

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

Elucidating nuclear reprogramming mechanisms: taking a synergistic approach



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

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Abstract

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

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

Cell differentiation and nuclear reprogramming

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

Wolffe, 1998). Such structures, formed via interactions between DNA and protein, are thought to compartmentalize chromatin into functional domains and, in some unknown way, stably maintain the differentiated state even when the cell divides.

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

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

Naturally induced nuclear reprogramming

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

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

Artificially induced nuclear reprogramming

In non-transformed somatic cells, once the differentiation programme of a cell has started, the process is normally irreversible. However, this programme may be reversed artificially. Using nuclear transfer (NT) (Wilmut *et al.*, 1997), cell fusion (Tada *et al.*, 1997), or even viral transduction of four specific stem cell genes (Takahashi and Yamanaka, 2006), it is possible to artificially and heritably alter a cell's gene expression and its functional identity. These techniques are collectively termed 'artificial induction of nuclear reprogramming' (Figure 1). The conversion of differentiated cells to pluripotent cells

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

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

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

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

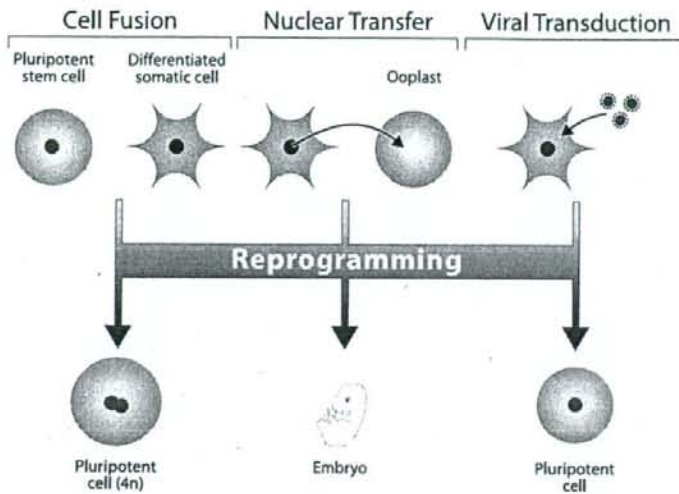


Figure 1. Artificially induced nuclear reprogramming. Cell fusion: a somatic cell fused with a pluripotent stem cell can be reprogrammed in the hybrid cell. These fused hybrid cells show similar features as embryonic stem (ES) cells; however the hybrid cell has a tetraploid karyotype and is unable to contribute to chimeras. Nuclear transfer: an adult somatic cell is transferred into an enucleated oocyte where it can produce ES cells which are pluripotent, contributing to all germ layers including the germ cell lineage. 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.

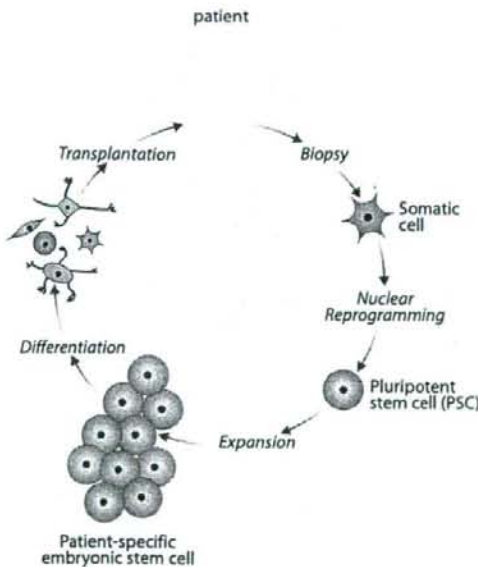


Figure 2. The ultimate goal of nuclear reprogramming research: controlled restoration of developmental potential. Once the mechanism by which nuclear reprogramming is understood, human somatic cells could be induced to dedifferentiate into pluripotent stem cells (PSC). PSC could then be expanded in culture 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 hybrid 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 x 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 *Msx1* expressing regions at the digit termini and more widely during early embryonic phases (Han et al., 2003). This process involves cell migration and a change in cell phenotype in response to the injury. There are certain caveats here, however. It is hard to dissect process important for reprogramming from other processes such as the innate immune response, cell migration, and other consequences of injury. It is also unknown to what extent these processes are conserved in mammals. Still, dedifferentiation of cells to form proliferating progenitor cells is interesting, and systems such as skeletal muscle, limb and tail regeneration or dorsal iris epithelium during lens regeneration should be studied further with screens designed to find the key players involved.

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

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

How can the study of this process be facilitated? One strategy is to use easily reprogrammable cells, such as cells differentiated from ES cells in culture (Bielloch 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 G₀/G₁ or G₂/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 G₂/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 G₂/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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Novel Cardiac Precursor-Like Cells from Human Menstrual Blood-Derived Mesenchymal Cells

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Key Words. Cardiomyogenesis human mesenchymal stem cell • Menstrual blood endometrial gland • Cell sheet technology cardiac precursors

ABSTRACT

Stem cell therapy can help repair damaged heart tissue. Yet many of the suitable cells currently identified for human use are difficult to obtain and involve invasive procedures. In our search for novel stem cells with a higher cardiomyogenic potential than those available from bone marrow, we discovered that potent cardiac precursor-like cells can be harvested from human menstrual blood. This represents a new, noninvasive, and potent source of cardiac stem cell therapeutic material. We demonstrate that menstrual blood-derived mesenchymal cells (MMCs) began beating spontaneously after induction, exhibiting cardiomyocyte-specific action potentials. Cardiac troponin-I-positive cardiomyocytes accounted for 27%–32% of the MMCs in vitro. The MMCs proliferated, on average, 28 generations without affecting cardiomyogenic transdifferentiation ability, and expressed mRNA of GATA-4 before cardiomyogenic induc-

tion. Hypothesizing that the majority of cardiomyogenic cells in MMCs originated from detached uterine endometrial glands, we established monoclonal endometrial gland-derived mesenchymal cells (EMCs), 76%–97% of which transdifferentiated into cardiac cells in vitro. Both EMCs and MMCs were positive for CD29, CD105 and negative for CD34, CD45. EMCs engrafted onto a recipient's heart using a novel 3-dimensional EMC cell sheet manipulation transdifferentiated into cardiac tissue layer in vivo. Transplanted MMCs also significantly restored impaired cardiac function, decreasing the myocardial infarction (MI) area in the nude rat model, with tissue of MMC-derived cardiomyocytes observed in the MI area in vivo. Thus, MMCs appear to be a potential novel, easily accessible source of material for cardiac stem cell-based therapy. *STEM CELLS* 2008;26:1695–1704

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Marrow-derived mesenchymal stem cells (MSCs) are a potential cellular source for stem cell-based therapy, since they have the ability to differentiate into cardiomyocytes [1, 2], use of MSCs presents no ethical problems, and autologous MSCs have been

injected into ischemic hearts clinically [3]. Direct injection of MSCs into the heart has been shown to be feasible in vivo [4–7], but with limited effect. The reason for this may be the extremely low rate of cardiomyogenesis exhibited by marrow-derived MSCs [2], with cardiac function improvement due to grafted MSC-induced neovascularization [7, 8] and an antiapoptotic

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effect on infarcted cardiomyocytes [9, 10]. To further improve prospects of restoring cardiac function, a search was initiated for another source of cells having high cardiomyogenic potential.

