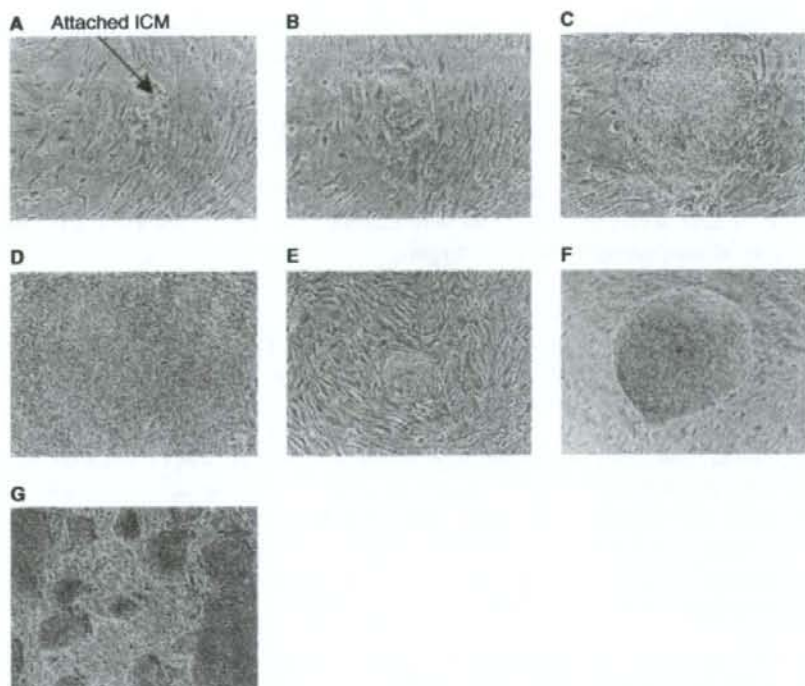


8. Place the dish back on the heated stage and observe the embryo for lysis of the trophoctoderm cells and the collapse of the embryo in on itself. This Complement-mediated lysis is commonly described as trophoctoderm 'bubbling' as the cells become large, transparent and bubble-like (See **Figure 5**). If no such 'bubbling' is evident, place the dish back in the incubator. Extend the incubation by intervals of 5 min until the embryo has collapsed and all trophoctoderm cells are lysed. The incubation time for the embryo in complement should not be longer than 30 min.
9. After washing the embryo a third time by transferring through all drops of the sixth row, it is carefully drawn through a thinly drawn pipette, the bore of which is just smaller than the diameter of the embryo. The ICM should detach from the lysed trophoctoderm cells with 1–2 draws up the pipette.
10. Transfer the detached ICM cell cluster to microdrops of human ES cell derivation medium and proceed as shown in **Figure 4**. Finally, transfer the ICM to a well of a four-well plate seeded with mitotically-inactivated feeders and containing pre-warmed human ES derivation medium. Place this plate back into the incubator and do not disturb for at least 48 hr, after which attachment and outgrowth should be evident.

### Breaking up the ICM, growing explants and analyzing colony morphology

At this stage of the derivation process, the goal is to increase 'putative human ES cell' number, and not to be too much concerned with removing differentiated from the outgrowths. However, explants should not be allowed to become over-confluent as all cells will begin to differentiate. Here now are some general notes on maximizing derivation efficiency:

- During the initial dispersion of the ICM-derived outgrowth, a part of the initial outgrowth should be left intact, in case the reseeded clumps fail to attach and/or grow after transfer to new wells.
- Do not be tempted to expand the cultures too quickly, or to treat the cells too harshly, breaking them into clumps that are too small to sustain proliferation.
- Remember not to seed the feeder layers too densely (should seed at  $>50,000$  cells/cm<sup>2</sup>) - MEFs form cell clusters that can easily be mistaken for early human ES cell outgrowths.
- Partially change medium when the original medium on the cells becomes acidic (medium becomes light pink/yellow). If changed too often, medium will have less auto-stimulatory factors secreted from the human ES cells themselves that encourage their proliferation.
- Mechanical disaggregation of human ES cell colonies is described in detail in **Chapter 5 (Routine Protocols)**.
- Colonies displaying a human ES cell morphology, are most readily identifiable as such, by their prominent and numerous nucleoli and their flattened shape of



**Figure 6** Identifying and subculturing colonies with a human cell morphology. (A–D) show the same colony with human ES cell morphology at days 5, 7, 10, 13. The colony is ready to mechanically disaggregate at day 13. (E) shows a P1 colony from this mechanical disaggregation. (F) shows a P2 colony. (G) shows a P5 culture successfully adapted to trypsinization.

their colonies, and the ability to distinguish individual cells within the colony (See **Figure 6**). Other morphologies that also arise from outgrowths include fibroblast-like cells (which are indistinguishable from feeders), spread out trophoblast cells, and epitheloid-like cells.

