

Embryonic Stem Cells: Discovery, Development, and Current Trends

Elias Theodorou and Michael Snyder

Abstract Murine embryonic stem cells were first derived almost 30 years ago from cultured blastocysts and have been primarily used as a tool to better understand development through targeted gene deletions. Only recently has the focus shifted toward embryonic stem cells themselves and the molecular mechanisms by which they choose a specific cell fate. Through rapid advances in cell culture and genomic modification techniques researchers are beginning to regularly utilize embryonic stem cells for in vitro gene function assays. More important, the mechanisms critical for establishing the pluripotent state of embryonic stem cells have been elucidated to the point that clinically beneficial stem cell-like counterparts can now be generated from nonembryonic sources.

Keywords Embryonic • Stem • Differentiation • Genomic • iPS

1 Embryonic Stem Cells

1.1 History

The events that lead to the research resulting in the isolation of the first mouse and human embryonic stem cells began in 1953 at the Jackson Laboratory in Bar Harbor, Maine. This initial research was performed by Leroy Stevens, who was funded by a tobacco company determined to prove that the harmfulness of cigarettes stemmed from the wrapping paper and not the tobacco. Stevens observed that male mice of the 129Sv strain would consistently develop teratomas of the testis [63]. Over the course

M. Snyder (✉)

Department of Genetics, Stanford University School of Medicine, MC: 5120,
300 Pasteur Dr., M-344, Stanford, CA 94305-2200, USA
e-mail:mpsnyder@stanford.edu

of almost two decades, Stevens’ work showed that cells giving rise to teratomas were even present as early as the blastocyst stage, within the inner cell mass [128]. From that observation, the term “embryonic stem cell” was coined. Teratomas were subsequently used to derive cell lines with the ability to differentiate into several cell types [80]. However, because these cell lines had abnormal properties (e.g., abnormal ploidy) and retained the ability to generate teratomas, they were named “embryonic carcinoma” cells. Steven’s groundbreaking discovery that pluripotent stem cells existed in early embryos resulted in multiple laboratories determining the proper culture conditions that would allow the propagation of mouse [33, 74] embryonic stem (ES) cells.

From the onset of culturing murine ES cells, it became apparent that having the technology to introduce heritable genetic changes in the germline would be a powerful asset to studying development and disease in the mouse. Because of that fact, the focus of embryonic stem cell research in the 1980s was not the basic biology of stem cells; instead, the greatest achievement of the ES cell field at that time was considered to be the advent of gene targeting and germline modification [26, 142]. Research conducted along these lines by Mario Capecchi, Martin Evans, and Oliver Smithies led to their joint award of the 2007 Nobel Prize in Physiology or Medicine.

A massive resurgence of interest in the properties of ES cells took place in the late 1990s after the efforts of John Hearn’s laboratory resulted first in the isolation of ES cells from rhesus monkey and marmoset ([144, 145], Table 1) and subsequently to the derivation of human ES (hES) cell lines [143].

Surprisingly, even though mouse ES cells were first isolated in 1981, rat ES proved to be much more difficult to derive. Rat ES cells were highly desirable because rats are easier than mice to manipulate for physiologic studies, yet no efficient method to precisely modify the germline existed. Rat ES cells eluded researchers until 2008, when two independent research groups succeeded in their derivation [16, 65].

Table 1 Embryonic stem cells derived to date

Mouse [33]
Hamster [27]
Sheep [96]
Rabbit [86]
Mink [131]
Pig [137]
Cow [37]
Rhesus monkey [144]
Zebrafish [132]
Chicken [102]
Marmoset [145]
Human [143]
Horse [114]
Rat [16]

1.2 Properties of Mouse Embryonic Stem Cells

Embryonic stem cells have several distinctive features that cumulatively set them apart from all known cell types. First, they are immortal and, like cancer cells, exhibit the ability to undergo limitless self-renewal. It has been shown in mouse ES (mES) cells through targeted deletion of the telomerase RNA component [93] that proliferative lifespan is directly dependent on telomerase activity. Second, ES cells possess the ability to form teratomas when transferred subcutaneously to syngeneic [117] or immunocompromised [35] mice. The final and most important characteristic of embryonic stem cells is their ability to contribute to all three major germ layers (ectoderm, mesoderm, and endoderm) either in vitro and, ideally, in vivo [25]. Testing differentiation capabilities of mES cells in vivo has typically been done by injecting mES cells into blastocysts and then examining the tissue contribution of those mES cells in the resulting chimeras [51, 159]. In recent years the gold standard test for the “stemness” of mES or ES-like cells has been the use of tetraploid blastocyst [40, 81, 154]. Embryos resulting from the outcome of such injections must be wholly derived from the 2N stem cells injected into 4N tetraploid blastocysts, making it the most stringent test of pluripotency available.

1.3 Self-Renewal of Embryonic Stem Cells

An important goal in the field of embryonic stem cells is determining what mechanism(s) allow ES cells to endlessly maintain their self-renewal potential. The exact mechanisms controlling stemness have not been determined, but research has revealed key extracellular and intracellular players. When mES cells were first derived they could only be maintained in their proliferative and pluripotent state by growing them on layer of permanently growth arrested mouse embryonic fibroblasts (MEFs). Almost a decade later it was found that leukemia inhibitory factor (LIF) was the soluble factor being produced by MEFs [108]. Part of the interleukin-6 (IL-6) superfamily of cytokines, LIF functions by binding to a heterodimeric receptor composed of gp130 and LIF receptor-beta (LIFR-beta). LIF and LIFR-beta are expressed in a complementary pattern in the developing blastocysts. As the LIF receptor is expressed in the inner cell mass of the blastocyst, LIF cytokine itself is expressed in the differentiated trophoblast layer [92]. Once LIF has bound the LIF/gp130 complex, the JAK-STAT pathway is activated, resulting in homodimers of Stat3 translocating to the nucleus and transcribing genes critical for self-renewal [78, 94]. IL-6 can substitute for LIF when present at high concentrations and complexed with soluble IL-6 receptor (sIL-6R) [166]. Although LIF is critical in culture, blastocysts are only altered in their ability to undergo delayed implantation during times of stress or when women conceive while still nursing, a phenomenon known as diapause [91]. The effects exerted by LIF on mES cells in culture can only occur in the presence of fetal bovine serum. Although serum has a complex mixture of