Our previous study showed that umbilical cord blood-derived mesenchymal stem cells (UCBMSCs) [11] and placental chorionic plate cells (PCPCs) [12] have a phenotype of mesenchymal cells and have higher cardiomyogenic differentiation ability *in vitro*. Since these materials are deemed medical waste and can be obtained without any ethical problems, they may be a suitable stem cell source for cardiac regenerative therapy. But the population of UCBMSCs in umbilical cord blood is scant [13] and there is also a problem in establishing PCPCs, since placental tissue contains a lot of maternal decidual-derived mesenchymal cells that could contaminate PCPCs. Therefore, it is difficult to obtain enough of these cells without using a limiting dilution method and/or massive *ex vivo* propagation, which may cause instability of the genome [14]. Consequently, material that contains a large amount of mesenchymal cells during the first few passages should be a highly suitable source of stem cells.

A previous paper suggests that endometrium contains an MSC-like population [15] and menstrual blood-derived mesenchymal (MMCs) cells have a pluripotent differentiation ability *in vitro* [16]. The data presented here demonstrate that human menstrual blood-derived mesenchymal cells and uterine endometrial gland-derived mesenchymal cells (EMCs) have a strong potential for cardiomyogenic transdifferentiation *in vitro* and *in vivo*. Moreover, large amounts of MMCs could be obtained from the first passage of menstrual blood culture, and MMCs have been shown to restore impaired cardiac function through marked cardiomyogenesis *in vivo*.

MATERIALS AND METHODS

Isolation of MMCs and EMCs

After informed consent was obtained, mesenchymal cells from approximately 10 ml of menstrual blood of six women (20–30 years old) were collected on the first day of menstruation. The samples were suspended in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% FBS, and split into two 10-cm dishes. The estimated adherent cell number at the start of culture was approximately 1×10^7 . The growth curve and phase-contrast microscopic view are shown in supplemental online Fig. 1. The results for MMCs obtained from six women were the same. A human endometrial tissue sample was also taken from a 52-year-old woman undergoing hysterectomy [17]. Individual endometrial glands were isolated under a microscope and then seeded. After the retroviral transfection of HPV16E6, E7, and hTERT [2], endometrial cell strains were generated by the limiting dilution method. Two strains exhibiting rapid cell division cycles were designated EMC100 and EMC214 (Fig. 3B and 3D, respectively). EMC100 and EMC214 showed adherent spindle shape morphology that proliferated for more than 250 population doublings without changing cardiomyogenic differentiation ability.

Isolation of Marrow-Derived Mesenchymal Stem Cells

Bone marrow-derived mesenchymal stem cells (BMMSCs) were obtained from a 41-year-old male as described previously [2].

Coculture with Murine Fetal Cardiomyocytes

MMCs, EMCs, and BMMSCs were infected with enhanced green fluorescent protein (EGFP) expressing adenovirus [2]. Fetal cardiomyocytes were obtained from hearts of day-17 mouse fetuses, as previously described [2]. The isolated cardiomyocytes were replated at $5 \times 10^4/cm^2$ on top of a floating athelocollagen membrane (CM-6, 40- μm thickness; Koken, Tokyo, http://www.kokenmpc.co.jp/english/products/collagen/cell_culture/cm-6_24/index.html) that

is permeable for only small molecules (less than 5,000 MW). The next day, the athelocollagen membrane was plated upside down on the culture dish. Harvested EGFP-labeled MMCs and EMCs were then seeded upon the athelocollagen surface (bottom surface) at $7 \times 10^4/cm^2$ (Fig. 1M). In several experiments (Figs. 1G–1L, 2, 3E, 3H, 3K–3M, 4, supplemental online Fig. 2, examination of chromosome chimeras), we did not use the athelocollagen membrane for the coculture system.

Immunocytochemistry and Immunohistochemistry

A laser confocal microscope (FV1000; Olympus, Tokyo, <http://www.olympus-global.com>) was used for immunocytochemical analysis. Samples were stained with mouse monoclonal anti-cardiac troponin-I antibody (4T21 Lot 98/10-T21-C2; HyTest, Euro, Finland, <http://www.hytest.fi/>) or with mouse monoclonal anti-sarcomeric α -actinin antibody (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>), or anti-connexin 43 antibody (Sigma-Aldrich) diluted 1:300 overnight at 4°C, then stained with TRITC-conjugated anti-mouse antibody (Sigma-Aldrich), TRITC-conjugated anti-rabbit antibody (Sigma-Aldrich), and Cy5-conjugated anti-mouse IgG (Chemicon, Temecula, CA, <http://www.chemicon.com>) diluted 1:100, containing 4'-6-diamidino-2-phenylindole (DAPI; Wako Chemical, Osaka, Japan, <http://www.wako-chem.co.jp/english>) at 1:300 for 30 minutes at 25°C–28°C. See also supplemental online data 1 for detail of method.

Functional Analysis

The method of action potential (AP) recording was as previously described [2] but with slight modification. A fluorescence inverted microscope (IX-70; Olympus) was used for AP recording. The microscope was equipped with a recording chamber and a noiseless heating plate (Microwarm Plate; Kitazato Supply, Fujinomiya, Shizuoka, Japan, <http://www.kitazato-supply.com>). A 10-mM volume of HEPES (Sigma-Aldrich) was added to the culture medium to stabilize the pH of the perfusate at 7.5. Standard glass microelectrodes having a direct current resistance of 15–25 M Ω when filled with pipette solution were used. Alexa 568 compound was dissolved to a concentration of 0.5 mM in 2 M of KCl solution in order to completely dissolve the Alexa 568 in the pipette solution. The electrodes were positioned with a motor-driven micromanipulator (PCS-5000; Burleigh Instrument, Inc., New York) under optical control. Spontaneously beating EGFP-positive cells were selected as targets, and after the APs of the target cells had been recorded, the dye was injected by iontophoresis (-7 nA for 10–20 seconds). The extent of dye transfer was monitored under a fluorescence microscope, and digital images were recorded with a digital photo camera (EOS-digital; Canon, Tokyo, <http://www.canon.com>) mounted on the microscope. The recording pipette was connected to a patch-clamp amplifier (MEZ-8300; Nihon Kohden, Tokyo, <http://www.nihonkohden.com>). The amplified signal was filtered with a 4-pole Bessel filter (NF-3625; NF electronic instrument; NF Corp., Tokyo, <http://www.nfcorp.co.jp/english/index.html>) set at 2 kHz, then digitized with an A/D converter with a sampling frequency of 10 kHz (Digitata 1.3224; Molecular Devices Corp., Union City, CA, <http://www.moleculardevices.com>). Pacemaker potential was defined by the slowly depolarizing membrane potential at phase IV of the AP.

