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Rats!

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The Chinese calendar year of the rat lived up to its name. By the end of the year, five papers describing methods to produce pluripotent stem cells from rats had been published. Some methods are capable of providing access to the germline of the rat, which should lead to valuable models of human disease (Buehr et al., 2008; Li et al., 2008; Li et al., 2009; Liao et al., 2009; Ueda et al., 2008). This marks the successful conclusion of efforts that span more than 15 years and lags almost 30 years behind the same achievement in the mouse. It is not an end but a beginning. The work now must establish the same robust targeting methodologies in the rat that are available for the mouse. The promise of induced pluripotent stem cell (iPS) technology for the rat needs to be more widely embraced. The methodology needs to be integrated into the large and expanded genomic toolbox for the rat. We think it is useful to consider the reasons that this advance may be important beyond the symbolism of prosperity that the rat embodies in the Chinese calendar.

Why the rat?

Given that a great deal of technology for functional genomics is available in the mouse, how important is the rat as a model organism? For the last 30 years, investigators have chosen to use mouse models because of the technologies that are available. Now that the same technologies are at hand with the rat, scientists will be able to choose the most appropriate model based on the biology, whether it is rat, mouse or both. Even though these species look similar, there are millions of years of evolution separating the rat and mouse, and there are significant differences between them.

As a model of human disease, the rat offers many advantages over the mouse and other organisms. In fact, rats were once the most widely used organism in medical research, and the successful isolation of rat ES (embryo-derived stem) cells will quickly expand their utility. The rat is an excellent model for cardiovascular disease, particularly for stroke and hypertension, and there are a variety of genetic stocks that are ideal for these studies. The physiology is easier to monitor in the rat and, over time, a volume of data has developed that will take years to be replicated in the mouse. Moreover, in many cases, the physiology is more like the corresponding human condition. In studies of cognition and memory, the rat is superior to other models because the physiological systems involved in learning and memory have been so extensively studied in this animal. The rat is more intelligent than the mouse and is capable of learning a wider variety of tasks that are important to cognitive research. The size of the animal enhances its use as a disease model, not just because of the ability to perform surgical procedures, but also because of the proportional size of important substructures in organs that affects both how much of the organ is involved in an experimental lesion and the distance effects of drug administration to specific anatomical areas. This is particularly important in the central nervous system. The rat models of breast cancer are superior to those in the mouse insofar as they are hormone responsive with histopathology and have premalignant stages that more closely resemble the human disease. The rat is the primary model for mechanistic studies of human reproduction. In models of diabetes, the rat model behaves more like the human disease in important ways, including the

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ability of environmental agents (e.g. toxins, stress, diet and vaccination) to modify the disease. For drug studies, the size of the rat enables serial blood draws. This is by no means a comprehensive list, but gives a sense of the depth and range of use for this animal model.

Recent PubMed data searches reveal over 1 million publications that use the rat. Information from more than 100 years of phenotyping experience is available. The rat has become a standardized physiological and toxicological model, particularly in the pharmaceutical industry. There is a wide range of strains supporting an extensive literature on comparative physiology and, more recently, comparative genomics. The range of models that are highly suited to the study of human disease, or more highly suited than the mouse, has been presented recently in great detail (Aitman et al., 2008). However, the production of specific mutations, conditional mutations and marker manipulations, such as with knock-in models, has been problematic in the rat. It is fair to say that functional genomic research into the various areas for which the rat is a valuable animal model of human disease, including those discussed above, has been prevented or hampered by the limitations in directed manipulation of the rat genome.

What's missing?

A series of priority meetings were held nearly ten years ago, sponsored by the National Institutes of Health (NIH), to determine how best to facilitate the use of rats as a model organism. The meetings grew from strong activity within the community and began with the biennial transplantation biology meetings, titled Alloantigenic Systems in the Rat. On alternate years, these meetings, attended by an international group of several hundred scientists, focused on genetic issues particular to the rat. Over a number of years, a consensus developed that a national repository guaranteeing the genetic and microbiological integrity of the many strains in worldwide use was needed badly. It was envisioned that such an organization would partner with similarly constituted labs from around the world, creating a robust and standardized source of specific strains, and could standardize the distribution of rat models.