soluble proteins, the presence of serum is only required to make available BMP4 to mES cells. BMP4 cytokine binds to the BMP receptor, BMPRI, which activates SMAD and induces the expression of the helix-loop-helix transcription factors known as inhibitors of differentiation (Id). As their name implies, Id proteins cooperate with Stat3 in maintaining self-renewal by repressing genes involved in neural differentiation [165]. In addition to Stat3 and Id factors, the proto-oncogene c-Myc has been shown to be a central player in maintaining the undifferentiated state. The levels of c-Myc are regulated through several mechanisms. Upon activation by the LIFR/gp130 complex of the JAK-STAT pathway, JAK phosphorylates and activates PI3 kinase (PI3K), which then phosphorylates the c-Myc inhibitor glycogen synthase kinase 3 (GSK3). The importance of PI3K's indirect maintenance of c-Myc's activity via GSK3 regulation is evident in cells that have been forced to differentiate in the presence of LIF by the addition of the PI3K inhibitor LY294002 [103]. Moreover, GSK3 inhibition of c-Myc can potentially be blocked by direct signaling through the Wnt pathway. The GSK3 inhibitor 6-bromindirubin-3'-oxime (BIO) allows mES cells to be propagated without the presence of LIF in the media [80, 116]. The importance of c-Myc in maintaining the self-renewal of mES cells is at least partially tied to c-Myc's upregulation of the catalytic subunit of telomerase [152], although telomerase alone is not sufficient to explain c-Myc's role in self-renewal.

Along with Stat3 and c-Myc, two other transcription factors, Oct3/4 and Nanog, have been shown to be important in maintaining ES cell self-renewal. The POU domain transcription factor Oct3/4 was initially cloned from mES cells and shown to be uniquely regulated, in that its expression would be turned off during mES cell differentiation [99, 112]. mES cells have been shown to be very sensitive to changes in levels of Oct3/4 protein, with reduction in levels resulting in dedifferentiation into trophoectoderm and upregulation driving differentiation into primitive endoderm and mesoderm [95]. While it has been known that absence of Sox2 protein influences the pluripotency of mES cells, it was only recently reported that Sox2 regulates the expression of several transcription factors that in turn are important for ensuring the appropriate levels of Oct3/4 [75]. Of the four transcription factors associated with the self-renewal of mES cells, the latest addition has been Nanog. Originally identified from a functional expression screen, Nanog has been shown to be highly expressed in mES cells and can maintain the self-renewal of mES cells without the addition of LIF or BMP to the media [17, 84]. Not surprisingly, when overexpressed, Nanog is not enough in and of itself to reprogram differentiated cells into ES-like cells. However, it is the only factor to date that can increase the efficiency of mES cell derivation. Likewise, ectopic expression of Nanog was able to boost the efficiency of fusions between mES cells and neural stem cells by over 100-fold in order to generate ES-like hybrid cell lines [123]. Boyer et al. [8] used chromatin immunoprecipitation (ChIP)-on-chip technology to conclude that Oct3/4, Nanog, and Sox2 share many overlapping transcriptional targets, including each other's promoters. Aside from the obvious implication of cross-regulation, the significance of the majority of the targets shared by the self-renewal trio remains to be discovered.

1.4 Differentiation of Mouse Embryonic Stem Cells

The first report describing the multilineage capability of mES cells in vitro with some detail was by Gail Martin, whose report shortly followed the landmark work by Evans and Kaufman [33]. By using embryoid bodies as had been done with mouse embryonic carcinoma cells, Martin was able to differentiate mES cells [74], although conditions were not defined for each observed lineage. A few years later a stand-alone landmark publication did describe the differentiation of embryoid bodies (EBs) in more detail, including the appearance of endothelial cells [28]. Surprisingly, more than 7 years after the initial isolation of mES cells passed before routine studies surfaced that attempted to use mES cells for modeling in vivo development events. Following the lead of work carried out by Evans and Martin, early studies relied on the use of EBs as well as later staged cystic embryoid bodies (CEBs), which resemble the 6- to 8-day cylinder stage of mouse development. Unlike early-stage mouse embryos, embryoid bodies could be generated much more easily and thus were utilized to mirror events observed in vasculogenesis and angiogenesis [110, 151] and globin switching [66]. Hematopoietic assays involving secondary reagents, such as methylcellulose [117], or the addition of cell lines, such as the coculture of a bone marrow stromal layer [41], expanded the repertoire of lineages obtainable in vitro.

The differentiation of mES cells into highly sought after cell types such as neurons [74] and myocardium [28] was observed when untreated embryoid bodies were allowed to adhere and differentiate. However, protocols that involved the use of specific reagents such as dimethylsulfoxide (for muscle) and retinoic acid (for neurons) were not employed for years, even though such reagents had proven successful in the directed differentiation of P19 EC cells [31, 32]. Instead, early methods used to generate cardiomyocytes and neurons, like those used for blood cell differentiation, focused on low-level manipulation of EBs. Emphasis was placed on how well the timing of EB differentiation paralleled embryonic development [82, 171]. As protocols became more refined, there was a shift to alter variables such as EB cell number and better define media conditions (e.g., lower serum content, insulin supplementation, etc.).