Alexa 568 was injected into cells via recording microelectrodes to stain the cells and confirm that the AP was generated by EGFP-positive cells (Fig. 1G–1I, 3E, 3H). Since the dye did not diffuse into the EGFP-negative murine cardiomyocytes, there were no tight cell-to-cell heterologous connections (i.e., gap junctions), at least in the *in vitro* condition. In some experiments, Alexa 568 diffused into the EGFP-positive satellite EMCs and MMCs, suggesting that a homologous cell-to-cell connection had been established at least 1 week after cocultivation. The measured parameters of the APs were averaged and are shown in Figure 1K.

The fluorescent image of the beating MMCs and EMCs was monitored using a CCD camera (Ikegami Tsushin Co., Ltd, <http://www.ikegami.co.jp>) and was stored using digital video. The video images (National Television Standards Committee format, 29.97 frame/second) of contraction of EMCs and MMCs were stored in a personal computer as MPEG-2 format files, then analyzed later.

Both edges of the EGFP-positive EMCs and MMCs along the line (Figs. 1L, 3K) were automatically detected, and the distance between both edges was measured from each video frame using an image edge-detection program using Igor Pro 4 (Wavemetrics Inc., Lake Oswego, OR) [11].

Calculation of Induction Rate

The MMCs and EMCs were exposed to 3 μ M 5-azacytidine (5-azaC; Sigma-Aldrich) for 24 hours to induce cell differentiation, or were left untreated. The 5-azaC-treated and nontreated MMCs or EMCs, cultivated with or without murine fetal cardiomyocytes, were enzymatically dissociated and stained, then observed by confocal laser microscope (supplemental online data 2 for detail of method). The cardiomyogenic induction rate (average of 10 separate experiments) was calculated as the fraction of cardiac troponin-I-positive cells in the EGFP-positive cells.

Examination of Chromosomes of MMCs or EMCs and Murine Cell Chimeras

To rule out cell fusion-dependent cardiomyogenesis, chromosomes from MMCs or EMCs cocultivated without separation by the athelocollagen membrane from murine cardiomyocytes for 1 week were stained using a human chromosome-specific probe and a mouse chromosome-specific probe (Chromosome Science Labo, Hokkaido, Japan, <http://www.chromosome-science.jp/en/probe/page01/page01e.html>) and spectral karyotyping with fluorescent *in situ* hybridization chromosome painting technique (Applied Spectral Imaging, Vista, CA, <http://www.spectral-imaging.com>), according to the manufacturer's protocol.

RNA Extraction and RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was done as described previously [2]. Primers for the following genes were used: cardiac transcription factors—Csx/Nkx-2.5 and GATA4; cardiac hormones—atrial natriuretic peptide and brain natriuretic peptide; cardiac structural proteins—cardiac troponin I, cardiac troponin T, myosin light chain-2a, myosin light chain-2v, and cardiac-actin; and ion channel—cyclic nucleotide-gated potassium channel 2 (supplemental online Table 1). The internal control was 18S rRNA. PCR primers were prepared such that they would amplify the human but not the mouse genes.

Flow Cytometric Analysis

The cells were analyzed using an EPICS ALTRA analyzer (Beckman Coulter, Fullerton, CA, <http://www.beckmancoulter.com>). Antibodies (anti-human CD10, CD13, CD14, CD24, CD29, CD31, CD34, CD44, CD45, CD54, CD55, CD59, CD71, CD73, CDw90, CD105, CD106, CD117, CD133, CD140a, CD166, CD309, HLA-ABC, and HLA-DR) [12] were purchased from Beckman Coulter, Immunotech (Luminy, France, http://www.beckmancoulter.com/products/pr_immunology.asp), Cytotech (Hellebaek, Denmark, <http://www.cytotech.dk/index.html>), Santa Cruz Biotechnology Inc. (Santa Cruz, CA, <http://www.scbt.com>), RDI (Research Diagnostics, Inc., Concord, MA, <http://www.researchdi.com>), and Pharmingen Pharmaceutical, Inc. (San Diego, http://wwwbdbiosciences.com/index_us.shtml).

In Vivo Cardiomyogenic Differentiation of EMCs

EGFP-labeled EMC tissue graft, made by a novel 3-dimensional cell sheet manipulation, was transplanted into male F344 nude rats (Clea, Tokyo, <http://www.clea-japan.com/>) (8 weeks of age). EMC100s and EMC214s ($2 \times 10^5/cm^2$) were plated onto fibrin polymer-coated culture dishes. Four days after plating, EMCs were detached as previously described [18], and transplanted onto the surface of the recipient heart (Fig. 5A) [19]. At 2 weeks after transplantation, immunohistochemical analysis was performed. EGFP-labeled EMC tissue graft on the fibrin polymer-coated culture dish did not show cardiomyogenic differentiation *in vitro*.

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MMC Transplantation in Myocardial Infarction Model In Vivo

Recipient male F344 nude rats (Clea) (6 weeks of age) were anesthetized with 2% isoflurane gas. After left thoracotomy, the left ventricle was exposed and left anterior coronary artery was ligated by 6-0 silk suture. The complete occlusion of the coronary artery was confirmed by the cyanotic color and dyskinetic motion of the left ventricular anterior wall. In some rats, we did not ligate the coronary artery (Sham). The chest was closed and animals survived for 2 weeks to create complete myocardial infarction.

Two weeks after the first operation, rats with myocardial infarction were randomized for the control myocardial infarction (MI) group, the MI+BMMSC group, and the MI+MMC group, and were blinded immediately before the cell injection. Echocardiograms were performed on the anesthetized (2% isoflurane) rats. Data were collected three times and averaged. Immediately before transplantation, $\sim 1-2 \times 10^6$ of EGFP-positive MMC or BMMSC suspension was drawn up into a 50- μ l Hamilton syringe (Hamilton Co., Reno, NV, http://www.hamiltoncompany.com/main_usa.asp) with a 31-gauge needle. A 10- μ l portion of the cell suspension was injected into the center and margin of the infarcted myocardium (MI+MMC, Fig. 7A). In the control MI group, culture medium or $\sim 1-2 \times 10^6$ of murine cardiac fibroblast was injected. Immediately before cell transplantation, 2-dimensional and M-mode echocardiographic (8.5 MHz linear transducer, EnVisor C; Phillips Medical System, Andover, MA, <http://www.medical.phillips.com/index.html>) images were obtained to assess left ventricular (LV) end-diastolic dimension and LV end-systolic dimension at the mid-papillary muscle level.

Two weeks after the transplantation, a similar echocardiogram was performed again; then after opening the abdomen, a blood sample was drawn from the abdominal great vein; then the left diaphragm was dissected to insert a 22-gauge manometer line into the left ventricle, which was connected to the transducer (model TP-400T; Nihon Kohden) to monitor left ventricular pressure. The electrocardiogram and measured pressure were digitized by PowerLab (ADInstruments, Milford, MA, <http://www.adinstruments.com>) at the sample frequency of 10 KHz and stored in a personal computer (Macintosh iBook G4; Apple, Cupertino, CA, <http://www.apple.com>).