The protocol outlined using mouse feeder layers has been described in detail here as it has been independently used to repeatedly derive human ES cells in several laboratories over three years and so we consider it our most established protocol.

The use of animal feeders has some disadvantages, such as risks of pathogen transmission and viral infection (Richards *et al.*, 2002; Amit and Itskovitz-Eldor, 2003; Amit *et al.*, 2004; Rosler *et al.*, 2004). Also, foreign sugars may be transferred from feeders to the glycoproteins of the human ES cells (Martin *et al.*, 2005). These concerns, however, will probably only be significant if cells or their derivatives are to be used therapeutically. For purposes of using human ES cells as a basic *in vitro* tool, this protocol has proved sufficient. While some laboratories are actively seeking alternatives to mouse feeders (human fibroblast feeders, human fallopian feeders, feeder conditioned medium, chemically defined medium), we feel these protocols are still

being optimized and have yet to be tested repeatedly in our hands. Should you wish to try one of these alternative protocols, we direct you to the papers cited.

## Adapting newly derived human ES cells to trypsin

Freshly derived lines can be adapted to trypsin as early as passage 3, when you have two wells of a six-well plate confluent with human ES cell colonies. Here are guidelines for successful adaptation of lines to trypsin:

- Initial splits with trypsin should be low i.e. 1:2–3. We trypsinize one of the two wells with trypsin and seed it into two or three new wells, while continuing to mechanically disaggregate cells the other well, in case there is a problem with enzymatic disaggregated cultures.
- Remember that cells are extremely prone of lysis by forces induced by rough pipetting when the cells are in serum or serum derivative containing medium. Do not make the mistake of scraping the cell cultures after the trypsin reaction has been stopped with serum or serum containing medium. Only scrape the wells when the trypsinization reaction is still proceeding, otherwise a large proportion of cells will lyse and seeding efficiencies will be very low.
- Ensure good plating efficiencies by gently pipetting the cells, and by carefully observing the cells under the microscope as they round up after trypsin has been added. As soon as all human ES cells have rounded up and become apparent as individual cells in the colony, wells should be scraped and the reaction stopped with serum or serum containing medium.
- We only trypsinize the cultures to clumps of ten cells or more during these early passages. If you have trouble with trypsinization, disaggregation with accutase or collagenase is recommended. When performing the second and third trypsinization on the culture, we gradually raise the split ratio to 1:2 and 1:4 respectively.
- Some have suggested that enzymatic disaggregation leads to a higher incidence of genetic abnormalities in lines of equivalent passage number [Mitalipova *et al.* 2005]. However, we point out that cultures can be expanded much more readily with enzymatic disaggregation and that comparisons between mechanically and enzymatically disaggregated cultures of equivalent passage is misleading (i.e. cells in trypsinized cultures will have undergone a substantially greater number of population doublings than mechanically disaggregated cultures of equivalent passage).

### Protocol for trypsinization

Routine trypsinization of human ES cells is covered in the Extended Protocols section of **Chapter 5**. For initial adaptation of human ES cell lines to trypsin we proceed as follows:

1. Warm aliquots of 0.05% trypsin EDTA solution and serum containing medium to 37°C in a waterbath.

2. Remove the medium from the well containing the culture to be disaggregated. Rinse the well twice with 1×PBS (1 mL per well of a four-well plate).
3. Add 1 mL warm 0.05% trypsin EDTA solution. Immediately transfer the plate to the heated platform of an inverted microscope and watch carefully as the human ES cells' morphology changes and these cells become refractile and spherical. When about 90% of the human ES cells round up, scrape the well until cell clusters (>10 cells or more) are floating in the medium. Do not wait until the feeders become refractile before stopping the reaction. Add 0.5 mL medium to stop the trypsin reaction.
4. Carefully remove the cell suspension with a P1000 pipette and transfer to a 15 mL centrifuge tube.
5. Pellet the human ES cells by centrifugation (300 g/~1,000 rpm for 5 min).
6. Carefully resuspend the pellet and seed the newly prepared wells according to the appropriate split ratio.
7. Half-change the medium in the well with fresh medium 24 hr after trypsinization. The human ES cell colonies should have adhered to the feeder layers and show signs of proliferation. Some cell death may also be evident and this medium change should remove most of the floating dead cells.

