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Cell

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Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

K. Takahashi and S. Yamanaka

Differentiated cells can be reprogrammed to an embryonic-like state by transfer of nuclear contents into oocytes or by fusion with embryonic stem (ES) cells. Little is known about factors that induce this reprogramming. Here, the authors demonstrate induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions. Unexpectedly, Nanog was dispensable. These cells, which we designated iPS (induced pluripotent stem) cells, exhibit the morphology and growth properties of ES cells and express ES cell marker genes. Subcutaneous transplantation of iPS cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPS cells contributed to mouse embryonic development. These data demonstrate that pluripotent stem cells can be directly generated from fibroblast cultures by the addition of only a few defined factors.

Journal of Neuroscience

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Activation of Gonadotropin-Releasing Hormone Neurons by Kisspeptin as a Neuroendocrine Switch for the Onset of Puberty

S. K. Han et al.

The authors examined the role of kisspeptin and its receptor, the G-protein-coupled receptor GPR54, in governing the onset of puberty in the mouse. In the adult male and female mouse, kisspeptin (10–100 nM) evoked a remarkably potent, long-lasting depolarization of more than 90 percent of gonadotropin-releasing hormone (GnRH)-green fluorescent protein neurons in situ. In contrast, in juvenile (P8 [postnatal day eight] to P19) and prepubertal (P26 to P33) male mice, kisspeptin activated only 27 and 44 percent of GnRH neurons, respectively. This developmental recruitment of GnRH neurons into a kisspeptin-responsive pool was paralleled by an increase in the ability of centrally administered kisspeptin to evoke luteinizing hormone secretion in vivo. To learn more about the mechanisms through which kisspeptin-GPR54 signaling at the GnRH neuron may change over postnatal development, the authors performed quantitative in situ hybridization for kisspeptin and GPR54 transcripts. Approximately 90 percent of GnRH neurons were found to express GPR54 mRNA in both juvenile and adult mice, without a detectable difference in the mRNA content between the age groups. In contrast, the expression of KiSS-1 mRNA increased dramatically across the transition from juvenile to adult life in the anteroventral periventricular nucleus (AVPV; $p < 0.001$). These results demonstrate that kisspeptin exerts a potent depolarizing effect on the excitability of almost all adult GnRH neurons and that the responsiveness of GnRH neurons to kisspeptin increases over postnatal development. Together, these observations suggest that activation of GnRH neurons by kisspeptin at puberty reflects a dual process

involving an increase in kisspeptin input from the AVPV and a post-transcriptional change in GPR54 signaling within the GnRH neuron.

Nature

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A Second Class of Chemosensory Receptors in the Olfactory Epithelium

S. D. Liberles and L. B. Buck

The mammalian olfactory system detects chemicals sensed as odors as well as social cues that stimulate innate responses. Odorants are detected in the nasal olfactory epithelium by the odorant receptor family, whose approximately one thousand members allow the discrimination of a myriad of odorants. Here the authors report the discovery of a second family of receptors in the mouse olfactory epithelium. Genes encoding these receptors, called trace amine-associated receptors (TAARs), are present in human, mouse, and fish. Like odorant receptors, individual mouse TAARs are expressed in unique subsets of neurons dispersed in the epithelium. Notably, at least three mouse TAARs recognize volatile amines found in urine: one detects a compound linked to stress, whereas the other two detect compounds enriched in male versus female urine—one of which is reportedly a pheromone. The evolutionary conservation of the TAAR family suggests a chemosensory function distinct from odorant receptors. Ligands identified for TAARs thus far suggest a function associated with the detection of social cues.

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Human Embryonic Stem Cell Lines Derived from Single Blastomeres

I. Klimanskaya et al.

The derivation of human embryonic stem (hES) cells currently requires the destruction of ex utero embryos. A previous study in mice indicates that it might be possible to generate embryonic stem (ES) cells using a single-cell biopsy similar to that used in preimplantation genetic diagnosis (PGD), which does not interfere with the embryo's developmental potential. By growing the single blastomere overnight, the resulting cells could be used for both genetic testing and stem cell derivation without affecting the clinical outcome of the procedure. Here the authors report a series of ten separate experiments demonstrating that hES cells can be derived from single blastomeres. Nineteen ES-cell-like outgrowths and two stable hES cell lines were obtained. The latter hES cell lines maintained undifferentiated proliferation for more than eight months, and showed normal karyotype and expression of markers of pluripotency, including Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, nanog and alkaline phosphatase. These cells retained the potential to form derivatives of all three embryonic germ layers both in vitro and in teratomas. The ability to create new stem cell lines and therapies without destroying embryos would address the ethical concerns of many, and allow the generation of matched tissue for children and siblings born from transferred PGD embryos.

