Stem Cells and Combinatorial Science

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Abstract: Stem cell-based technologies have the potential to help cure a number of cell degenerative diseases. Combinatorial and high throughput screening techniques could provide tools to control and manipulate the self-renewal and differentiation of stem cells. This review chronicles historic and recent progress in the stem cell field involving both pluripotent and multipotent cells, and it highlights relevant cellular signal transduction pathways. This review further describes screens using libraries of soluble, small-molecule ligands, and arrays of molecules immobilized onto surfaces while proposing future trends in similar studies. It is hoped that by reviewing both the stem cell and the relevant high throughput screening literature, this paper can act as a resource to the combinatorial science community.

Keywords: Stem cell, array, self–assembled monolayer, purmorphamine, myoseverin, TSW119, cardiogenol C, reversine, SC1, neuropathiazol, complex phenotypic screen, pluripotency, multipotency, differentiation, self-renewal, hematopoietic stem cell, embryonic stem cell, mesenchymal stem cell, transdifferentiation, dedifferentiation, cytophobic, cytophillic, multiplex screening.

INTRODUCTION

It is thought that stem cells could provide an unlimited source of feed stock for cell-based therapies to reverse diseases caused by cellular degeneration. Work on stem cell-focused technology may eventually, through the development of robust *ex vivo* techniques, solve the problem of the limited supply of tissue for organ transplants. Additionally, stem cell research could provide a valuable window into the forces triggering the development of organisms from a single cell into an adult. This insight could, in the future, result in technologies and clinical applications not only currently unforeseen but also unfathomable.

At the moment, however, multiple challenges exist before the potential of stem cell research is to be realized. First, conditions are required that allow these cells to grow and divide *in vitro* and thereby permit the production of cellular precursors for various technologies. These conditions must be both cost effective and safe for future transplantation and clinical applications. Therefore these conditions should be completely defined and should have no components derived from any non-human sources (*vide infra*). Second, conditions need to be developed that allow these precursor cells to differentiate into desired cell types. These conditions not only need to be cheap, defined, and humanized, they also need to provide the required cells in a high yield with few other types of cells as "impurities". Efficient differentiation is essential so that few costly and time-consuming isolation techniques are necessary to obtain the desired differentiated cells. Third, it is necessary to identify and characterize undifferentiated, partially differentiated, and fully differentiated cells. Characterization methods are essential to monitor the process of differentiation and to ensure quality control of any developed technology.

Small molecules could aid in overcoming these challenges. Stem cells within their natural niche receive signals from their environment from a number of sources. These signals initiate when the cell binds to soluble protein factors, to the extra-cellular matrix, or to other cells. Small molecules could functionally mimic these signaling niches to cause stem cells either to differentiate or to self-renew in an undifferentiated state. Moreover, small molecules could act to inhibit or activate key enzymes in a differentiation pathway to provide a non-biomimetic niche. Additionally, these molecules, using a chemical genetic approach, could provide valuable insight into the mechanism of cellular differentiation. A strength of small molecule-based approaches is that they could be directly applicable to drug therapies or drug-supported cell-based therapies. Proteins are readily denatured, are expensive to produce in a purified form, and are not easily transported across membranes by passive mechanisms. Beyond this, specific small molecule binders of unique cell-surface proteins could be part of affinity labels to identify key stepping stone cell types in a differentiation pathway, as well as to better characterize the undifferentiated state.

Combinatorial science sets out to search libraries of molecules for those possessing a desired activity. It is thought that through the exploration of directed or random swaths of diversity space, an active molecule can be discovered more efficiently than through more "rational" or iterative methods. Combinatorial techniques are beginning to be unleashed on stem cell science to provide small molecules that either direct differentiation or promote undifferentiated self-renewal. Moreover, combinatorial arraying technology has been applied to find materials and combinations of molecules to which stem cells adhere, and either proliferate or differentiate. This review sets out to chronicle current advances in how combinatorial science has been applied to

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the stem cell field. This topic has been reviewed previously [1-3], however, in this case, along with providing an update on the recent literature, it expands the scope to include arraying-based research and provides an historical context to stem cell literature. It is hoped that this review can act as a resource for the combinatorial community interested in conducting high throughput screens involving stem cells.

Stem cells are found in most tissues throughout the body of an adult organism. These cells are referred to as "adult stem cells" and help maintain homeostasis by replenishing old cells and playing a role in tissue repair. Additionally, stem cells are involved in the development of an organism from a small collection of cells (morula) into an adult. Stem cells, to different degrees, have the power to differentiate into other cell types. This transformation can involve the formation of fully differentiated adult cells or partially differentiated precursors to these adult cells. The differentiation power possessed by a stem cell can be described as "totipotent", "pluripotent", and "multipotent". Multipotent describes the potential of adult stem cells that can differentiate into a limited number of cell types and precursors. Multipotent stem cells are usually thought to be restricted to differentiation within a single developmental germ layer (ectoderm, endoderm, mesoderm) in an organism. "Pluripotent" describes cells that can differentiate into most cell types from all three germ layers. Embryonic stem cells are thought to be pluripotent and their differentiation power seems to be limited only by their inability to produce cells that make up extra-embryonic tissue found in the placenta during embryonic development. The only known totipotent cells are the cells that make up the morula. Totipotent stem cells have the power to differentiate into all the cell types necessary to generate an adult organism. They can differentiate into placental tissue along with the three germ layers. In addition to differentiation potential, most stem cells have the ability to self-renew. This power allows the production of more undifferentiated stem cells.