## Troubleshooting

***When performing immunosurgery, the trophoctoderm fails to exhibit bubbling and the embryo does not collapse? What has happened?***

There is a problem with the antibody and/or the complement. Ensure the correct dilutions were made when making up the solutions and that fresh preparations of both solutions are used in the next derivation.

***The ICM fails to attach or proliferate. Why?***

This could be due to overexposure the blastocyst to complement, thereby damaging the ICM cells. It could also be due to use of poor quality feeder layers or medium.

***After trituration through a drawn glass capillary, some trophoctoderm is still attached to the ICM. Should I triturate further at risk of damaging the ICM?***

No, do not triturate further. Removal of all trophoctoderm is not essential. In fact, having some trophoctoderm still present can facilitate attachment of the ICM to the feeder layer and promote outgrowth.

***I have an ICM outgrowth but after mechanical disaggregation it stopped growing. What happened?***

This may be due to the cells being manipulated too roughly, breaking the colonies into two small clumps to support initial proliferation. Be more gentle, pipetting the cells more slowly next time.

*None of my outgrowths produced a line displaying human ES cell morphology that continued to proliferate. What can I change to improve derivation frequencies in the next attempt.*

You should probably change serum replacement/FBS batches. Screening serum batches for human ES cell growth is very important. Seed previously established human ES cell lines at different densities using media supplemented with different batches. Culture the cells for two weeks and assess the cultures morphologically and by immunoFACS (see **Chapter 9** for characterizing human ES cell cultures in this way). Use the serum batch that supports proliferation (but not differentiation or cell death) of human ES cells.

- Feeders provide a matrix and growth factors for your ICM outgrowths. Poor feeder support of the outgrowth can also be the reason the putative human ES cells cease proliferating, always ensure feeders are seeded at the appropriate density ( $\sim 50,000$  cells/cm<sup>2</sup>) are of high quality (e.g. with a passage number  $\leq 3$ , an upright, slender cell morphology, and no evidence of senescence).
- Poor embryo development and explant outgrowth can be due to microbial contamination. Filter and screen all solutions and media prior to using them for derivation of new lines. Microbial contamination and the avoidance of it are discussed in detail in **Chapters 1 and 2**. The media used to derive and culture human ES cells are extremely rich and so are susceptible to becoming contaminated with microbes. Routine use of antibiotics masks contamination which slows cell growth and as these cells are very sensitive the presence of antibiotics can be toxic to the cells. Remember feeders are a common source of contamination. Feeders should always be screened for microbes including mycoplasma as outlined in **Chapter 1**.
- The problem could also be due to physical changes in the cell culture, i.e. temperature, humidity, pH of medium. Make sure your incubator and heated plates are functioning and are set to the correct temperature (37°C). Make sure to keep the water tray in the incubator full of autoclaved water. Change the medium on the cells before it becomes too acidic (i.e. yellow).
- Remember organic solvents and other toxic aerosols are not introduced to the derivation facility. Other harsh chemicals like PFA also should not be used within the derivation facility, refrain from using perfume/aftershave. Human embryonic cultures are very sensitive to such aerosols and they can perturb cell proliferation if present in the atmosphere.

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

### Elucidating nuclear reprogramming mechanisms: taking a synergistic approach



Dr Hidenori Akutsu

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.

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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 trophoblast) become morphologically distinct from the inner cell mass (ICM). Cells of the trophoblast 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 trophoblast cells have only the potential to form extraembryonic cells or the placenta.

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) (Wilmot *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

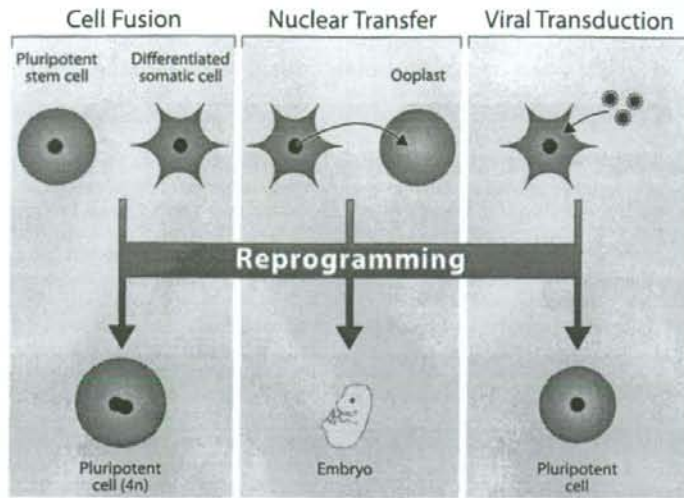
illustrates that cells do not permanently lose the ability to be pluripotent during differentiation.