Nature Genetics

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Molecular Analysis of Flies Selected for Aggressive Behavior

H. A. Dierick and R. J. Greenspan

Aggressive behavior is pervasive throughout the animal kingdom, and yet very little is known about its molecular underpinnings. To address this problem, the authors have developed a population-based selection procedure to increase aggression in *Drosophila melanogaster*. They measured changes in aggressive behavior in the selected subpopulations with a new two-male arena assay. In

only ten generations of selection, the aggressive lines became markedly more aggressive than the neutral lines. After twenty-one generations, the fighting index increased more than thirty-fold. Using microarray analysis, the authors identified genes with differing expression levels in the aggressive and neutral lines as candidates for this strong behavioral selection response. They tested a small set of these genes through mutant analysis and found that one significantly increased fighting frequency. These results suggest that selection for increases in aggression can be used to molecularly dissect this behavior.

NeuroRehabilitation

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Drug-Induced Arousal from the Permanent Vegetative State

R. Clauss and W. Nel

Background: Zolpidem is an omega-1-specific indirect GABA [gamma aminobutyric acid] agonist that is used for insomnia, but may have efficacy in brain damage. The long-term efficacy of zolpidem in the permanent vegetative state is described in three patients. *Method*: Two motor-vehicle-accident patients and one near-drowning patient, all of them in the permanent vegetative state for at least three years, were rated according to the Glasgow Coma and Rancho Los Amigos scale before and after zolpidem application. Long-term response to daily application of this drug was monitored for three to six years. *Results*: All patients were aroused transiently every morning after zolpidem. Glasgow Coma Scale scores ranged from 6/15–9/15 before zolpidem to 10/15–15/15 after zolpidem. Rancho Los Amigos Cognitive scores ranged from I–II before zolpidem to V–VII after. Drug efficacy did not decrease, and there were no long-term side effects after three to six years' daily use. *Conclusion*: Zolpidem appears an effective drug to restore brain function to some patients in the permanent vegetative state.

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ES Cells Derived from Cloned and Fertilized Blastocysts Are Transcriptionally and Functionally Indistinguishable

T. Brambrink et al.

Reproductive cloning is uniformly rejected as a valid technology in humans because of the severely abnormal phenotypes seen in cloned animals. Gene expression aberrations observed in tissues of cloned animals have also raised concerns regarding the therapeutic application of "customized" embryonic stem cells derived by nuclear transplantation (NT) from a patient's somatic cells. Although previous experiments in mice have demonstrated that the developmental potential of embryonic stem cells derived from cloned blastocysts (NT-ES cells) is identical to that of embryonic stem cells derived from fertilized blastocysts, a systematic molecular characterization of NT-ES cell lines is lacking. To investigate whether transcriptional aberrations, similar to those observed in tissues of cloned mice, also occur in NT-ES cells, the authors compared transcriptional profiles of ten mouse NT- and fertilization-derived embryonic stem cell lines. They report here that the embryonic stem cell lines derived from cloned and fertilized mouse blastocysts are indistinguishable based on their transcriptional profiles, consistent with their normal developmental potential. Their results indicate that, in contrast to embryonic and fetal development of clones, the process of NT-ES cell derivation rigorously selects for those immortal cells that have erased the "epigenetic memory" of the donor nucleus and, thus, become functionally equivalent. Their findings support the notion that embryonic stem cell lines derived from cloned or fertilized blastocysts have an identical therapeutic potential.

