentiation cultures [63,65,67]. Similarly, two groups have reported macaque PSC to PGC differentiation in EBs [64,89]. There are no reports of PSC to spermatogonial stem cell (SSC) differentiation, but several studies have reported PSC differentiation to haploid germ cells [63,77,78], suggesting a transient transition through an SSC-like intermediate. Thus, direct differentiation of PSCs to SSCs would provide a source of transplantable cells that could be used to ask important questions about the safety and efficacy of PSC-derived cells.

Interestingly, the postnatal mammalian testis itself may provide an alternative source of PSCs that bypasses the need for an embryonic intermediate or genetic manipulation. Several groups have shown the ability of germ cells in the mouse postnatal testis to produce PSCs *in vitro* [90-96]. Several recent studies have also provided evidence for PSCs derived from the adult human testis [97-100]. These cells arise *in vitro* from spermatogonia and can give rise to tissues of all three embryonic germ layers. Given that germ cells are responsible for initiating embryogenesis, it seems possible that germ cell factors could influence their ability to become pluripotent (for example, including expression of genes associated with pluripotency). Among the genes that are thought to form a core regulatory network in ESCs (OCT4, SOX2, and NANOG) [101], only OCT-4 is expressed by a few postnatal germ cells or cultured SSCs. Several reports have described a relatively small group of normal mouse spermatogonia that express OCT-4, including those in the adult testis, which could potentially be those that have the capacity to produce PSCs *in vitro* [102-107]. In human spermatogonia, though, only a few postnatal spermatogonia retain embryonic-expressed OCT-4, and this expression is lost after the first few months of infant life except in pathological conditions [108,109]. In cultured SSCs, *Oct-4* mRNA and protein can be detected, albeit at substantially lower levels than in ESCs [90,95,110,111], and this feature may be required for long-term survival of SSCs in culture [112]. Thus, the mechanisms that predispose spermatogonia (presumably SSCs) to acquire a pluripotent phenotype in a culture dish are unclear, but may involve similar gene expression features with other pluripotent cells (ESCs).

Gene expression in non-human primate ESCs

There are great challenges working with human ESC lines rather than ESC lines from pedigreed animals. While working with human ESC lines involves some ethical as well as religious issues, use of non-human primate ESC (nhpESC) lines alleviates many of these concerns. Our two groups have recently derived a large number of nhpESC lines, including from rhesus macaques and baboon, using both fertilized blastocysts and NT [9,113-118]. In addition, others have derived other monkey ESC lines, such as from marmoset [119]. There are many other advantages in working with nhpESC compared to hESC lines: namely, the use of prime quality embryos as compared to left over human IVF embryos donated to science that usually either have genetic disorders [120] or lag in their development. In addition, since the donors of primate gametes are not anonymous, we have much information on the parents, as opposed to the anonymity of human donors. For these reasons we have closely examined the factors that define a stem cell

line, including genes expressed, and compared them to the literature on gene and mRNA expression of human ESCs.

Using the newly derived nhpESC lines, we examined their gene expression [9] and found that they are very similar (>97%) to each other. We next compared the gene expression of these lines to that of two types of fibroblast: skin fibroblasts from the parents from which gametes were taken to derive the lines; and fibroblasts derived from teratomas generated by the injection of nhpESCs into severe combined immunodeficiency (SCID) mice [1]. We found a unique set of genes that is differentially expressed between the nhpESCs and the two types of fibroblast. Interestingly, many of these genes were membrane-bound proteins and receptors [1] (Table 1). We have also shown that there are chromosomes that show an overabundance of over-expressed genes, such as chromosomes 16, 19 and X, which correlate to human chromosomes 17, 20 and X, respectively.

Our second study [114] has shown that there is indeed a unique set of genes in nhpESCs that maintains pluripotency, and that these differentially expressed genes are involved in many pathways. As expected, both studies revealed the over-expression of the 'classic' stem cell factors OCT-4, Nanog and Sox-2 (Table 1). Interestingly, when imprinting was examined, the nhpESCs were found to have aberrant imprinting, the implications of which are unknown [121].