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 Egan, 2007).

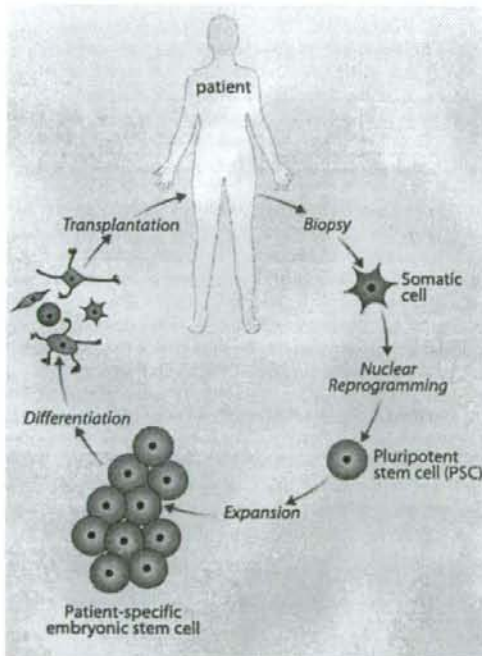
### Nuclear transfer: the oldest and still the most reliable reprogramming technique

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 (Wilmot *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 a tetraploid 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 Eggen *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  $\times$  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 (Kozioł *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 *Mx1* 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<sub>2</sub>/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.

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## Possible Involvement of CD81 in Acrosome Reaction of Sperm in Mice

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**ABSTRACT** Tetraspanin CD81 is closely homologous in amino acid sequence with CD9. CD9 is well known to be involved in sperm–egg fusion, and CD81 has also been reported to be involved in membrane fusion events. However, the function of CD81 as well as that of CD9 in membrane fusion remains unclear. Here, we report that disruption of the mouse CD81 gene led to a reduction in the fecundity of female mice, and CD81<sup>-/-</sup> eggs had impaired ability to fuse with sperm. Furthermore, we demonstrated that when CD81<sup>-/-</sup> eggs were incubated with sperm, some of the sperm that penetrated into the perivitelline space of CD81<sup>-/-</sup> eggs had not yet undergone the acrosome reaction, indicating that the impaired fusibility of CD81<sup>-/-</sup> eggs may be in part caused by failure of the acrosome reaction of sperm. In addition, we showed that CD81 was highly expressed in granulosa cells, somatic cells that surround oocytes. Our observations suggest that there is an interaction between sperm and CD81 on somatic cells surrounding eggs before the direct interaction of sperm and eggs. Our results may provide new clues for clarifying the cellular mechanism of the acrosome reaction, which is required for sperm–egg fusion. *Mol. Reprod. Dev.* © 2007 Wiley-Liss, Inc.

**Key Words:** CD9; acrosome reaction; fertilization; mice; zona pellucida

### INTRODUCTION

Fertilization is accomplished by the direct interaction of sperm and eggs, a process mediated primarily by predicted, but yet unidentified gamete membrane proteins. In fertilization, the acrosome reaction is a change in sperm that is required for penetration into the zona pellucida, the egg coat, and facilitates the subsequent fusion with the egg plasma membrane (Moreno and Alvarado, 2006). Zona pellucida protein 3 (ZP3), one of the components forming the meshwork of the zona pellucida, has been considered to be the prime

physiological inducer of the acrosome reaction in sperm, although the frequency of acrosome reaction is low after incubation with recombinant ZP3 (Beebe et al., 1992). This discrepancy suggests that, besides ZP3, unknown major factor(s) might be responsible for the acrosome reaction. To date, despite the importance of the acrosome reaction in fertilization, the underlying cellular mechanisms that regulate the acrosome reaction remain unclear.