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Glucocorticoid Enhancement of Memory Requires Arousal-Induced NoradrenergicActivation in the Basolateral Amygdala

B. Roozendaal et al.

Considerable evidence indicates that glucocorticoid hormones enhance the consolidation of long-term memories for emotionally arousing experiences but not for less arousing or neutral information. However, previous studies have not determined the basis of such arousal-induced selectivity. Here the authors report the finding that endogenous noradrenergic activation of the basolateral complex of the amygdala (BLA) induced by emotional arousal is essential in enabling glucocorticoid memory enhancement. Corticosterone administered immediately after object recognition training enhanced twenty-four-hour memory of naive male rats but not that of rats previously habituated to the training context in order to reduce novelty-induced emotional arousal. The beta-adrenoceptor antagonist propranolol administered either systemically or into the BLA blocked the corticosterone-induced memory enhancement. Further, in habituated rats, corticosterone activated BLA neurons, as assessed by phosphorylated cAMP response element binding (pCREB) immunoreactivity levels, and enhanced memory only when norepinephrine release was stimulated by administration of the alpha(2)-adrenoceptor antagonist yohimbine. These findings strongly suggest that synergistic actions of glucocorticoids and emotional-arousal-induced noradrenergic activation of the BLA constitute a neural mechanism by which glucocorticoids may selectively enhance memory consolidation for emotionally arousing experiences.

Volume 103, Number 35
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Spermatozoa and Spermatids Retrieved from Frozen Reproductive Organs or Frozen Whole Bodies of Male Mice Can Produce Normal Offspring

N. Ogonuki et al.

Cryopreservation of male germ cells is a strategy to conserve animal species and strains of animals valuable to biomedical research. The authors tested whether mouse male germ cells could be cryopreserved without cryoprotection by simply freezing epididymides, testes, or whole bodies. The reproductive organs were isolated from killed mice and were frozen for one week to one year at -80 degrees C before spermatozoa and spermatids were collected and injected into mature oocytes. Normal pups were born irrespective of strains tested (ICR and C57BL/6). Epididymides and testes frozen and transported internationally to another laboratory by air could produce pups of inbred C57BL/6 mice. Testicular spermatozoa retrieved from the bodies of male mice (BALB/c nude and C3H/He strains) that had been kept frozen (-20 degrees C) for fifteen years could also produce normal offspring by microinsemination. Thus, freezing of either male reproductive organs or whole bodies is the simplest way to preserve male germ cells. Restoration of extinct species could be possible if male individuals are found in permafrost.

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Rats Produced by Interspecies Spermatogonial Transplantation in Mice and In Vitro Microinsemination

Takashi Shinohara et al.

Spermatogonial transplantation has demonstrated a unique opportunity for studying spermatogenesis and provided an assay for spermatogonial stem cells. However, it has remained unknown whether germ cells that matured in a xenogeneic environment were functionally normal. In this investigation, the authors demonstrate the successful produc-

tion of xenogeneic offspring by using spermatogonial transplantation. Rat spermatogonial stem cells were collected from immature testis and transplanted into the seminiferous tubules of busulfan-treated nude mouse testis. Using rat spermatids or spermatozoa that developed in xenogeneic surrogate mice, rat offspring were born from fresh and cryopreserved donor cells after microinsemination with rat oocytes. These offspring were fertile and had a normal imprinting pattern. The xenogeneic offspring production by interspecies germ cell transplantation and *in vitro* microinsemination will become a powerful tool in animal transgenesis and species conservation.

Reproductive BioMedicine Online

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Unsuccessful Derivation of Human Embryonic Stem Cell Lines from Pairs of Human Blastomeres

C. Y. Fong, M. Richards, and A. Bongso

Human embryonic cells that differentiate into all three primordial germ layers have been established. Differentiation of these cells into desirable lineages offers hope for future transplantation therapies. Currently, hESC lines are derived from the inner cell mass (ICM) of blastocysts, leading to destruction of the embryo, and thus the process is ethically controversial. Successful attempts at deriving hESC lines from blastomeres without destruction of the ensuing embryo have not been reported. One or two blastomeres are routinely biopsied from eight-cell embryos for preimplantation genetic diagnosis. In this study it was therefore attempted to derive hESC lines from paired blastomeres. Of sixty-six pairs of eight-cell stage blastomeres, four pairs produced two morula and two blastocyst-like structures. When plated on mitomycin-C-treated mouse embryonic fibroblasts, one morula and one blastocyst-like structure separately produced small colonies containing hESC-like cells with prominent nucleoli and high nuclear-cytoplasmic ratios. When these colonies were detached and plated onto fresh feeders, there was no further colony formation or ensuing hESC lines. The results showed that it might not be possible to derive hESC lines directly from paired blastomeres. A minimum number of blastomeres in close contact with one another may be required to successfully generate an hESC line, as blastomeres, like ICM and hESC cells, may be "social" cells.