Early neuronal differentiation studies had demonstrated that EC cells could be differentiated into cells that displayed morphologic characteristics of neurons [49, 121]. The transition to studying neuronal differentiation of mES cells took off in the mid-1990s with more detailed characterization of the “neuron-like” cells that were produced from EB differentiation protocols. In research published by David Gottlieb’s laboratory it was shown by several tests, namely immunostaining and voltage-clamp readings in response to excitatory and inhibitory agonists, that neuron-like cells possessed key characteristics of true neurons [1]. Furthermore, they were the first group to quantitate the effectiveness of the embryoid body differentiation protocol and to implement the now widely used 4–/4+ retinoic acid differentiation protocol. Almost in parallel, Fraichard et al. [38] demonstrated that they too could differentiate mES cells into neurons that passed several stringent criteria.

Differentiated neurons derived through an EB protocol involving much longer incubation postplating were found to be immunopositive for neuronal cytoskeletal marker beta III tubulin. Neuron-like cells also displayed current induction when treated with glycine, *N*-methyl-D-aspartate (NMDA), and kainite and furthermore were shown to express glutamic acid decarboxylase (GAD) and acetylcholinesterase (AChE), two enzymes involved in neurotransmitter metabolism. Previous work had demonstrated that cells expressing the marker Nestin shared characteristics to neuroepithelial precursor cells that give rise to both neurons and glial cells [63]. Although the percentage of glial cells derived from embryoid bodies was not high, there was a subpopulation that expressed either the astrocyte marker GFAP or oligodendrocyte marker O4. Research using EB formation to differentiate mES cells into neurons continued; meanwhile, generating cells representing different developmental stages gained momentum. In 1996 it was found that the Nestin-positive neural precursor cells present in differentiating mES cells culture could be expanded with the strong mitogen bFGF so that “cleaner” populations of either glial cell or neurons could be obtained [98].

Following preliminary studies involving the derivation of postmitotic neurons, there was a steady progression in determining ways to differentiate mES cells into different types of neurons and supportive cells, as well as identifying basic mechanisms of mES differentiation. After gaining experience in differentiating mES cells into postmitotic neurons, some groups were able to enhance or direct the differentiation mES cells into specific neuronal subtypes. Renoncourt et al. first demonstrated the potential to generate cranial and somatic motoneurons as interneurons. The main limitation of the study was that the evidence for differentiation was based on cells in culture expressing homeodomain proteins associated with certain cell types such as HB9 or Isl-1 for motoneurons [109]. It was later demonstrated that functional motoneurons could be derived from mES cells based on the ability of the motoneurons to incorporate into the spinal cords of chick embryos and form synapses with muscle [157]. The authors were able to make specific neurons by using a combination of a HB9-GFP transgenic mES reporter line (derived from transgenic mice) and retinoic acid as well as sonic hedgehog (Shh) differentiation of EB-derived neuronal precursors. The systematic derivation of dopaminergic midbrain neurons and serotonergic hindbrain neurons was also achieved through the use of a multistep protocol involving EBs [61]. To eliminate the large variation seen in most mES cell neuronal differentiation protocols, neuronal differentiation methods have been devised that center around the use of stromal cells [2]. Stromal cell-induced neuronal differentiation of mES cells using defined media results in the reliable production of beta III tubulin-positive neurons and, after lengthy culture, tyrosine hydroxylase positive dopaminergic neurons ([54]; E. Theodorou, unpublished observations). The work done by Barberi et al. demonstrated that by using defined media along with a bone marrow stromal cell feeder layer, mES cells could be induced to differentiate into key neuronal lineages of interest: neural stem cells (NSCs), dopaminergic, serotonergic, GABAergic, and spinal motor neurons. Significant progress has also very recently been made in differentiation of mES cells using the tried and proven 4-/4+ retinoic acid differentiation protocol.

When embryoid bodies taken through a 4-/4+ differentiation protocol were dissociated and plated in well-defined neural precursor survival media followed by neuronal maintenance media, yields of neurons were brought to a very high level [6]. A surprising finding that has only begun to be explored is that, if grown in minimal media, mES cell will by default differentiate into neuroectodermal precursors. These findings were first described in work detailing how autocrine bFGF signaling was necessary for mES cells to go down this default pathway, without the use of cellular aggregation or specialized stromal cell layer [164]. The results of Ying et al. were later used as the basis for forming neural stem cells without the aid of neurospheres, but by proliferation of neuroectodermal precursors treated with EGF and FGF-2 [21]. Protocols involving exploitation of this default differentiation pathway have only recently entered the mainstream in the mES cell field but will very likely increase the options available to study differentiation once detailed protocols have become available.

Just as highly sought as the neurons themselves, supportive glial cells were found to have been present in EBs that had been subjected to the 4-/4+ differentiation protocol. Although the cell populations were mixed, the oligodendrocytes that were present had the ability to myelinate neuronal axons in culture (as judged by scanning electron microscopy). The oligodendrocyte precursors could be further enriched with the proper supportive media and to a limited degree could differentiate into myelinating oligodendrocytes in a rat model [68]. In the same year this work was surpassed by two independent groups able to derive not only oligodendrocytes, but also astrocytes [15, 87]. From a technical point of view, what separated these latter publications was that they did not simply age EBs in order to allow production of oligodendrocytes. Instead, mES cells were differentiated into glial-restricted precursors (GRPs) that were self-renewing and could be maintained in culture. Although glial cell differentiation of mES cells has received far less attention than differentiating neurons, significant headway has been made toward producing impressive numbers of oligodendrocytes through the use of aggregate protocols [52].