Two tetraspanins, CD9 and CD81, are known to be important in the membrane fusion events in various biological systems. In virus–host cell fusion, human CD81 has been identified as a co-receptor for hepatitis C virus (Higginbottom et al., 2000; Cormier et al., 2004). Both CD9 and CD81 have been implicated in myoblast fusion (Tachibana and Hemler, 1999; Schwander et al., 2003) and monocyte/macrophage fusion in mice (Takeda et al., 2003). Recent studies using gene-targeting techniques demonstrated that female mice carrying a deletion of the CD9 gene produce eggs that mature normally but are defective in sperm–egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Takahashi et al., 2001). CD81 has also been reported to be expressed on the plasma membrane of unfertilized mouse eggs (Takahashi et al., 2001). Furthermore, CD81<sup>-/-</sup> mice have been reported to have defects in reproduction after several generations of backcrossing (Deng et al., 2000).

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Recently, Rubinstein et al. (2006) provided more detailed data showing that eggs of CD81<sup>-/-</sup> mice are unable to be fertilized with sperm, although the degree of the defect appeared not to be severe compared with that of CD9<sup>-/-</sup> eggs. Moreover, injection of CD9<sup>-/-</sup> eggs with mouse CD81 mRNA revealed that mouse CD81 was only moderately effective at reversing the infertility of CD9<sup>-/-</sup> eggs (Kaji et al., 2002). These findings taken together indicate that CD81 and CD9 each have different roles in fertilization.

Here we studied the role of CD81 in fertilization by *in vitro* fertilization (IVF) and immunohistochemical analysis, and propose a possible role of CD81 in the acrosome reaction in sperm.

## MATERIALS AND METHODS

### Animals

CD81<sup>-/-</sup> mice (Miyazaki et al., 1997) were kindly provided by Dr. Miyazaki and were backcrossed to C57BL/6 mice. Genotyping was carried out using polymerase chain reaction as previously described (Miyazaki et al., 1997). To visualize acrosome-intact sperm, EGFP-transgenic mice expressing EGFP in the acrosomes were generated by pronuclear injection of constructs carrying the EGFP gene driven by the mouse acrosin promoter (Nakanishi et al., 1999) and the DsRed2 gene tagged with a mitochondrial transport signal and driven by the CAG promoter into fertilized eggs of BDF1 mice (unpublished information). After the sperm were acrosome-reacted, EGFP was lost from the sperm heads and DsRed remained in the mitochondria of the mid-piece region. All animal procedures were performed according to protocols approved by the National Center for Child Health and Development and use committees.

### Egg Collection

Female mice (aged 8–15 weeks) were injected with 5 U of hCG (Gonotropin; Aska Pharmaceutical Co., Ltd, Tokyo, Japan) 48 hr after administration of 5 U of PMSG (Serotropin; Aska Pharmaceutical Co., Ltd). Ovulated eggs were collected from the oviductal ampulla 13.5–15 hr after hCG injection, and placed in 100- $\mu$ l drops of TYH medium equilibrated with 5% CO<sub>2</sub> in air at 37°C. Cumulus cells were removed with 300 IU/ml of hyaluronidase (H-3506, Sigma-Aldrich, Missouri, MO), and eggs were incubated with a defined number of sperm.

### Sperm Preparation and *In Vitro* Fertilization

Sperm were collected by squeezing two cauda epididymides of 8- to 10-week-old B6C3F1 or transgenic male mice in a well containing 100- $\mu$ l of TYH medium. Sperm were incubated at 37°C in 5% CO<sub>2</sub> for 90 min before being mixed with eggs derived from wild-type or CD81<sup>-/-</sup> female mice. The final concentration of sperm added to an egg-containing drop was  $1.5 \times 10^5$  sperm/ml. To examine the rate of fertilization, we counted the number of eggs at the two-cell stage 24 hr after incubation with the sperm. For counting the number of

fused sperm, the zona pellucida was removed from the eggs by a brief incubation in acid Tyrode solution, and sperm were incubated with eggs preloaded with 4',6-diamidino-2-phenylindole (DAPI) for counting the number of sperm fused with eggs (Yamagata et al., 2002). For counting the number of acrosome-intact sperm, EGFP-expressing sperm were incubated with zona-intact CD81<sup>+/+</sup> or CD81<sup>-/-</sup> eggs. The eggs were all subjected to confocal microscopic analysis for the presence of sperm exhibiting red and green fluorescence or red fluorescence alone within the perivitelline space 4 hr after incubation.