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In-vitro Developmental Potential of Individual Mouse Blastomeres Cultured With and Without Zona Pellucida: Future Implications for Human Assisted Reproduction

K. Illmensee, K. Kaskar, and P. M. Zavos

This study was designed to compare the developmental potential of individual blastomeres derived from two-, four-, six-, and eight-cell mouse embryos cultured with and without zona pellucida (ZP). In the first series, one, three, five and seven blastomeres were biopsied from two-, four-, six-, and eight-cell embryos, respectively, and inserted individually into empty ZP recipients, leaving the remaining blastomere within its original ZP. In the second series, the same protocol was used except that the biopsied blastomeres were cultured without ZP and were compared with the remaining blastomere within its original ZP. For the first series, individual blastomeres derived from two-, four-, six-, and eight-cell embryos cultured with ZP showed blastocyst development of 82.4, 68.6, 44.4, and 23.1 percent respectively, with corresponding hatching rates of 70.6, 60.0, 25.9, and 7.7 percent. For the second series, individual blastomeres cultured without ZP progressed with blastocyst development of 73.3, 64.5, 35.7, and 22.7 percent respectively. Blastocyst multiplication was achieved most efficiently when using individual blastomeres from four- and six-cell

embryos. This is the first report on comparative in vitro propagation of single blastomeres derived from various cleavage stages in a mammalian species. Blastomere cloning with its multiple applications may be envisaged for human assisted reproductive technologies.

Science

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Detecting Awareness in the Vegetative State

A. M. Owen et al.

The authors used functional magnetic resonance imaging to demonstrate preserved conscious awareness in a patient fulfilling the criteria for a diagnosis of vegetative state. When asked to imagine playing tennis or moving around her home, the patient activated predicted cortical areas in a manner indistinguishable from that of healthy volunteers.

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Cancer Regression in Patients after Transfer of Genetically Engineered Lymphocytes

R. A. Morgan et al.

Using adoptive transfer of lymphocytes given after host immunodepletion, it is possible to mediate objective cancer regression in patients with metastatic melanoma. However, the generation of tumor-specific T cells in this mode of immunotherapy is often limiting. Using a retrovirus encoding a T cell receptor, the authors report here the ability to specifically confer tumor recognition by autologous lymphocytes from peripheral blood. Adoptive transfer of these transduced cells in fifteen patients resulted in durable engraftment at levels exceeding ten percent of peripheral blood lymphocytes for at least two months post infusion. The authors observed high sustained levels of circulating, engineered cells at one year post-infusion in two patients, that both demonstrated objective regression of metastatic melanoma lesions. This study suggests the therapeutic potential of genetically engineered cells for the biologic therapy of cancer.

Stem Cells

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Equivalency of Nuclear-Transfer-Derived Embryonic Stem Cells to Those Derived from Fertilized Mouse Blastocyst

S. Wakayama et al.

Therapeutic cloning, whereby nuclear transfer (NT) is used to generate embryonic stem cells from blastocysts, has been demonstrated successfully in mice and cattle. However, if NT-ESCs have abnormalities, such as those associated with the offspring produced by reproductive cloning, then their scientific and medical utilities might prove limited. To evaluate the characteristics of NT-ESCs, the authors established more than one hundred fifty NT-ESC lines from adult somatic cells of several mouse strains. Here, they show that these NT-ESCs were able to differentiate into all functional embryonic tissues in vivo. Moreover, they were identical to blastocyst-derived ESCs in terms of their expression of pluripotency markers, in the presence of tissue-dependent differentially DNA methylated regions, in DNA microarray profiles, and in high-coverage gene expression profiling. Importantly, the NT procedure did not cause irreversible damage to the nuclei. These similarities of NT-ESCs and ESCs indicate that murine therapeutic cloning by somatic cell NT can provide a reliable model for preclinical stem cell research.