A rough outline of the repository and what was missing in rat research were discussed thoroughly at a meeting held in Lansdowne, VA in 1998 (http://www.nhlbi.nih.gov/meetings/model/index.htm). At that time, a genetics toolbox for the rat was discussed. In addition to the standardization and distribution of common rat strains, it was very clear that rat work was hampered by a lack of complete genome data and difficulty in genetically modifying rat strains. Many of the pieces needed to successfully sequence the rat genome were available already, but targeted genetic modification was impossible. At that time, there were no proven germline-competent stem cells and even basic methods for the in vitro culture of early rat embryos were not available. Back then, even making transgenic rats was relatively inefficient.

The Lansdowne meeting was followed in 1999 by an NIH-sponsored priority meeting that reaffirmed the principal goals for enhancing rat research. Support was organized for a national strain repository, efforts toward genetic modification and the completion of the genome sequence by strengthening genomic tools. Both meeting reports emphasized the need for a broader involvement of the research community who use this model and interaction with other model system-based research communities (http://www.nhlbi.nih.gov/resources/docs/ratmtgpg.htm). Since the draft genome sequence of the rat was published in 2004, the genomic tools have continued to expand. This is, in a large measure, because of the organization of international efforts by the National Institutes of Health and the European Union (EU) to complete the goals set forth in the reports, as well as to address new genomic priorities. Most recently, this is

being accomplished as part of the EU's sixth Framework Programme in its EURATools module (http://www.euratools.eu).

A remarkable amount of work has been accomplished since those meetings. The Rat Resource and Research Center is now a robust national repository. Methods have been developed that allow the rat embryo to be cultured from one cell into a blastocyst (Iannaccone et al., 2001; Yang et al., 2004; Zhou et al., 2003). A high-quality draft sequence of the rat genome is available. Annotation and comparative genomic tools are now distributed widely (e.g. Rat Genome Database, RGD, http://rgd.mcw.edu). Furthermore, lentiviral transformation techniques make transgenic rats readily available. We now have an elegant scheme that allows selection of specific mutant strains of rat following *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (Zan et al., 2003). The results of the EU consortium were published recently in a dedicated issue of Nature Genetics (May, 2008) showing the power of cooperative effort in genome research. What remained, was for a stem cell methodology to be developed that would allow for more advanced access to the germline for site-directed knockouts, knock-ins, conditional mutations, cellular marker systems and other approaches that would be made possible with homologous recombination techniques.

Stem cell beginnings

In the 1960s and 1970s, Pierce, Stevens and others observed the stages of development of teratocarcinoma in mouse testes and ovarian teratomas in specific strains of mice. Their studies concluded that the parthenogenic division of oocytes in the ovaries produced embryonic structures that were indistinguishable morphologically from the cleavage stages, morulae and blastocyts; furthermore, they argued that nests of undifferentiated cells were stem cells giving rise to the tumor with all of its differentiated tissues. Thus, the idea that embryo-like, pluripotent stem cells resided in these tumors led Solter, Mintz, Gardner, Papaioannou and others to purposefully create teratocarcinomas by ectopic transplantation of embryonic tissues (Evans, 1981). The isolation, in culture, of stem cells called EC (embryonal carcinoma) cells quickly followed, and those cells were shown to participate in chimera formation. Evans, Martin and others then tried to isolate stem cells by culturing blastocysts directly in vitro and succeeded in making cultures of ES cells that were shown to produce functional gametes in chimeras. The purine-salvaging enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was mutated with retroviral insertion and became the first null mutation in mice to be produced with ES cells, providing an important model of Lesch-Nyhan syndrome (Kuehn et al., 1987).

Scientists tried diligently to isolate the same ES cells from various strains of rat embryos but were unsuccessful (Brenin et al., 1997). The primary germ cell tumor in mice is teratocarcinoma and this is thought to correlate with the ability to isolate ES cells from blastocyst outgrowths. In the rat, the primary germ cell tumor is yolk sac carcinoma and, consistent with this, many labs report that blastocyst outgrowths are endoderm-like in appearance and express endoderm markers. Recently, the precursor pool for extraembryonic endoderm (XEN-P) has been isolated from rat blastocysts (B. Debeb, V. Galat and J. Epple-Farmer et al., unpublished), but the in vitro cell culture equivalent of epiblast precursor cells or ES cells remained elusive, until now.