Muscle cells of the heart, called cardiomyocytes, are another highly desirable cell type because of their immediate relevance to disease, with the ability to incorporate into postinfarcted hearts in rats and canines [67, 83]. The presence of cardiomyocytes was noted for many years in teratocarcinoma cells [130] well before mES cells were first derived. It was even determined that varying the levels of retinoic acid, typically used to signal differentiation of neurons, could induce cardiomyocyte differentiation [31]. The first indications of EBs differentiating into cardiac muscle were the presence of Z-bands in histologic sections. Along with reverse transcription-polymerase chain reaction (RT-PCR) for cardiac myosin, Western blots for alpha and beta cardiac proteins indicated that cardiac-like muscle in EBs was reminiscent of some aspects of heart development at the E9-E10 stage [111]. Later it was confirmed by immunostaining, in situ hybridization, and RT-PCR of markers of differentiation that indeed cardiomyocytes were being produced in embryoid bodies [82]. Unlike the differentiation of neurons from EBs, the size of the EBs used to produce cardiomyocytes had a bearing on the efficiency of differentiation. Generally, EBs that were formed through the use of the hanging drop method (in which the

specific number of cells per EB can be controlled) and comprised approximately 500–1000 cells gave the highest percentage of cells staining positive for alpha-cardiac myosin heavy chain [72]. Dissociation of the same EBs through enzymatic treatment allowed the authors to identify the presence of sinusnodal, atrial, and ventricular cell types. Evidence that mES cell–derived cardiomyocytes did indeed resemble their *in vivo* counterparts was independently demonstrated by researchers who used EB dissociation combined with cellular electrophysiology [29]. More recently, the use of EBs has allowed certain questions to be asked about cardiogenesis without the difficulty of having to culture live embryos. In addition, recent studies suggest the existence of a cardiovascular progenitor in differentiating EBs [53,160]. With the ability to isolate a cardiovascular progenitor, the cardiomyocyte lineage exemplifies how mES differentiation has allowed for a better understanding of the basic biology of a cell type with intrinsic medical value. The lineages covered in this review represent only a sampling of the cell types derived to date. A more extensive overview is presented by Keller [55].

2 High-Throughput Functional Assays

2.1 *Large-Scale Differentiation Studies*

Both ethical/social impact and experimental practicality must be taken into consideration when choosing a stem cell line as a model system. Murine ES cells naturally lend themselves to high-throughput functional screening for several reasons: (1) mES cells can easily be grown to large quantities and differentiated in defined media; (2) mES cells have been shown to differentiate into all tissues of the adult mouse [88, 101]; (3) unlike other mammalian ES cell lines, mES cells have been genetically manipulated for many years [9]; and (4) to prove *in vivo* efficacy of differentiated cells, they can be transplanted into mouse models of disease [15, 56]. Other embryonic and adult stem cell lines have potential benefits that could be taken advantage of once studies with an established system such as mouse ES cells have taken place. To date no other system has surpassed murine ES cells in overall utility.

In 2003 the power of cDNA functional screening in mES cells was brought into the mainstream with work done by Chambers et al. [17] that culminated in the cloning of the mES cell self-renewal factor *Nanog*. Oddly enough, the screen yielded one of the most critical and aggressively studied stem cell self-renewal factors, yet *Nanog* was the only hit from the screen. While the expression cloning of *Nanog* was an important step forward, it also served to highlight some of the limitations of relying on cDNA libraries, such as the absence of positives due to clones that are low abundance or that are not represented as in-frame, full-length cDNAs.

More recently, several groups have taken strides to bring functional ES cell screening up to speed with genomics technology. In an effort to logically go from a model of differentiation to systematic identification of genes responsible for the observed phenotype, Ihor Lemischka's laboratory combined a unique selection

method with microarray technology [106]. The method involved the transfection of an unbiased episomal cDNA library into mES cells that were transgenic for the large T antigen. The episomal library was based on the same vector as the library used in Chambers et al. and replicated in the presence of large T antigen. Once the mES cells were placed through a functional screen, the episomal library was extracted and labeled for microarray analysis. cDNAs that were selected for or against (depending on the functional screen being performed) were then used in one-gene-per-well assays for confirmation. Using microarrays to take a focused approach to screening paid off with a dramatic increase in positives. However, the use of cDNA libraries for focused screening brought about problems previously, seen such as false positives resulting from the expression of incomplete cDNAs. One more obstacle that arose was an increase of false positives as a result of incomplete alternate splice variants misidentified by microarray as full-length transcripts. A second large-scale cDNA functional screen in mES cells [34] was the first instance of (1) a screen involving full-length ORFs and (2) a screen directed at the differentiation of mES cells into a specific cell lineage—neurons. While the assay relied on a sensitive and rapid method for hit detection, it did not yield many positives. In addition, the type of assay conducted by Falk et al. reflected those previously published [18, 105] in which an enormous number of cDNAs were transfected in small pools or individually. Thus, the cost feasibility of such a screen would be outside the reach of many laboratories. What was lacking was a collection of stable mES cell lines, each expressing a defined factor, preferably in an inducible fashion. Theodorou et al. [141] delivered such a system when they generated more than 700 mES cell lines, each line transgenic for a unique transcription factor, which was expressed upon withdrawal of tetracycline from the culture media. While the collection of mES cells was generated through screening thousands of individual colonies, it represents the first such large-scale stable collection made available to the scientific community and which can be propagated long term. The collection pieced together by Theodorou et al. would not have been feasible without the foundation work involving targeted modification of mES cells.