When the two studies are compared, as depicted in Table 1, many of the top differentially expressed genes are similar. Taking into account that these were two separate studies, the resemblance between the two gene lists is striking. While Ben-Yehudah and colleagues [1] compared the gene expression of nhpESCs to fibroblasts and generated a list of genes over-expressed in ESCs, Mitalipov and colleagues [117] generated a list of genes that were highly expressed in a number of nhpESC lines. Out of the top 25 genes over-expressed in nhpESCs in both studies, 6 (24%) were found on both lists. As expected, genes known to be involved in maintenance, such as *OCT-4* and *Nanog*, can be found on both lists. Additionally, *PTPRZ1* is found on both lists; this gene has been shown to be expressed by hESCs and is down-regulated upon differentiation. Depletion of *PTPRZ1* resulted in decreased colony formation and lower recovery of hESCs. However, the lists include genes that have yet to be associated with pluripotency, such as *TACSTD1*. *TACSTD1*, also called Ep-CAM, is an epithelial adhesion molecule that was originally identified as a marker of carcinomas and is also expressed by rat SSCs [122-124]. We found this gene to be the most differentially expressed gene between stem cells and fibroblasts, indicating that it might have other functions in signaling rather than solely adhesion. In addition, it

should be pointed out that 40% of the genes on the list in Ben-Yehudah and colleagues [1] are hypothetical; these genes may also play a significant role in pluripotency.

When we compiled the data in Ben-Yehudah and colleagues [1] using Ingenuity software to identify system networks responsible for the regulation of the pluripotent state in nhpESCs, we were able to create many gene networks. Some of these networks contained anticipated candidate genes, including *SOX2*, *OCT-4* and *NANOG*, as we have shown previously [1]. In addition we could identify networks that have been shown to be differentially expressed between stem cells and fibroblasts, as depicted in Table 1. These genes participate in networks that have yet to be associated with pluripotency. Although most of the genes depicted in Figure 1 and Table 1 are unidentified or have not been associated with pluripotency, some were found to play roles in regulating the transition from pluripotency to differentiation; for example, the gene *TACSTD1* is included in both Figure 1 and Table 1.

Since ESCs can serve as a method of studying development [125], much research has been carried out to understand the mechanisms that underlie regulation of this specific process, such as the gene regulatory networks that control pluripotency. These regulatory networks have been studied in mice [126] and have revealed the importance of key regulators of the pluripotent state, including OCT-4, Sox-2 and Nanog. A comprehensive review described similar findings in humans [127,128] and has also been discussed by us [1]. It should be pointed out that although many genes have been implicated in the networks controlling pluripotency, little is known about the networks controlling this process. An exception is the OCT-4/Sox-2/Nanog network, which has been shown to be invaluable for maintaining pluripotency. In our hands, we could identify the pluripotent genes and networks [1,24], but could not fit all the differentially expressed genes into these networks or form new ones.

Imprinted genes in nhpESCs

While genes involved in pluripotency can be identified and even gene regulatory networks can be described, other mechanisms controlling the expression of genes in pluripotent cells can be established - for example, epigenetic mechanisms that control gene expression in pluripotent cells. One such epigenetic mechanism is DNA methylation, which is considered a key factor in the formation of cellular memory and identity [129]. A comprehensive review summarized the key features of the regulatory mechanisms that control the transcriptional regulatory features in hESCs [130,131], which complemented their work with CHIP-chip in mESCs [132].