Tissue samples were obtained by fixing and slicing along the short axis of the left ventricle, for every 1-mm depth of the ventricle. After Masson's trichrome staining, digital images of samples were collected using a light microscope (IX-70; Olympus). The images were digitized and analyzed using Igor Pro 4 (Wavemetrics Inc.). The pixel area of blue color (fibrosis area) was defined as the infarcted area, and the pixel area of red color was defined as "survived" myocardium. The data on each pixel area from each slice were collated and the percentage fibrosis area was calculated as follows: % Fibrosis = $100 \times (\text{Pixel area of blue color})/(\text{Pixel area of blue color and red color})$.

Statistical Analysis

All data are shown as the mean value \pm SE. The difference among mean values was determined with analysis of variance. The posthoc test (Bonferroni) was used when three or more groups were compared. Student's *t* test was used when two values were compared. Statistical significance was set at $p < .05$.

RESULTS

Cardiomyogenic Transdifferentiation of MMCs

To exclude cell fusion-dependent cardiomyogenesis [20], EGFP-labeled MMCs were cocultured in the same dish with mouse cardiomyocytes, separated by a 40- μ m high-density athelocollagen membrane (Fig. 1M). The two cell types were never in direct contact. On day 5 after cocultivation commenced, approximately half of the MMCs were beating strongly in a synchronized manner (supplemental online Video 1). Im-

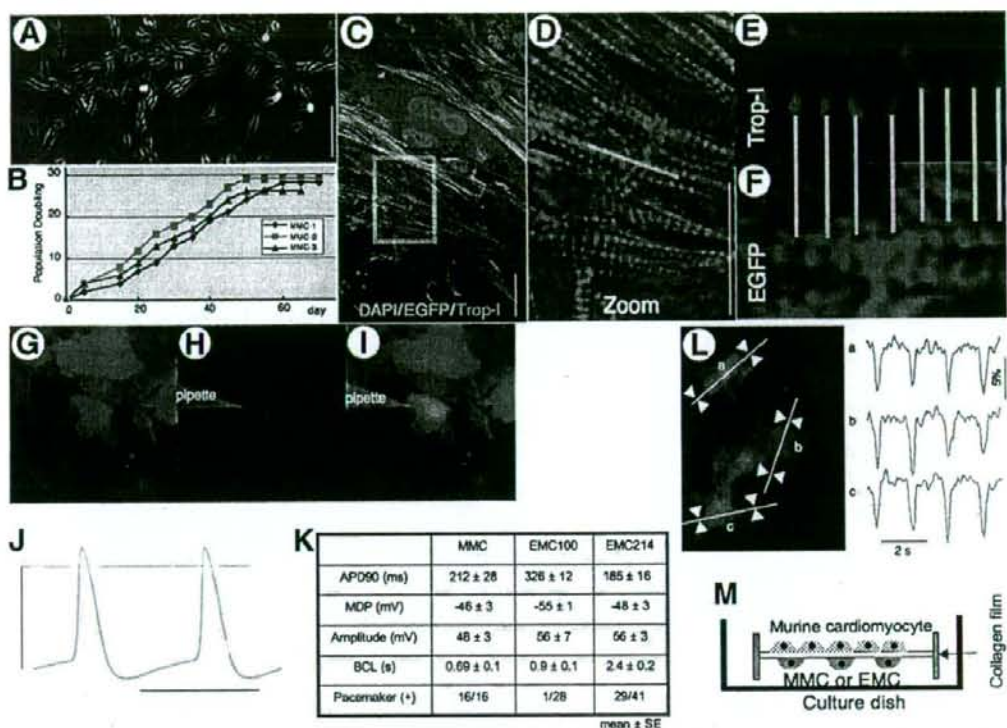


Figure 1. Cardiomyogenic differentiation of menstrual blood-derived mesenchymal cells (MMCs) in vitro. (A): Phase-contrast microscopic view of MMC (bar denotes 100 μ m), regarded as being PD1, or day 2. (B): The representative growth curves of MMCs as a function of time after the culture. The growth curves from all three donors are linear over at least 25 population doublings. (C–F): Laser confocal microscopic view of immunocytochemistry of differentiated MMCs with anti-cardiac troponin-I (Trop-I) antibody. Enhanced green fluorescent protein (EGFP)-positive (green) human MMCs expressed Trop-I (red). Scale bar denotes 20 μ m. (D): Expansion of area within the white box in (C). Clear striation pattern of Trop-I is observed. Trop-I and EGFP images along the yellow line are shown in (E, F). (E, F): Trop-I and EGFP staining was observed alternately in striated manner, suggesting Trop-I is expressed in the EGFP-positive cell. (G–I): EGFP-labeled MMCs were injected with Alexa 568 solution (red) through a microelectrode to confirm that the recorded signal was obtained from MMCs. (J): Representative action potential traces are shown (horizontal line denotes 500 ms). The vertical line denotes 50 mV, and dotted horizontal line denotes 0 mV. (K): Action potential parameters. (L): A representative still image (left panel) and detected fractional shortening (% FS) along the white line obtained from sites a, b, and c are shown in right panel. (M): Experimental schema. Abbreviations: ADP, action potential duration; BCL, basic cycle length; DAPI, 4',6-diamidino-2-phenylindole; MDP, maximum diastolic potential.

munocytochemistry revealed that the MMCs were stained positive by the anti-cardiac troponin-I antibody (Fig. 1C–E). Clear striations of red fluorescence of troponin-I in the differentiated MMCs (Fig. 1D, 1E) were observed. Troponin-I and EGFP staining appeared alternately in a striated manner, suggesting troponin-I expressed in the EGFP-positive cell (Fig. 1E, 1F). Clear striations were observed with red fluorescence of α -actinin in the differentiated MMCs (Fig. 2B) and diffuse dot-like staining pattern of connexin 43 around the margin of each EGFP-positive cardiomyocyte (Fig. 2C–2F), suggesting that these human transdifferentiated cardiomyocytes have tight electrical coupling with each other. APs were recorded from spontaneously beating MMCs. The APs obtained from MMCs showed clear cardiomyocyte-specific sustained plateaus and slowly depolarizing resting membrane potentials—so-called “pacemaker potentials” (Fig. 1J, 1K)—and were, therefore, determined to be APs of cardiomyocytes, not of smooth muscle cells, nerve cells, or skeletal muscle cells. The fractional shortening (% FS) of the MMCs was analyzed (Fig. 1L) using a cell edge detection program. The EGFP-positive cells contracted simultaneously within the whole visual field. The % FS was $5.9 \pm 0.5\%$ ($n = 19$).

The percentage of cardiac troponin-I-positive cells was calculated to determine the cardiomyogenic transdifferentiation rate. Whereas MMCs without cocultivation did not show any troponin-I expression (supplemental online Figs. 1A–1D, 2A, 2B), 27%–32% of MMCs became positive for cardiac troponin-I antibody as a result of the cocultivation (Figs. 1C–1F, 4A, supplemental online Fig. 2C, 2D). A cytosine analog, 5-azaC, has a remarkable effect on cell transdifferentiation and has been shown to induce transdifferentiation of BMSCs into cardiomyocytes in mice by nonspecific demethylation of the genome [1]. Cardiomyogenic transdifferentiation was observed in the cocultivated MMCs without any 5-azaC pretreatment, meaning that 5-azaC was not essential for cardiomyogenic transdifferentiation. Nuclear fusion between the cocultivated MMCs and murine cardiomyocytes without separation of the athelocollagen membrane was observed in only 0.16% (3/1846).