2.2 Mouse Embryonic Stem Cell Modification and Expression Systems

Over the years there have been multiple attempts to genetically modify ES cells so that they could serve as a platform for large-scale gene expression studies. The difficulty has been in developing a methodology that could satisfy most of the requirements for an ideal expression system: (1) ease of inserting transgenes into a defined chromosomal locus, (2) inducible expression, (3) ubiquitous and constitutive expression, and (4) expression that is trackable. Inserting a transgene into a defined locus in a predictable and efficient manner has proven to be the most difficult requirement. Several traditional methods for making defined chromosomal modifications and insertions exist, but they all share a low success rate [12].

More advanced systems have taken advantage of unique restriction sites [20, 24] or improved electroporation and culture conditions [141]. The method that has proven most successful uses standard positive selection along with a counterselection cassette coding for either wild-type or attenuated diphtheria toxin [162, 163]. All of these methodologies rely on the delivery of large, linearized plasmids carrying fragments of modified genomic DNA via electroporation into high numbers of mES cells and therefore do not lend themselves to high throughput. As a way to circumvent repeated modification of large plasmids, large-scale DNA preparations, and electroporations, several groups have made advances using homologous recombination simply as a “priming” step. With this new method, a specific genomic locus is primed with recombination sites such as loxP or frt so that by standard transfection methods a plasmid coding for a corresponding recombination site could be introduced into cells [3, 76]. Such systems have been referred to as recombinase-mediated cassette exchange (RMCE). The locus of choice for RMCE priming via homologous recombination is ROSA26, located on chromosome 6 in mice. It has been shown the ROSA26 locus [169] allows for ubiquitous and constitutive expression of an inserted transgene. An added bonus to utilizing the ROSA26 locus is that it is readily targeted with high efficiency [127].

There have been multiple instances in the literature where mES cell lines were primed for RMCE in order to increase the efficiency of transgenic line derivation. Some systems relied on the endogenous ROSA26 locus directly driving the expression of an artificial transactivator for which a corresponding promoter and associated transgene were inserted elsewhere [59, 148, 161]. Invariably, this involves the picking of many colonies and screening (at best) with PCR and, in some cases, by Southern blot to check for the proper copy number of the transgene. A few reports described mES cells retrofitted with knock-in vectors dependent on unique loci other than the tried-and-proven ROSA26 locus [4, 70, 170]. In such proof-of-concept papers there is usually insufficient evidence provided to warrant the transfer of a large clone collection. One example involved the use of a ubiquitous Col1A collagen promoter to drive the expression of a small molecule-controlled transactivator, which could then transactivate a gene of interest [3]. However, even with a strong promoter, the system ultimately was ineffective at expressing the GFP transgene in the entire brain as well as skeletal muscle. This could have been a result of the Col1A promoter being inactivated in these tissues at the epigenetic level, although this was not determined. A more likely possibility is that transgene expression was lessened because of the incorporation of the bacterial elements of the inserted plasmid, which were previously shown to interfere with transgene expression [42, 147]. Yet one more promising system that has been shown to be effective *in vitro* as well as *in vivo* incorporated the use of Cre-lox recombination as well as doxycycline-inducible expression to control transgene timing and expression levels [73, 167]. While the tightly regulated expression from the ROSA26 locus is a desirable characteristic, it was outweighed by several key points: (1) Cell lines would have to be screened for positives by PCR, (2) expression induced by hydroxytamoxifen was mosaic and took 48 hours to achieve, and (3) monitoring of transgene expression was not incorporated into the system.

Building off of an earlier generation system, Masui et al. were able to take an inducible promoter system [95] that had been used for so-called “supertransfections” [38] and modify it so that it would satisfy many requirements that would make it amenable to high throughput ([76]; E. Theodorou, unpublished observations). Not only did their system incorporate the ROSA26 allele to allow for constitutive and ubiquitous expression, it also primed the ROSA26 allele with modified loxP recombination sites for RMCE. The loxPV (originally referred to as lox2272) site used for RMCE acted as a substrate for Cre recombinase but could not recombine with a wild-type loxP site. This allowed for recombination that was directional and would always insert the transgene in the proper configuration relative to the promoter element. An added set of modifications increased the ease and rapidity for which correctly targeted clones could be selected. Instead of employing the more typical strategies employed of screening for inserts by PCR and Southern blotting, Masui et al. [76] employed a rapid two-tiered approach. Clones were initially screened for resistance to puromycin antibiotic since a puromycin resistance cassette was shuttled into the modified ROSA26 allele. However, simply screening for puromycin resistance would identify the clones having undergone successful RMCE as well as clones containing the puromycin resistance cassette incorrectly inserted in the genome near an active promoter. Puromycin clones could be quickly tested for correct targeting by (1) growing cells and assaying for a renewed sensitivity to hygromycin or (2) inducing transgene expression and screening clones by fluorescent microscopy for the presence of Venus. This was a marked improvement over other available systems. The use of the IRES-Venus element added the benefit of not only making transgene-expressing clones easier to identify, but also to select clones with higher fluorescence, corresponding to higher expression of upstream transgene (E. Theodorou, unpublished observations). The ability to simultaneously detect correctly targeted clones and clones that have the highest levels of expression is another distinct advantage over more cumbersome methods such as RT-PCR or Western blots [170].