The rhesus monkey is the only primate in which SCNT has been successful so far [133]. Therefore, this model

Table 1. Twenty-five genes over-expressed in Ben-Yehudah and colleagues [1] and Mitalipov and colleagues [117]

Top 25 over-expressed genes in [1]		Top 25 over-expressed genes in [117]	
Affymetrix ProbeSet ID	Gene symbol	Affymetrix ProbeSet ID	Gene symbol
1	MmuSTS.2870.1.S1_at	MmuSTS.3741.1.S1_at	PTPRZ1
2	MmugDNA.35532.1.S1_at	MmugDNA.32128.1.S1_at	NANOG
3	MmuSTS.4178.1.S1_at	MmugDNA.33796.1.S1_s_at	FLJ16517
4	MmugDNA.17159.1.S1_at	MmugDNA.12465.1.S1_at	LIN28
5	MmugDNA.20158.1.S1_at	MmuSTS.1454.1.S1_at	MAL2
6	MmugDNA.11043.1.S1_at	MmuSTS.2862.1.S1_at	SPP1
7	MmunewRS.431.1.S1_at	MmuSTS.3364.1.S1_at	PDZK1
8	MmuSTS.2285.1.S1_at	MmugDNA.37987.1.S1_at	SALL1
9	MmunewRS.475.1.S1_at	MmuSTS.1929.1.S1_at	MYCN
10	MmugDNA.24757.1.S1_at	MmugDNA.20158.1.S1_at	NELL2
11	MmuSTS.3573.1.S1_at	MmuSTS.2870.1.S1_at	TACSTD1
12	MmuSTS.3621.1.S1_at	MmugDNA.17017.1.S1_at	OTX2
13	MmuSTS.4813.1.S1_at	MmugDNA.24774.1.S1_s_at	APOA1
14	MmugDNA.38382.1.S1_at	MmuSTS.1037.1.S1_at	SH3GL3
15	MmugDNA.41477.1.S1_at	MmugDNA.11977.1.S1_at	MBD2
16	MmugDNA.17159.1.S1_s_at	MmugDNA.33242.1.S1_at	PODXL
17	MmugDNA.19721.1.S1_at	MmugDNA.6117.1.S1_at	CECR2
18	MmuSTS.3827.1.S1_at	MmuSTS.4090.1.S1_at	EBAF
19	MmugDNA.32128.1.S1_at	MmugDNA.36148.1.S1_at	CYP26A1
20	MmugDNA.27729.1.S1_at	MmuSTS.2285.1.S1_at	POU5F1
21	MmuSTS.3741.1.S1_at	MmugDNA.3748.1.S1_at	LOC112868
22	MmugDNA.7641.1.S1_at	MmugDNA.32848.1.S1_at	ST8SIA4
23	MmugDNA.33796.1.S1_s_at	MmuSTS.214.1.S1_at	ZIC3
24	MmugDNA.26523.1.S1_s_at	MmuSTS.1436.1.S1_at	LCK
25	MmugDNA.31842.1.S1_s_at	MmuSTS.4824.1.S1_at	GDF3

Underlined genes are those differentially expressed in both studies; genes in bold are as yet uncharacterized genes.