Cardiomyogenic Transdifferentiation of EMCs

We hypothesized that the origin of cardiomyogenic cells in the MMCs was the endometrial gland, since MMCs have a high content of detached endometrial glands, whereas circu-

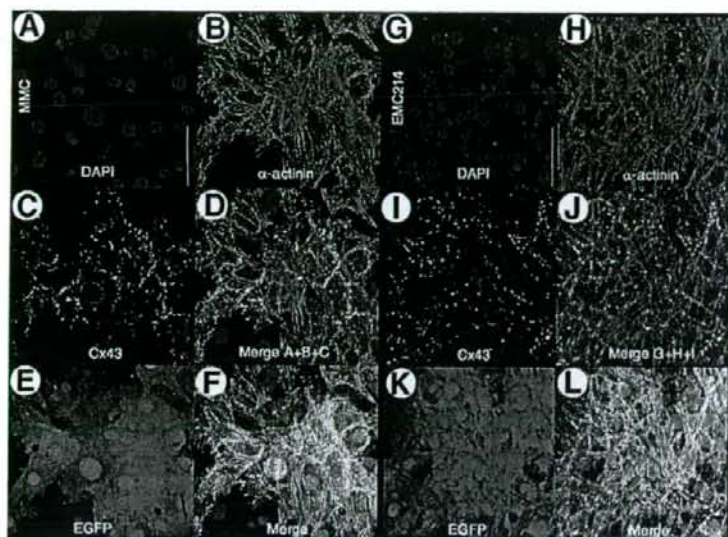


Figure 2. Immunocytochemical analysis of menstrual blood-derived mesenchymal cells (MMCs) and EMC214s stained with anti-sarcomeric α -actinin and connexin 43. (A–L): Laser confocal microscopic view of immunocytochemistry of differentiated MMCs and EMC214s with anti-sarcomeric α -actinin (α -actinin) and connexin 43 (Cx43) antibody. (A–F, G–L): Enhanced green fluorescent protein (EGFP)-positive (E, K; green) human MMCs and EMC214s express α -actinin (B, H; red) and Cx43 (C, I; cyan). Nuclei are stained with 4'-6-diamidino-2-phenylindole (DAPI) (A, G; blue). Clear striation patterns of α -actinin and diffuse Cx43 dot-like staining around the margin of the MMCs and EMC214s were observed. Scale bars in the figure denote 50 μ m.

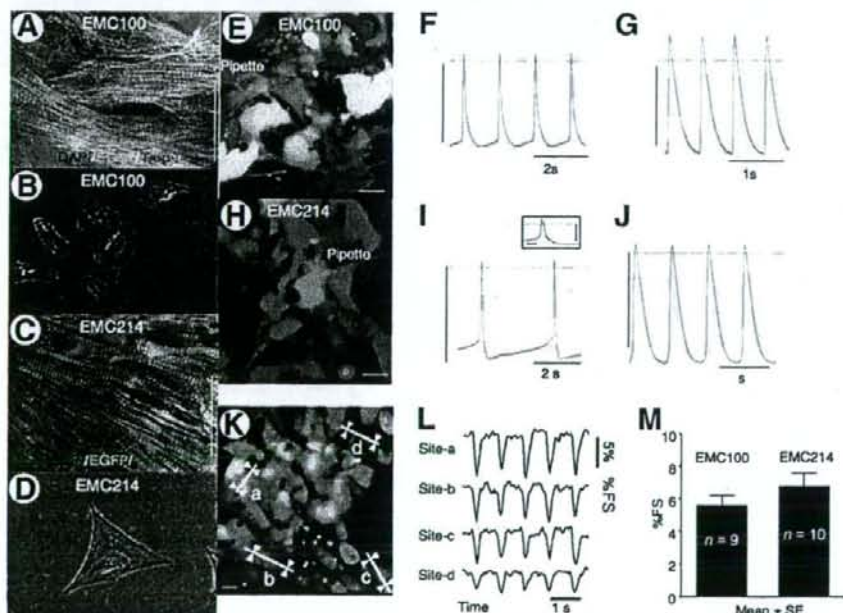


Figure 3. Cardiomyogenic differentiation of endometrial gland-derived mesenchymal cells (EMCs) in vitro. (A, C): Immunocytochemistry of differentiated EMC100s (A) and EMC214s (C) with anti-cardiac troponin-I (Trop-I) antibody. The cells were stained with 4'-6-diamidino-2-phenylindole (DAPI; blue), and anti-cardiac troponin-I antibody (red). Enhanced green fluorescent protein (EGFP)-positive (green) human EMCs expressed Trop-I (red). Please note clear striation staining pattern of Trop-I (A, C) in EMCs. Scale bar denotes 20 μ m. (B, D): Phase-contrast images of EMC100s (B) and EMC214s (D) before the cardiomyogenic induction. (E, H): EGFP-labeled EMC100s and EMC214s (green) were injected with Alexa 568 solution (red) through a microelectrode (E, H), and a recorded signal was obtained from the cells. Representative action potential traces are shown (F, G: EMC100; I, J: EMC214). Action potential of E is expanded in the inset (the vertical line denotes 100 ms). The vertical line denotes 50 mV and dotted horizontal line denotes 0 mV levels. (K–M): A representative still image (K) and detected fractional shortening (% FS) along the white line obtained from sites a, b, c, and d in (L) are shown in (M). (M): The measured % FS was averaged and is shown.

lating blood-derived endothelial progenitor cells [21] or marrow-derived MSCs [2] do not have such high cardiomyogenic differentiation ability. We consequently established a line of

EMCs (Fig. 3B, 3D) with a lifespan prolonged by a cell cycle-mediated gene to ensure a supply of cells for analysis. Almost all EMCs beat strongly in a synchronized manner