### Immunostaining

For immunostaining of cryostat sections, ovaries from 8- to 10-week-old wild-type C57BL/6 females were fixed in 2% paraformaldehyde in PBS (-) for 2 days at 4°C, and then immersed in 30% sucrose in PBS (-) for more than 2 days at 4°C, embedded in Tissue-Tek OCT compound (Sakura Finetek Co., Tokyo, Japan), and finally frozen before serial cryostat sectioning (8  $\mu$ m in thickness). Slides were fixed in an acetone and incubated with anti-CD81 antibody (Eat-1) diluted 1:300 in PBS (-) containing 0.1% bovine serum albumin (BSA), BSA/PBS (-), or anti-ZP3 antibody diluted 1:300 in BSA/PBS (-), overnight at 4°C. After washing three times with BSA/PBS (-), the samples were incubated with Alexa 546-conjugated goat anti-hamster IgG (A-21111, Invitrogen, California, CA) or Alexa 488-conjugated goat anti-rat IgG (A-11006, Invitrogen) for 2 hr at room temperature. After extensive washing, the slides were inspected for fluorescence using LSM 510 META confocal microscope.

### Immunoblotting

Samples containing equal amounts of eggs were dissolved in nonreducing sample buffer and subjected to 12% SDS-PAGE according to procedures described previously (Miyado et al., 2000). After electrophoresis, the gels were transferred to PVDF membranes for immunoblot analysis. The blots were blocked in 1% nonfat dry milk, and were probed with the primary antibodies, anti-mouse CD81 antibody (Eat-1, BD Biosciences, California, CA) or anti-mouse CD9 antibody (KMC8, BD Biosciences). After washing in TBS-Tween buffer, the membranes were incubated with HRP-labeled secondary antibodies; goat anti-rat antibody or goat anti-hamster antibody. The expression level of immunoreacted products was determined by treatment of the blots with an ECL or ECL Plus Detection Kit (GE Healthcare Bio-Science Co., New Jersey, UK) and exposure to X-ray film at room temperature.

### Statistical Analysis

Data from different groups were tested by the *t*-test for the significance of the difference between the means of two independent samples using the computer software KaleidaGraph (version 3.6, Synergy Software, Inc., Pennsylvania, PA).

## RESULTS

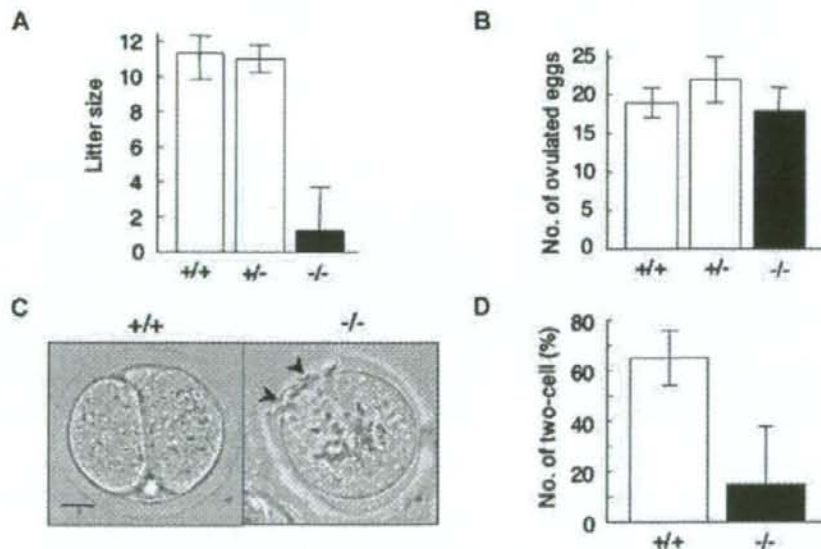
## Female Fertility Impaired by CD81 Deficiency

Figure 1A depicts the average litter size in matings of three genotypes of females, wild-type, CD81<sup>+/-</sup> and CD81<sup>-/-</sup> mice, with CD81<sup>+/-</sup> males over a period of 6 months. Although these females displayed normal mating behavior with the males (data not shown), the average litter size of CD81<sup>-/-</sup> females was markedly reduced relative to those of CD81<sup>+/-</sup> and wild-type mice (on average,  $1.3 \pm 2.5$  vs.  $11.3 \pm 1.3$  and  $11.0 \pm 0.8$ ) (Fig. 1A). To examine the oocyte maturation and ovulation, we also collected the eggs from mice superovulated by stimulation with exogenous gonadotropin. The eggs collected from CD81<sup>-/-</sup> mice were indistinguishable with regard to morphology and number (on average,  $18.0 \pm 2.8$ ) from those wild-type and CD81<sup>+/-</sup> mice (on average,  $19.9 \pm 1.7$  and  $21.7 \pm 2.8$ ) (Fig. 1B). Therefore, the reduction in fertility of CD81<sup>-/-</sup> females did not seem to be due to defects of ovulation or oocyte maturation.