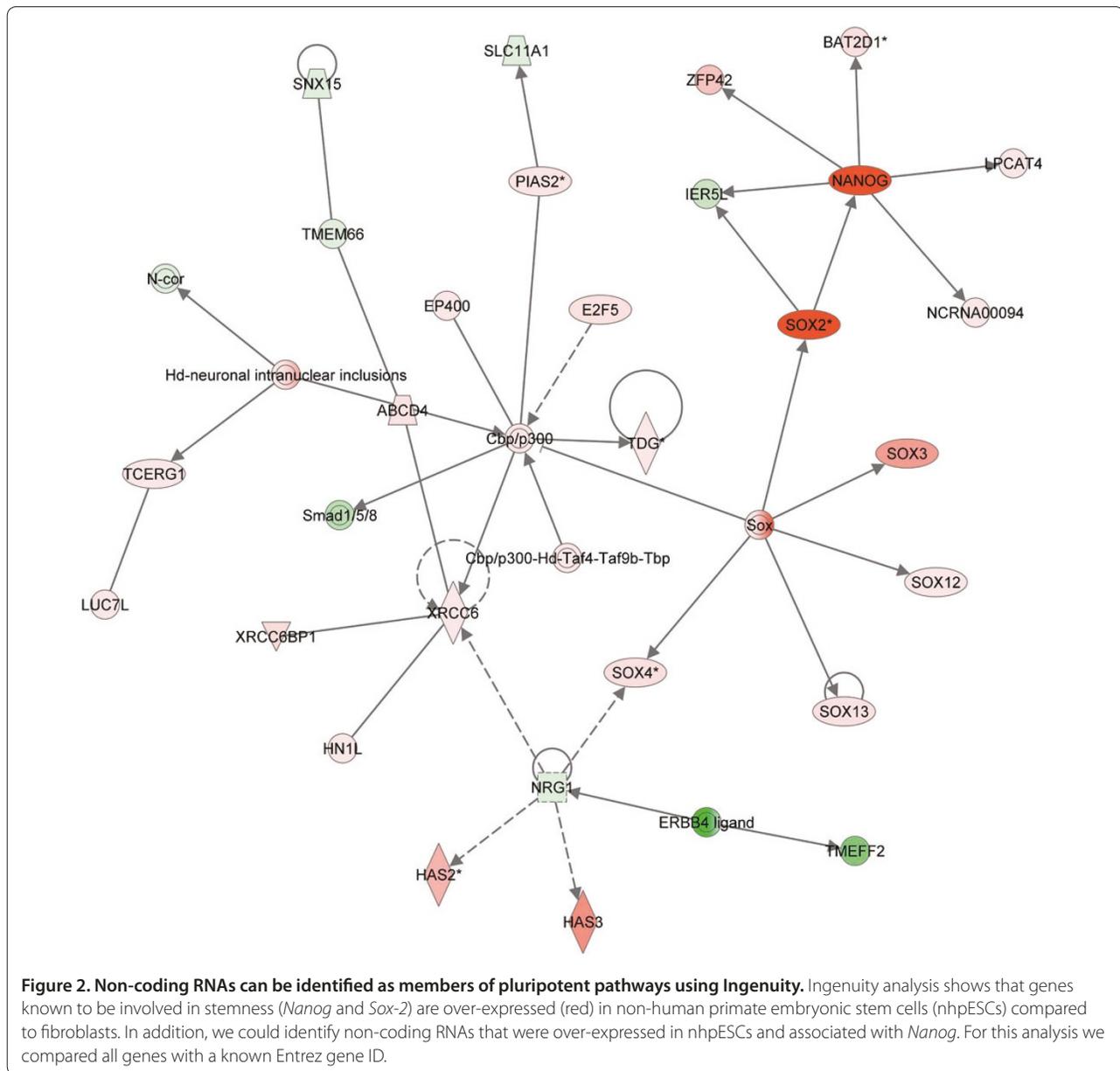
can help answer questions on the epigenetic state of cells that undergo reprogramming - for example, whether they are closer to ESCs or to the somatic cell from which they originate. The answer to this question might shed light on why it is very difficult for primate cells to undergo NT compared to mice and other animals. This could lead to improvements in primate NT.

We have recently compared DNA methylation in native ESCs, fibroblasts, and ESCs generated by SCNT [129]. We wished to examine if the SCNT cells undergo changes in methylation state that would resemble a stem cell rather than a somatic cell. We have identified and compared epigenome programming and reprogramming. Based on our previous knowledge, we have characterized hundreds of regions that are hyper- or hypomethylated in fibroblasts compared to native ESCs. We found that these regions are conserved in human cells and tissues. When ESCs were compared to the SCNT cells, we found to our surprise that the vast majority of these regions were

reprogrammed in SCNT ESCs. The meaning of these phenomena is that these cells do indeed undergo reprogramming of their DNA methylation during SCNT. This reprogramming leads to an almost perfect correlation between the epigenomic profiles of the native (ESC) and reprogrammed (NTSC) lines.

We also found that at least 58% of these changes are correlated *in cis* to transcription changes, Polycomb repressive complex-2 occupancy, or binding by the CTCF insulator [129].