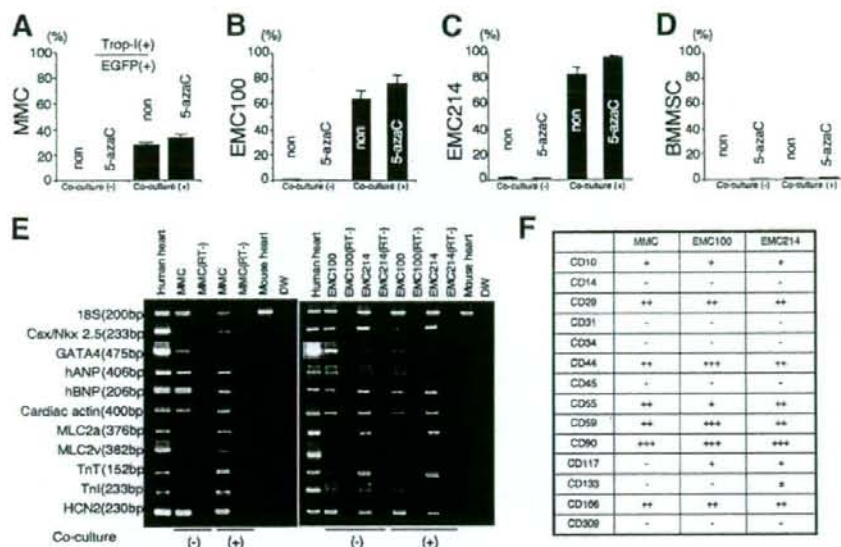


Figure 4. Cardiomycogenic transdifferentiation rates and expression of cardiomyocyte-specific genes and cell surface markers of menstrual blood-derived mesenchymal cells (MMCs) and endometrial gland-derived mesenchymal cells (EMCs). (A–D): Cardiomycogenic transdifferentiation rates of MMCs, EMCs, and bone marrow-derived mesenchymal stem cells (BMMSCs). The character in each column denotes pretreatment with 5-azacytidine (5-azaC) or the lack of treatment (non). (E): Reverse transcriptase polymerase chain reaction (PCR) was performed with PCR primers with specificity for human genes encoding cardiac proteins but not for the corresponding murine genes (supplemental online Table 1). Human heart and mouse heart cells were used as a positive control and negative control, respectively. Most human cardiac genes were constitutively expressed in the default state of MMCs and EMCs. (F): Summary of flow cytometric analysis of MMCs and EMCs with fluorescein isothiocyanate–coupled antibodies against human surface antigens. Abbreviations: DW, distilled water; EGFP, enhanced green fluorescent protein; hANP, human atrial natriuretic peptide; hBNP, human brain natriuretic peptide; HCN2, cyclic nucleotide-gated potassium channel 2; MLC2a, myosin light chain 2a; MLC2v, myosin light chain 2v; TnI, Trop-I, cardiac troponin I; TnT, cardiac troponin T.

(supplemental online Video 1), and 76.4%–96.5% became positive for cardiac troponin-I antibody as a result of cocultivation (Figs. 3A, 3C, 4B, 4C, supplemental online Fig. 2E–2L). EMCs were also positive for sarcomeric α -actinin and connexin 43 (Fig. 2G–2L). APs were recorded from EMCs. The APs obtained from EMCs showed clear cardiomyocyte-specific sustained plateaus and, in some cells, pacemaker potentials (Fig. 3E–3J). The EGFP-positive EMCs contracted simultaneously within the whole visual field (Fig. 3L, 3M). Nuclear fusion between the cocultivated EMC100s or EMC214s and murine cardiomyocytes without separation of the athelocollagen membrane was observed in only 0.57% (6/1058) or 0.28% (5/1758), respectively.

Expression of Cardiomyocyte-Specific Genes and Surface Markers of EMCs and MMCs

The RT-PCR was performed with primers that hybridized with human cardiomyocyte-specific genes but not with the murine orthologs. Differentiated MMCs and EMCs expressed cardiac-specific genes (Fig. 4D). Interestingly, most of the analyzed genes were expressed in the cells before the induction of transdifferentiation by cocultivation.

There is no difference between surface markers of the MMCs and EMCs. Both cells were positive for CD29 (integrin β 1), CD59, and negative for CD14, CD34, CD45, CD309 (Flk-1), etc. (Fig. 4E, supplemental online Fig. 3A–3C).

Cardiomycogenic Effects In Vivo

An EGFP-labeled EMC tissue graft made by a novel 3-dimensional cell sheet manipulation [18] was transplanted into male F344 nude rats to ensure in vivo cardiomycogenic transdifferen-

tiation ability. The EGFP-positive cell layer (green) was observed at the epicardial surface of the host heart (Fig. 5B–5D). Whole EMCs throughout the layer expressed a clear striation staining pattern of sarcomeric α -actinin (Fig. 5B–5G), suggesting extremely high cardiomycogenic transdifferentiation ability of EMCs in situ.

MMCs or BMMSCs were transplanted into the nude rats with MI in vivo. Echocardiography showed that the left ventricular fractional shortening (% LVFS) in the MI+MMC group was significantly greater than it in the MI+BMMSC group at 2 weeks after transplantation (Fig. 6A–6I, supplemental online Fig. 4). The MI area was digitized and every 1-mm depth of tissue section stained with Masson's trichrome (Fig. 6J–6O); averaged data are shown in Figure 6P. The MI area was significantly lower in the MI+MMC group than in the MI+BMMSC group. The EGFP-positive mass of MMCs observed in the MI area expressed a clear striation staining pattern of cardiac troponin-I (Fig. 7) and sarcomeric α -actinin (supplemental online Fig. 5), suggesting an extremely high in situ cardiomycogenic transdifferentiation ability of MMCs, which contributed to improvement in cardiac function.

DISCUSSION

Mechanisms of Highly Cardiomycogenic Transdifferentiation Ability of MMCs and EMCs

The gene expression pattern of MMCs and EMCs before cardiomycogenic transdifferentiation is quite different from that of marrow-derived MSCs [2]. GATA-4 expression in the MMCs and EMCs, and Csx/Nkx 2.5 expression in EMCs with the

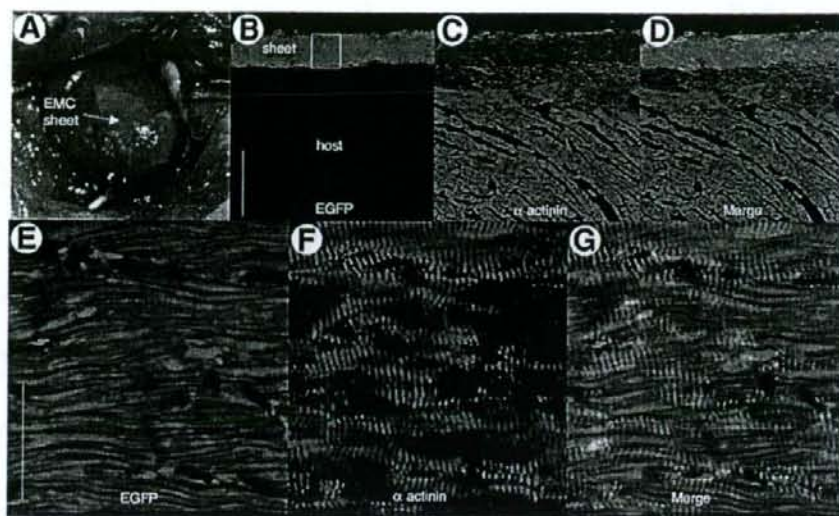


Figure 5. In vivo cardiomyogenesis of endometrium-derived mesenchymal cells (EMCs) in cell sheet tissue graft on host heart. (A): Macroscopic view of enhanced green fluorescent protein (EGFP)-labeled EMC tissue graft (sheet) on the epicardial surface of the recipient's heart. (B–D): Two weeks after transplantation, immunohistochemistry revealed survival of EMC tissue layer (green) on the recipient heart. Scale bar denotes 100 μm . (C): Engrafted EMCs stained positive with anti-sarcomeric α -actinin (red; α -actinin). (E–G): The area in the white box in (B) is shown in greater detail in (E–G). (F): The clear striation pattern of α -actinin staining was observed throughout the entire layer of engrafted EMCs, suggesting extremely high cardiomyogenic potential of EMCs in situ. Scale bar denotes 20 μm .