2.3 *Large-Scale Gene Transfer*

Traditional cloning of open reading frames (ORFs) into plasmid vectors typically requires one or more restriction enzymes, and success rate can vary greatly. A recently developed cloning system has alleviated the need for restriction enzyme cloning [150]. The Gateway system of recombinational cloning is based on site-specific recombination mediated by the λ -phage integrase family [44]. Recombination involves two different reactions and results in one relevant product after each reaction. As shown in Fig. 1c, the consensus att recombination sites are 25 nucleotides long (and thus create a short peptide linker whenever N- and/or C-terminal fusions are introduced). Step one of the cloning (Fig. 1a) involves incubating a PCR product amplified with attB primers with a donor vector (pDONR221) in the presence of λ -integrase (Int) and *Escherichia coli* integration host factor (IHF).

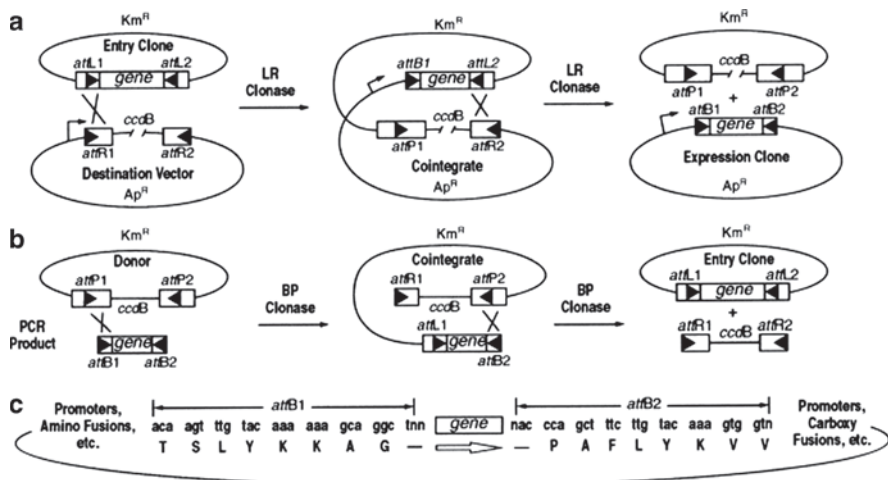


Fig. 1 Overview of the Gateway system. **a:** The entry clone allows transfer of the ORF into any destination vector with the appropriate recombination sites via an LR reaction. **b:** An entry clone containing a full-length open reading frame (ORF) is generated through the recombination of the donor vector with a polymerase chain reaction product with *attB*1/*attB*2 overhangs. **c:** Destination vectors may be designed to produce N- or C-terminal fusions proteins that link to the translated ORF through either an N- or C-terminal eight-amino acid linker peptide. (From Hartley, J.L., Temple, G.F., and Brasch, M.A. (2000) DNA cloning using in vitro site-specific recombination. *Genome Res.* 10, 1788–1795.)

Site-specific recombination occurs between the PCR product *attB* sites and donor vector *attP* sites [hence the name BP Clonase for the enzyme mix (Fig. 1a)]. The BP Clonase reaction is subsequently transformed into *E. coli* strain DH5 α or any *ccdB*-sensitive strain and spread on antibiotic-containing agar plates. Of the colonies that grow, the majority will be correctly recombined entry clones because of the potency of the *ccdB* counterselection. The donor vector will not grow in DH5 α because the *ccdB* gene blocks the growth of DH5 α through the inhibition of topoisomerase. Individual plasmid minipreps of colonies can be screened for correct insert size by performing Taq polymerase PCR with primers identical to the *attB* sites produced from the LR Clonase reaction or by digestion with restriction endonuclease BsrG1 (New England Biolabs, Ipswich, MA), whose restriction site is present in all *att* recombination sequences.

Shortly after the Gateway system of cloning was first unveiled, several groups reported the beginnings of human ORF collections using Gateway [11,124]. As of yet, no complete Gateway clone collection exists, although some companies, such as OriGene Technologies (Rockville, MD) and Invitrogen Corporation (Carlsbad, CA), have made a large percentage of the estimated 25,000 human ORFs [19] available for purchase and modification. Although it is not cost-feasible for most laboratories to purchase large-scale ORF collections, the situation has improved thanks to the efforts of the National Institutes of Health (NIH) to clone every human ORF as part of the Mammalian Gene Collection (MGC) [129]. The availability of the MGC

collection has enabled academic laboratories to become involved in clone generation and to significantly reduce the cost per clone [39,60,113]. Furthermore, the NIH has recently taken notice of the utility of the Gateway system and as part of the newly established ORFeome Collaboration will attempt to transfer all human ORFs into the Gateway system (<http://www.orfeomecollaboration.org/>). Lastly, it will soon be possible for most laboratories to shuttle ORFs without the high cost associated with the Gateway system. This will be possible because of the MAGIC clone shuttling system, which relies on phage recombination occurring within bacteria rather than depending on the use of purified recombinant proteins [64].

3 Transcription Factor Studies

3.1 *Transcription Factor Functional Determination in Murine Embryonic Stem Cells*

Murine ES cells have been used for more than a decade to study transcription factor function. Two of the earliest targeted knockouts in mES cells were the noted transcription factors pRb [50] and p53 [30]. Although in many cases ES cells were used as a tool to generate mice with null mutations, in some cases the differentiation properties of the mES themselves were more informative than the knockout mice [10, 156]. For example, overexpression of transcription factors can, in some instances, effectively force the differentiation of ES cells. In a study conducted by Fujikura et al. [38] cDNAs for 10 transcription factors were delivered (by transfection or electroporation) into wild-type mES cells. Six factors produced no phenotypic changes, while 4 other transcription factors induced the formation of extraembryonic endoderm in some colonies. Thus, there is evidence to show that using mES cells as in vitro tools to model transcription factor lineage commitment is feasible; however, better methods of cDNA delivery to allow homogeneous expression throughout a cell culture, as well as combining such systems with reporter cell lines, would be desirable.