As expected, since the process of adding or removing a methyl group from the DNA must be a complex process, we found that while epigenomic reprogramming is extensive and globally accurate, the efficiency of adding and stripping DNA methylation during reprogramming is regionally variable. In several cases, this variability results in regions that remain methylated in a fibroblast-like pattern even after reprogramming [129].



Small RNAs and other RNAs

We have carried out many systems analyses using Ingenuity, as described in Figure 1. These usually identified networks not directly associated with pluripotency. However, we could occasionally identify a connection between genes associated with pluripotency and unique genes. One example is depicted in Figure 2. While *Sox2* and *Nanog* are expressed in pluripotent cells (red), they are also associated with the gene *NCRNA00094*. This gene has been shown previously to be a non-coding RNA with unknown activity that is expressed in ESCs [134].

NCRNA00094 is an example of a large number of non-coding RNAs that might play a role in maintaining

pluripotency. One subtype of non-coding RNAs that has been shown to participate in this process is the miRNAs. miRNAs are short non-coding RNA sequences that control gene expression by inhibiting the translation of specific mRNAs or causing their degradation [135,136]. Hence, several studies have been carried out to find miRNAs involved in the maintenance of pluripotency in ESCs. Using mathematical and statistical tools, a recent study [137] identified miRNAs that might be involved in pluripotency. A similar study was carried out in mESCs [138,139] and hESCs [140-142]. When different types of human adult and ESCs are compared, a number of miRNAs seem to be involved in this process, such as miR302 [53,136,142-144]. Specifically, one study showed

a connection between the 'stem cell factors' and miRNAs that inhibit them [145]. However, more work has to be done to identify their specific targets and actions, such as the crosstalk between stem cell factors and miRNAs, as in the case of Lin-28 and Let7, for example [139].

A comprehensive comparison of gene and RNA profiles of mouse fertilized and SCNT lines has been carried out recently [146]. They found that the two types of ESCs have similar miRNA and protein expression profiles. They conclude that this phenomenon is consistent with their similar developmental potentials and might result from their similar transcriptional profiles.

While much research has been conducted on miRNA involvement in pluripotency in hESCs and mESCs, this has been little studied in the monkey. A recent study by some of us [137] has computationally searched the rhesus genome to identify novel miRNAs involved in pluripotency by homology to human miRNAs. This study identified 383 novel miRNAs: 173 have 100% homology to human miRNAs and 281 have >90% homology in the seed sequence of the miRNAs [137]. This study also identified miRNAs that are involved in human ESC pluripotency, such as miR302, as described above.

Conclusions

In this review we have summarized our and other results from the past decade on the generation of nhpESCs, SCNT, the generation of iPSCs and our work on primordial germ cells. All these fields of research cumulatively enhance our understanding of the early stages of human development. These exciting results, together with our results on gene expression in rhesus macaques and other primates, open the possibility of studying the gene expression and its control by miRNAs that results in the undifferentiated state of ESCs. Moreover, these studies may lead to better understanding of the mechanisms behind processes such as induced pluripotency, the knowledge of which could be used to test cellular therapies in nonhuman primates before introduction in humans.

Abbreviations

BMP = bone morphogenetic protein; EB = embryoid body; EGC = embryonic germ cell; ESC = embryonic stem cell; hESC = human embryonic stem cell; invPGC = *in vitro*-derived primordial germ cell; iPSC = induced pluripotent stem; IVF = *in vitro* fertilization; mESC = mouse embryonic stem cell; miRNA = microRNA; nhpESC = non-human primate embryonic stem cell; NT = nuclear transfer; PGC = primordial germ cell; PSC = pluripotent stem cell; SCNT = somatic cell nuclear transfer; SSC = spermatogonial stem cell.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The support of this research by the NIH is acknowledged gratefully.

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Published: 5 August 2010

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doi:10.1186/scrt24

Cite this article as: Ben-Yehudah A, *et al.*: Systems biology discoveries using non-human primate pluripotent stem and germ cells: novel gene and genomic imprinting interactions as well as unique expression patterns. *Stem Cell Research & Therapy* 2010, **1**:24.