ability of self-renewal suggest that MMCs and EMCs both have cardiogenic potential and may be termed "cardiac precursor cells" due to their biological features. Cardiac mRNA but not cardiac protein (i.e., troponin-I) was expressed at the default state in the present study, suggesting that both genetic and epigenetic factors may be essential to cause physiologically functioning cardiomyogenic differentiation in MMCs and EMCs. The mechanism of the drastic improvement in the transdifferentiation rate of MMCs and EMCs may be attributable to the default characteristics (expression level of cardiomyocyte-specific mRNA) of MMCs and EMCs in culture compared to marrow-derived MSCs. Highest cardiomyogenic transdifferentiation efficiency was observed in EMC214s (96.5%), EMC100s (76.4%), UCBMSCs (44.9%) [11], MMCs (33.2%), PCPCs (15.1%) [12], and BMMSCs (0.3%, Fig. 4D) [2] in that order. In the practical point of view, EMCs and UCBMSCs are difficult to obtain in enough numbers during the first few passages. MMCs are, therefore, the most suitable cellular source for cardiac stem cell therapy, having a high cardiomyogenic transdifferentiation efficiency. MMCs, EMCs, UCBMSC, and PCPCs are derived from the organ that is related to the pregnancy, therefore the high cardiomyogenic transdifferentiation ability of mesenchymal cells may be caused by a pregnancy-related environmental condition.

Origin of the MMCs and EMCs

Cell surface marker analysis revealed that MMCs are neither encirculating endothelial progenitor cells [22] nor macrophages, but are mesenchymal phenotype cells. We speculated that MMCs may originate in uterine endometrial glands since a lot of detached endometrial glands were observed in menstrual blood and EMCs have the same surface marker as the MMCs, as well as an extremely high cardiomyogenic potential (76.4%–96.5% and 33.2%, respectively). As has been reported, MSCs cannot be detected in circulating blood and all tissues have MSC

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reservoirs localized in the perivascular niche [23], so EMCs and MMCs do not seem to originate from BMMSCs.

Clinical Contribution

In the present study, MMC transplantation improved impaired cardiac function in vivo. Since MMCs were transplanted at 2 weeks after coronary occlusion, when myocardial necrosis had been completed, the improvement of cardiac function is not due only to transplanted MMC-induced neovascularization [7, 8] or an antiapoptotic [9] effect on infarcted cardiomyocytes. Since they display high cardiomyogenic transdifferentiation ability in vitro and massive cardiomyogenic transdifferentiation in vivo, MMC-derived cardiomyocytes may play a role in the improvement of cardiac function in the present study. Myocardial infarction is known to suppress contraction ability of cardiomyocytes even at normal zone by left ventricular remodeling. Therefore MMC-derived paracrine factors may also play an important role in recovery of % LVFS by prevention of development of LV remodeling.

Neovascularization and the antiapoptotic effect are important for improving cardiac function to some extent. However, the feasible effect is dependent on the number of residual host cardiomyocytes in the infarcted myocardium. To achieve further improvement of cardiac function, a stem cell source that can be expected to exhibit powerful cardiomyogenic transdifferentiation in situ is required. MMCs can be transdifferentiated into cardiomyocytes in situ on the recipient heart, suggesting that they are a promising source for cardiac stem cell-based therapy material, significantly more efficient for cardiomyogenesis than BMMSCs.

MMCs can be readily obtained in a noninvasive manner from young female volunteers, and stored. It should therefore be possible to obtain MMCs of all the HLA types, possibly enabling the establishment of an MMC bank system to facilitate cardiac stem cell-based therapy.

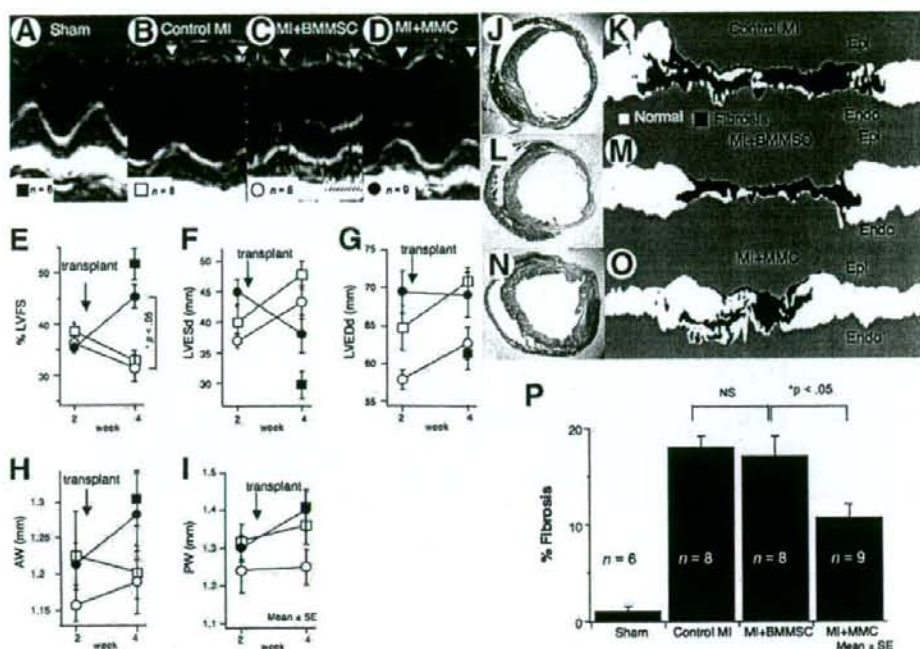


Figure 6. The effect of menstrual blood-derived mesenchymal cell (MMC) transplantation on cardiac function. (A–D): Representative M-mode echocardiographic images. The contraction of the left ventricular (LV) anterior wall was improved by transplantation of MMCs (white arrows). The symbol and number in each group is depicted at the bottom left of each image. (E–I): Measured LV parameters are averaged and shown at 2 weeks and 4 weeks after the myocardial infarction (MI). The significant improvement of (F) LV end-systolic diameter (LVESd) and (E) % fractional shortening (% LVFS) were observed. The diameter of (H) anterior left ventricular wall thickness (AW), and (I) posterior left ventricular wall thickness (PW). There is no statistical significance. (J–O): Representative Masson's trichrome stain images (J, L, N) and digitized images (K, M, O) of control MI group, MI + bone marrow-derived mesenchymal stem cell (BMMSC), and MI + MMC group are shown. (P): The calculated % fibrosis areas are summed and averaged. The MMC transplantation showed significant reduction of % fibrosis area. Abbreviations: Endo, endocardium; Epi, epicardium; NS, not significant.