## Involvement of CD81 in Fertilization

To clarify the cause of the reduced fertility of CD81<sup>-/-</sup> females, the function of CD81<sup>-/-</sup> eggs was further examined by IVF. When cumulus oocyte complexes (COCs) collected from CD81<sup>-/-</sup> or wild-type control mice were incubated with the wild-type sperm,

the sperm could disperse cumulus cells, somatic cells surrounding eggs, and reach and apparently penetrate the zona pellucida of CD81<sup>-/-</sup> and wild-type eggs. However, the average rate of eggs developing to the two-cell stage was substantially decreased for CD81<sup>-/-</sup> eggs (on average,  $15.0 \pm 2.5\%$ ) compared with that for wild-type eggs (on average,  $65.0 \pm 10.8\%$ ) 24 hr after incubation with the sperm (Fig. 1D). Furthermore, in CD81<sup>-/-</sup> eggs, several sperm were observed in the perivitelline space (Fig. 1C). The delayed formation of two-cell embryos and the accumulation of more than one sperm within the perivitelline space in CD81<sup>-/-</sup> eggs demonstrate that CD81<sup>-/-</sup> eggs have impaired ability of fertilization. Subsequently, to examine the cause of the impaired fertilization, we performed IVF for CD81<sup>-/-</sup> eggs and wild-type eggs after the zona pellucida was removed using acid Tyrode solution (Fig. 2A,B). To measure the number of sperm fused with eggs, both types of eggs were preloaded with DAPI before incubation with wild-type sperm (Yamagata et al., 2002). One hour after insemination, estimation of the average number of sperm fused with one egg by measurement of DAPI fluorescence revealed that CD81<sup>-/-</sup> eggs showed a decreased number of fused sperm (on average,  $1.21 \pm 0.23$ ) in comparison with the wild-type eggs (on average,  $1.95 \pm 0.27$ ). Those results suggest that CD81 is involved in sperm-egg fusion, either directly or indirectly.



**Fig. 1.** Female infertility caused by CD81 deficiency. **A:** Average litter sizes of crosses between CD81<sup>+/-</sup> males and three types of females, wild-type, CD81<sup>+/-</sup> and CD81<sup>-/-</sup> mice. Each of the mating pairs was kept in a separate cage, and births over a 6-month period were monitored. Data of births during successive 2-month periods were grouped together, and the average litter size of wild-type, CD81<sup>+/-</sup> and CD81<sup>-/-</sup> females was calculated from data recorded for five mating pairs 8–15 weeks of age at the start of the experiment. **B:** Average

number of ovulated eggs from wild-type, CD81<sup>+/-</sup> and CD81<sup>-/-</sup> female mice. The eggs were collected 13.5–16 hr after hCG treatment, and counted. **C:** Representative micrographs of CD81<sup>+/-</sup> and CD81<sup>-/-</sup> eggs. The eggs were obtained 24 hr after incubation with the wild-type sperm. **D:** Average number of eggs that developed to the two-cell stage 24 hr after incubation with the wild-type sperm. The black bars show the results for CD81<sup>-/-</sup> eggs (A,B,D). Error bars represent SEM (A,B,D). Scale bar, 20  $\mu$ m (C).

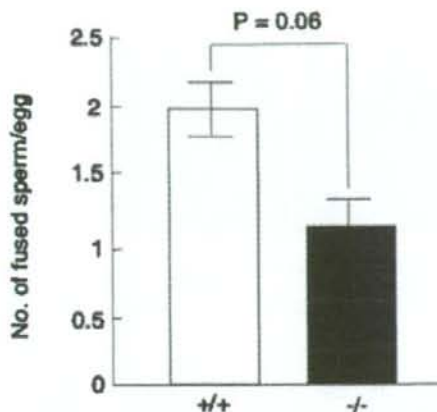


Fig. 2. In vitro sperm-egg fusion. Average number of sperm fused with wild-type or CD81<sup>-/-</sup> eggs after 3 hr of incubation. Error bars represent SEM. Data from different groups were tested by the *t*-test for the significance of the difference between the means of two independent samples ( $P = 0.06$ ).