3.2 *Forward Differentiation of Murine Embryonic Stem Cells by Ectopic Expression of Defined Factors*

The power of exogenous gene delivery in ascertaining protein function and deriving a desired cell type has been realized for decades. First on the scene was MyoD [138], the canonical “forced differentiation” transcription factor that was able to transdifferentiate a variety of cell lines simply through ectopic expression [155]. PPARgamma also followed suit with its ability to convert mouse fibroblasts into adipocytes when ectopically expressed in the presence of the appropriate ligand [14, 146].

The use of transient transfections to establish stable cell lines for studying differentiation progression naturally first occurred with embryonic carcinoma (EC) cells because of their availability, prior characterization, and ease of culture. EC cells have been adopted for experiments involving transgene induction [127] and inhibition [86] of differentiation. In addition, the high efficiency of EC cell transfection made it possible in one instance to test a handful of bHLH transcription factors for induction of neuronal differentiation [36].

mES cells very quickly took the lead over EC cells for differentiation studies due to their normal karyotype and development *in vivo*, potentially making any discovery of greater relevance to basic biology. Most often the genes of choice in ectopic expression assays were transcription factors, although the overall gene choice and target cell type varied. The earliest attempt to test the ability of a factor to promote the differentiation of mES cells into a specific cell lineage began with the well-known differentiation-inducing factor MyoD. Ectopic expression of MyoD did promote muscle differentiation in mES cells partially differentiated via embryoid bodies [121]. Teratomas formed from transgenic MyoD mES cells, however, failed to show any added increase in muscle formation. Nonetheless, the work marked the first use of mES cells for examining the function of a specific protein, specifically a transcription factor. Most transcription factors, unlike MyoD, did not have successful track records of effectively differentiating multiple cell lines, and as a result likely candidates were often chosen from factors with very potent *in vivo* phenotypes. For instance, only mild effects were seen in the case of Pax4, a transcription factor essential for pancreatic beta cell development. The overexpression of Pax4 did lead to an increase in Nestin-positive pancreatic precursors as well as Pdx-1-positive cells but failed to form fully functional insulin-producing beta cells [7]. In another instance a protein critical for eye formation in mice, the Rx/rax transcription factor [78], had an effect on morphology of mES cells and resulted in their displaying characteristics indicative of retinal neurons [134]. The mES cell differentiation reports mentioned thus far involved the use of screening for stably expressing clones from pools of cells transfected with randomly integrating expression vectors. Although the method of individual clone testing is tedious, it has proven to be effective with transcription factors, possibly because of the potency of transcription factors even if expressed at low levels. In at least one instance transgenic mES cell stable clones were generated as pools with the transcription factor Osterix. Osterix had been shown to be critical for generation of osteoblasts *in vivo* [91], so it was believed that overexpression would lead to differentiation. Pools of clonal mES cell lines transfected with Osterix were tested, and it was found that 46% of cells expressed the transgene [136]. Although clones were pooled and varied in expression, Osterix managed to stimulate osteogenic differentiation in transgenic cells. By far the most impressive demonstration of single-factor differentiation guided by *in vivo* data is Sox9. Sox9 was the first factor shown to be essential for cartilage formation [5]. Ectopic expression of Sox9 as a stable mES transgenic cell line [58] or adenoviral delivery with related family members Sox5 and Sox6 [48] resulted in direct differentiation of mES cells into mature chondrocytes.

Single-factor induction of mES cell differentiation continues, but systems for expression have become progressively more sophisticated. Zebrafish with deficiencies in the caudal homeobox transcription factor *cdx4* fail to specify blood progenitors [23]. By using the Ainv15 tet conditional expression system, Wang et al. [153] were able to conditionally increase mesoderm specification and hematopoietic progenitor formation. While the Ainv15 system was based on the use of an established knock-in cell line, some groups have achieved the same effect by using multiplasmid transfections. Recently, it was shown that the nuclear protein Chibby (Cby) could induce cardiogenesis in a tetracycline-inducible fashion [125]. Lastly, after performing two rounds of knock-in electroporations, Serafimidis et al. [119] generated a cell line that was capable of inducible *Ngn3* expression. The use of an inducible system allowed the authors to carry out a lengthy and complex differentiation protocol with precise timing for *Ngn3* expression. Not only was the expression system and protocol more sophisticated than many of the previous stable cell line experiments executed in mES cells, but it also allowed for the generation of difficult-to-obtain endocrine pancreas progenitors.

3.3 Reverse Differentiation of Murine Embryonic Stem Cells by Ectopic Expression of Defined Factors

What has turned out to be the highest-impact discovery in stem cell biology since the derivation of stem cell lines has been the use of a defined set of cDNAs for cellular dedifferentiation. Research done in the laboratory of Shinya Yamanaka proved that differentiated cells could be reprogrammed into ES-like cells called induced pluripotent stem (iPS) cells through overexpression of a small number of cDNAs. While seemingly isolated and completely without precedent, efforts to dedifferentiate fully differentiated or partially differentiated cells has a firm foundation in the literature. The earliest interest in the biology of cellular reprogramming (likely) originated from observations of animals such as newt [45] and starfish [118] that have the ability to regenerate severed limbs. Such observations fueled work done in the 1980s and 1990s that began to apply biochemistry and molecular biology to the study of regeneration [13, 58, 69]. The work carried out in newts specifically encouraged Mark Keating's group at Harvard Medical School to go beyond studying reprogramming in tissues and focus on the level of single cells. By ectopic expression of the transcription factor *msx1*, Keating's group managed to convert a small percentage of multinucleated myotubes into dividing, mononucleated cells [98]. Not only was this work pivotal in being the first to show that differentiation could be induced by a gene normally expressed in progenitor cells, but it also accomplished this feat in mammalian cells. Equally as impressive, the mononucleated cells had regained the ability of their parental cell line, C2C12, to differentiate into several lineages, including adipocytes, myocytes, and chondrocytes. Prior to Keating's work, the only success in reprogramming in mammalian cells was in the complex milieu of the oocyte during cloning [149]. Of course, embryonic stem cells were