Role of Established Cardiomyogenic EMC Cell Line for Determining Cardiomyogenic Factors

Several stem cell types are used for clinical patients. Of these, MSCs are reported to show cardiomyogenesis *in vitro*. Thus, the analysis of key mechanisms for cardiomyogenic differentiation in the human mesenchymal cell is extremely important in order to expand the efficacy of current cardiac stem cell therapy. However, it is very difficult to specify the key factor of cardiomyogenesis by *in vivo* experiment only. Establishment of EMCs and an *in vitro* cardiomyogenic differentiation assay system are essential. Stable and high cardiomyogenic transdifferentiation ability in our established system enables us to observe, with wide dynamic range, the effects of treatment for cardiomyogenesis. Moreover, the primary culture condition of murine cardiomyocytes usually fluctuates due to variations in environments, the skill of individual researchers, and institutional differences in isolation protocols. Our established EMCs may provide a good positive control for a cardiomyogenic assay system *in vitro* to check whether the feeder cell condition is suitable for cardiomyogenic assay. When feeder conditions are suitable, we can survey for possible cardiomyogenic assistant factors or appropriate culture conditions for human BMMSCs by applying various agents or modifying culture conditions systematically. Thus, by using our EMCs and cocultivation system, we may be able to expand the cardiomyogenic differentiation potential of marrow-derived MSCs. Consequently, we

may be able to increase the efficacy of cardiac stem cell-based therapy dramatically.

Neither passive stretching of EMCs nor an application of the supernatant of murine cardiomyocyte culture medium to the EMCs alone caused cardiomyocyte differentiation. Taking these findings into account, the multiple environmental factors, including mechanical stretching and/or feeder cardiomyocyte-derived humoral factors, seem to contribute to cardiomyogenic transdifferentiation in human mesenchymal cells. Further experiments should be done.

Study Limitations

Cell fusion between the human cells (MMCs or EMCs) might be a major cause of EGFP-positive cardiomyocytes in the present study. However, EGFP-positive cardiomyocytes could be observed, even when human cells and murine cardiomyocytes were cocultured separately by the athelocollagen membrane that is permeable for only small molecules (less than 5,000 MW)—thus allowing no possible penetration of cells or organelles through the membrane (supplemental online Fig. 6). Furthermore, even if the cells were cocultured without the athelocollagen membrane, nuclear fusion between EMC100s, EMC214s, or MMCs and fetal murine cardiomyocytes was less than 1% in the present study. Moreover, transdifferentiated EMCs at the external layer of the cell sheet graft on the epicardial surface did not directly contact the host cardiomyocytes (Fig. 5). Taking these results

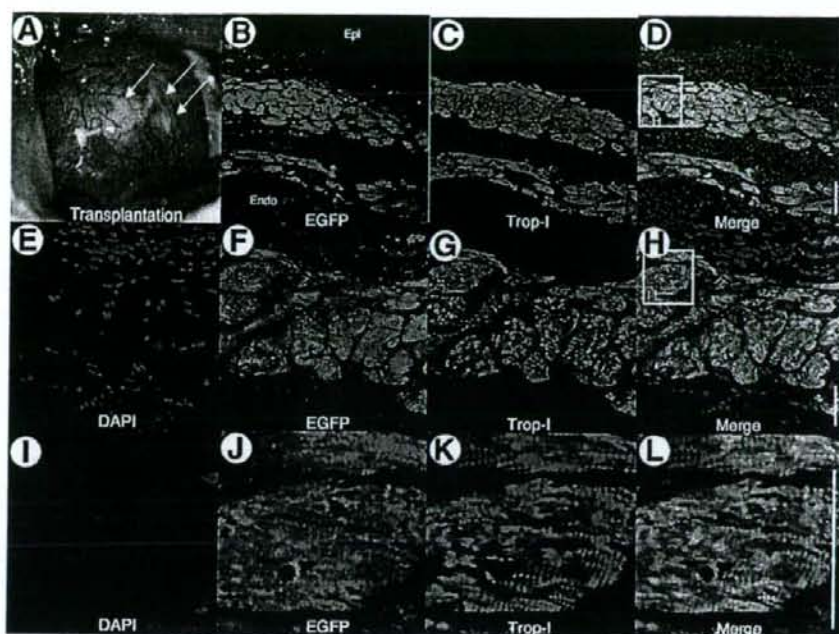


Figure 7. Cardiomycogenesis of engrafted menstrual blood-derived mesenchymal cells (MMCs) in vivo. (A): Macroscopic view of the recipient's heart immediately after enhanced green fluorescent protein (EGFP)-labeled MMC transplantation (white arrows) into the myocardial infarction area of the recipient's heart. (B–L): Two weeks after transplantation, immunohistochemistry revealed survival of the MMC tissue layer (green) on the treated heart. (B–D): Engrafted MMCs stained positive with anti-cardiac troponin-I (red; Trop-I). Scale bar denotes 100 μ m. (E–H, I–L): The area in the white box in (D) was observed in higher resolution (E–H) and the white box in (H) was also observed in higher resolution (I–L). (K): The clear striation pattern of Trop-I staining was observed throughout the whole layer of engrafted MMCs, suggesting extremely high cardiomyogenic potential of MMCs in situ. Scale bar denotes 20 μ m. Abbreviations: DAPI, 4'-6-diamidino-2-phenylindole; Endo, endocardium; Epi, epicardium.

into account, we concluded that the cell fusion did not play a major role in the observed significant cardiomyogenic potential of MMCs and EMCs in the present study.

Infarcted heart tissue may increase auto-fluorescence in some fixative conditions and such auto-fluorescence of host cardiomyocytes might be confused as EGFP-positive like cells. However, autofluorescence of the host myocardium adjacent to the infarcted area was not significant in our present condition (Figs. 5B, 6B, supplemental online Fig. 5B, 5F). Therefore, EGFP-positive tissue in the present study can be defined as of human cell origin and easily distinguished from the host heart by the EGFP fluorescent intensity.

The transfection of the cell cycle-mediated gene may increase cardiomyogenic differentiation to some extent. However, our previous study in human BMMSCs, [2] with the same combination of cell cycle-mediated gene transfection, did not show any increase in efficiency. Furthermore, non-gene-transfected MMCs have an extremely high cardiomyogenic efficiency compared to gene-transfected BMMSCs. Taking these results into account, we concluded that transfection of those genes does not play an essential role in causing such high cardiomyogenic differentiation efficiency in EMCs.

In comparison to previous papers, there was no observable effect of BMMSC transplantation on cardiac function in the present study. This discrepancy may be caused by different experimental conditions, that is, species difference between BMMSCs and the host animal [24], transplantation at acute myocardial infarction [25–27], and usage of immunosuppressive agents, etc [24–27].

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In the present study, we did not use a pressure-tipped catheter, therefore the LV dp/dt value may be underestimated.

SUMMARY

MMC transplantation decreased fibrosis area and restored the LV systolic function in the MI-model in vivo. Engrafted MMC transdifferentiated into cardiomyocyte within MI area. MMC can be a major cell source for stem cell therapy to achieve cardiomyogenesis.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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