#### Expression of CD9 in CD81<sup>-/-</sup> Eggs

The mechanisms by which CD81 acts are still unclear. However, CD81 tends to form multimolecular complexes in which tetraspanins associate with specific proteins depending on the type of cell. In B cells, CD81 directly associates with CD19, taking part in the CD19-CD21-CD81 signaling complex (Pileri et al., 1998), which accords with the evidence that the expression of CD19 in bone marrow, spleen, and peripheral B cells is reduced in CD81<sup>-/-</sup> mice (Miyazaki et al., 1997). As previously mentioned, CD9 on the egg plasma membrane is required for fusion with sperm, and the impaired fusibility of CD81<sup>-/-</sup> eggs with sperm may likely be dependent on the expression of CD9. To investigate whether CD81 deficiency may cause downregulation of CD9 expression, the expression level of CD9 was examined (Fig. 3). We collected three types of eggs,

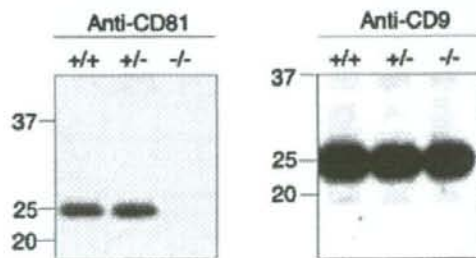


Fig. 3. The expression of CD9 in wild-type, CD81<sup>+/-</sup> and CD81<sup>-/-</sup> eggs. Proteins were isolated from the types of eggs indicated and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions. The proteins were electrophoretically transferred to a membrane, where they were probed with antibodies to CD81 (panel A) and CD9 (panel B). The proteins corresponding to each 110 egg (panel A) and 10 eggs (panel B) were analyzed.

wild-type, CD81<sup>+/-</sup> and CD81<sup>-/-</sup> eggs, 13.5–15 hr after hCG injection into mice, and examined the expression level of CD9 in comparison with that of CD81 by SDS-PAGE. The amounts of CD81 were invariable in wild-type and CD81<sup>+/-</sup> eggs, but CD81 was lost in CD81<sup>-/-</sup> eggs. By contrast, there were no significant differences in the expression of CD9 among these eggs. Therefore, the impairment of fertilization caused by CD81 deficiency cannot be attributed to decreased expression of CD9 in eggs.

#### Expression of CD81 During Folliculogenesis

The expression and localization of CD81 in ovarian tissues were immunohistochemically assessed using cryostat sections of adult wild-type ovaries. The follicles consist of immature eggs and granulosa cells that surround the egg; a single follicle usually grows to the preovulatory stage and releases the egg for potential fertilization (Buccione et al., 1990). Immunohistochemical staining with anti-CD81 mAb demonstrated that CD81 was continuously expressed in the egg and surrounding follicles (Fig. 4), and in cumulus cells surrounding ovulated eggs (data not shown). These data indicate that the sperm may encounter CD81 on the somatic cells surrounding eggs before direct interaction of sperm and eggs.

#### Possible Involvement of CD81 in Acrosome Reaction

Based on the localization of CD81 and the impaired fertilization of CD81<sup>-/-</sup> eggs, we speculated that the inability of wild-type sperm to fuse CD81<sup>-/-</sup> eggs might be due to impairment of prefusional stages, including the acrosome reaction. To examine the involvement of CD81 in the acrosome reaction of the sperm, CD81<sup>-/-</sup> eggs or wild-type eggs were incubated with the sperm collected from transgenic mice specifically expressing enhanced green fluorescent protein (EGFP) in the acrosomes (Fig. 5). The acrosome corresponds functionally to a lysosome and thus contains lysosomal enzymes (Moreno and Alvarado, 2006), and acrosin is a sperm acrosomal serine proteinase that is lost from the sperm head after the acrosome reaction is completed (Baba et al., 1994). Therefore, sperm expressing EGFP at the acrosomes in the heads are useful for the detection of acrosome-intact sperm. After 3 hr of incubation, we counted the number of acrosome-intact sperm within the perivitelline spaces. To count the sperm that had penetrated into the zona pellucida, the eggs were incubated with  $3.0 \times 10^5$  sperm/ml. When the number of sperm within the perivitelline space were counted 3 hr after incubation with the eggs, we observed that an increased percentage ( $8.5 \pm 2.3\%$ ) of the sperm that had penetrated into the perivitelline space of CD81<sup>-/-</sup> eggs exhibited EGFP fluorescence in their head portion. In contrast, very few sperm that had penetrated into the perivitelline space of wild-type eggs exhibited green fluorescence ( $1.4 \pm 1.0\%$ ). These results suggest that the sperm that penetrated into the zona pellucida of the CD81<sup>-/-</sup> eggs were impaired in the acrosome reaction.