known to have greater differentiation potential than the immortalized C2C12 cell line and represented the next logical step in dedifferentiation research. Although some progress was made demonstrating that mouse ES cells could reprogram adult cells to a degree [131], it was only recently shown that fibroblasts fused to human ES cells could result in cells with 4N DNA content yet all the major characteristics of hES cells [22]. Reprogramming of differentiated cells with hES cells was independently reproduced with the aid of hES cells expressing an OCT4-GFP reporter [167]. Once it was determined that fibroblasts could be essentially reprogrammed into fully functioning hES-like cells, the difficulty lay in finding a way to deconvolute which proteins were essential to the dedifferentiation process. Using a brute force approach, Takahashi et al. [136] first used microarray analysis to examine which transcription factors had the highest levels of expression in mES cells. They then performed a series of batch retroviral infections into Fbx15-Neo reporter tail tip fibroblasts until the fibroblasts were infected with all 24 transcription factors. Reprogrammed ES-like cells (referred to as iPS) were generated, and further analysis determined that the minimal combination of factors needed for dedifferentiation was Oct2/4, Myc, Sox2, and Klf4 [136]. Remarkably, mouse iPS cells were identically grown as mES cells and were successfully used to generate EBs, displayed markers of various lineages when differentiated, and produced teratomas, but when injected into blastocysts they could only produce embryonic mouse chimeras.

Rapid progress has continued to be made in the area of dedifferentiation via overexpression of defined transcription factors. As a direct follow-up to the original work in Yamanaka's laboratory, Okita et al. delivered the set of four transcription factors into fibroblasts to generate iPS cells. As previously mentioned, ectopic Nanog has shown the ability to increase the percentage of mES-neural stem hybrids [123]. Taking advantage of this, Okita et al. [100] took the published iPS protocol one step further by selecting out clones that had high levels of Nanog expression. The iPS clones high in Nanog expression were able to generate chimeras and, in one instance, transmit through the germline. A major drawback of the work done by Okita et al. was that the presence of the Myc proto-oncogene resulted in approximately 20% of the germline pups rapidly developing tumors. The problem of tumor formation was eventually circumvented by using a conditionally expressed Myc transgene [43] so that iPS-derived myeloid precursors were rendered safe before transplantation into a mouse model of sickle cell anemia. Another report from the same laboratory demonstrated that drug selection of retrovirus-generated iPS cells was not necessary to select reprogrammed clones, and that iPS clones generated through a gentler, morphology-based protocol could contribute to adult chimeras [81].

The most exciting results surrounding iPS cells have come from reprogramming experiments carried out with human cell lines. Defying expectations [107], several groups were able to apply similar techniques used to derive mouse iPS cells to derive human iPS or hES-like cells. Most fascinating is that each research group managed to contribute to at least one unique finding to the generation of human iPS cells. The first group to publish success at generating human iPS from fibroblasts proved that the human orthologs OCT4, SOX2, MYC, and KLF4 could work across

species, and the iPS cells they generated were able to differentiate and form EBs and teratomas [136]. In the same year Yu et al. [168] was able to accomplish the same goal but managed to do it without using transcription factors identical to those of Takahashi et al. Instead, a miniature screen involving 14 transcription factors highly expressed in human oocytes was carried out. The 14 transcription factors were narrowed down to OCT4, SOX2, NANOG (not essential, but improved efficiency), and LIN28. This demonstrated that reprogramming fibroblasts into iPS was not limited to the originally published four transcription factors. A recent report focused on the translational applications of iPS by using human orthologs of the original four factors to convert primary biopsied skin cells into iPS cells [104]. Finally, in an effort to eliminate any chance of Myc inducing tumors, Nakagawa et al. [89] were able to generate both mouse and human iPS without the use of Myc. The three-factor mouse iPS cells were the first and only reported instance of iPS cells being transmitted through the germline without inducing tumors in resulting offspring. One additional interesting find by Nakagawa et al. was that homologous transcription factors could substitute, to a lesser degree, for the three programming factors (e.g., Oct1 and Oct6 could substitute for Oct4).

Early work done by the Keating laboratory and very recently by the Yamanaka and other laboratories relied on the use of defined cDNAs to induce cellular reprogramming in a defined and controlled manner. Indeed, it has been the use of full-length cDNAs that has allowed researchers in the field of dedifferentiation to achieve what others previously could not through indirect methods involving cell fusion [22, 134] or whole-cell extracts [139]. The crowning achievement of cDNA reprogramming has obviously been the creation of iPS cells, which will not only prove invaluable in the laboratory, but also are a significant step forward in stem cell therapy without the ethical issues posed by embryonic stem cells. After iPS cell derivation was reproduced, the floodgates opened, and the race has been on to generate iPS cells solely through the use of non-viral-based reagents such as chemical compounds [46, 71, 122], excisable transgenes [158], and transducible proteins [171]. As of this writing, no single method has superseded all others for the generation of ES-like iPS cells. It is likely that in the exploding field of cellular reprogramming we will not have to wait too long.

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