Au courant

The most convincing of the five new publications describing methods to isolate ES cells is from Austin Smith's lab (Buehr et al., 2008). Smith and his colleagues studied crucial signal transduction pathways and concluded that epiblast precursor cells were being

forced to differentiate. Since they knew that fibroblast growth factor 4 (FGF4) mediates differentiation through MAP kinase kinase (MEK)-extracellular regulated kinase (ERK) signaling, they used a known inhibitor of FGF receptors (FGFRs) to prevent it. When FGFR inhibition alone proved inadequate, they added inhibitors of MEK and glycogen synthase kinase-3 (GSK-3). This allowed pluripotent undifferentiated cells to grow in culture. The cells were capable of making chimeras [visualized with a green fluorescent protein (GFP) marker], a key characteristic of ES cells. However, the lab could not make offspring from the ES cell-derived functional gametes in the chimeras, which is a necessary step to prove the validity of ES cells. The team reasoned that the pleiotropic effects of shutting down FGF signaling resulted in a cell population that was somehow inadequate and the appearance of abnormalities in the chimeras led them to remove the FGFR inhibitors. This new culture regimen with two inhibitors allowed ES cells to be isolated from rat blastocysts that made germline-competent chimeras. The offspring of one chimera were shown, by microsatellite analysis, to have the genetic fingerprint of the ES cells. By adding leukemia inhibitory factor (LIF), which is sufficient to prevent ES cell differentiation in mice but not in rats, they developed ES cells capable of long-term passage. These ES cells were also transformed by transfection with a GFP lineage marker that allowed visualization of the ES cell contribution to the tissues of the chimeras. It would have been desirable to see the GFP-marked ES cells in the offspring of the chimeras, since this would have provided an unequivocal external marker of the origin of the offspring and evidence that genetically modified ES cells are still germline competent. This experiment is coming, no doubt. Similar results were published in Li et al. (Li et al., 2008). The experiments discussed in the other manuscripts either did not use the cells to make chimeras or, where chimeras were made, they did not produce offspring; therefore, these ES cells must be considered unproven, but promising at this time.

Transfection of cells with several key transcription factors is sufficient to reprogram adult cells to an embryonic state and produce proven ES cells in mice. These induced pluripotent stem cells (iPS) have all the characteristics of ES cells. An interesting, and possibly important, approach that was published recently used signal transduction inhibitors from Smith's work in the culture of iPS cells (Li et al., 2009). This resulted in a population of stem cells that were capable of forming chimeras, but no offspring have yet been reported. iPS technology could overcome strain barriers that might exist in the isolation of rat ES cells. This method could also facilitate the isolation of rat stem cells from rare animals.

It is not surprising, in retrospect, that the rat and mouse blastocyst are different and the long time to develop rat ES cells reflects the effort it took to understand those key differences. The inability of LIF alone to prevent differentiation of cultured epiblast cells is only one difference. Our information about ES cells and cultured extraembryonic ectoderm precursor cells tells us that the inner cell mass is a heterogeneous population of loosely committed cells. In different species they are differently balanced or biased.

Complimenting stem cell accomplishments are promising results using zinc finger nucleases, where genes can be targeted for mutation in the rat without producing ES cells. This technology may also allow for knock-in models. The field is buzzing with unpublished reports of knockout results in rats that used the same system that was published recently using zebrafish (Ekker, 2008). Following a long and hard-fought struggle, multiple methods may target rat genes and greatly improve the tractability of the rat as a model organism.

In 'The Discoverers', Boorstein quotes Turgenev who wrote in a letter to Tolstoy, 'a system is like the tail of truth, but truth is like a lizard; it leaves its tail in your fingers

and runs away knowing full well that it will grow a new one in a twinkling.' Experimental systems constantly evolve and using the most appropriate system for a given experiment is the best way to the truth. But, using a system like targeted mutations in the rat requires the necessary tools. The successful isolation of ES cells from rat blastocysts provides a key tool to this long-standing and important model system.

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