Near the sixteen cell stage, the developing embryo begins to differentiate into the blastocyst which is composed of the inner cell mass (ICM) and the surrounding trophoblast. The cells making up the ICM maintain pluripotency whereas the trophoblast can only form placental tissue. Embryonic stem (ES) cells are undifferentiated pluripotent cells that can be isolated from ICM of the blastocyst, and can be maintained in culture in a self-renewing state under suitable conditions [4]. ES cells can be used to generate chimeric organisms which, in turn, develop into normal, fertile adults. Currently, chimeras have been developed only from mouse ES cells [5].

As embryonic development continues from the blastocyst stage, multipotent progenitors are formed. Multipotent cells lose some of their differentiation potential and are committed to a specific lineage. Therefore they can only give rise to a limited number of specific cell types. For example, hematopoietic stem cells can develop into several types of blood cells and are multipotent. It was therefore thought that hematopoietic stem cells should not be able to form cells of other lineages. However, recent studies [6] have challenged this tenet by demonstrating that hematopoietic stem cells can form neuron-like cells, suggesting that cells that were thought to be lineage-committed can be reprogrammed. After a series of cell divisions terminally differentiated cells are formed. These cells have been thought to be permanently committed to a specific function and unable to give rise to any other cell types. This long-held belief has also been brought into question. Recently it has been shown that differentiated skin cells can be dedifferentiated, employing surprisingly simple techniques, to generate cells that exhibit a high degree of pluripotency [7-9].

PLURIPOTENT STEM CELLS

Historically, three types of pluripotent cell lines have been established from mouse embryos: embryonic stem (ES) cells, embryonic carcinoma (EC) cells, and embryonic germ (EG) cells. EC cells are cultivated from teratocarcinomas, which either arise spontaneously or through experimental induction, or are produced when the ICM of the blastocyst is transferred to extrauterine sites of the organism. EG cells are derived from primordial germ cells isolated from the genital ridge of embryos. Only ES and EG cells are able to give rise to all somatic and germ line cell types [10, 11]. Permanent lines of murine ES cells were first established in 1981 by the cultivation of ICM material from early embryos [5, 12]. Pluripotent EG cells were isolated from primordial germ cells in 1994 [13].

Research into pluripotent cell lines, however was precipitated by work involving teratomas, teratocarcinomas, and ECs which began more than fifty years ago, and many innovations and themes persist from this work into the modern day. Teratomas and teratocarcinomas are benign and malignant tumors, respectively, found in the gonad and occasionally at extragonadal sites. Historically they were considered a simple medical oddity in that these tumors consist of a menagerie of cell types, lineages, differentiated adult tissues, and strangely shaped partially defined organs such as teeth, bone, and muscle within the tumor itself [14, 15]. Following their malignant nature, teratocarcinomas, when transplanted either subcutaneously or intraperitoneally, demonstrate the ability to grow rapidly. Naturally occurring teratomas/teratocarcinomas are quite rare and are therefore difficult to study experimentally. In the 1950's, mice strain 129 was developed, exhibiting a 1% incidence of spontaneous testicular carcinoma. This discovery provided a source of cells and allowed their eventual study [16]. In 1964 it was found that grafted fetal genital ridges result in a higher tumor incidence, suggesting that tumor formation arises from a nest of cells within the fetal testes; this led to the conclusion that these cells resemble stem cells [17]. Furthermore, it was found that intraperitoneal injection of a single teratocarcinoma cell from this nest can form multiple cell types in a resulting tumor [18]. Taken together, these data indicate that teratocarcinomas possess a unique type of stem cell that has a wide degree of differentiation power giving rise to multiple adult cell types and has the ability to self-renew as is exhibited by the tumor malignancy.

Parallels between teratocarcinomas and embryos were established in 1970, when it was demonstrated that retransplantable teratocarcinomas could be obtained when early mouse embryos were subjected to extrauterine transplantation [19, 20]. Further research into embryonic carcinomas was accelerated when it was found that mouse chimeras could be established by injecting embryonic carcinoma cells into the blastocyst cavity [21]. With the discovery that these

tumors could be grown *ex vivo* on feeder cells, mass cultures of these pluripotent teratocarcinomas could be sub-cloned, thereby establishing EC cell lines [1,2]. These stem cells can proliferate indefinitely and give rise to teratocarcinomas upon subcutaneous injection. Human EC lines were established in the late 1970's [24]. To characterize and identify mouse and human EC cells, monoclonal antibodies that recognize unique antigens were established. Stage-specific embryonic antigen 1 (SSEA1), which is a Le^x-like (α1-3 fucosylated N-acetyllactosamine) carbohydrate antigenic determinant, is expressed on the surface of EC cells and has been shown to be useful in the monitoring of cell differentiation in these cell lines and in the isolation of primordial germ cells [25, 26]. As early as 1978 it was demonstrated that pluripotent cells can be differentiated chemically when it was shown that mouse and human EC cells can be induced to differentiate in culture upon the addition of retinoic acid [27-29].

Mouse ES cell lines were derived in 1981 by growing cells from blastocysts on a feeder layer of mouse fibroblasts [5, 30]. Although the feeder layer is important for the isolation of the ES cells, the soluble leukemia inhibitory factor (LIF) is also required for the maintenance of the established culture in its undifferentiated state [31, 32]. These mouse ES cells were shown to produce chimeras more efficiently than EC cells [10, 33]. In the mid-1980's technological advancements allowed mouse ES cells to be employed in transgenesis [34], and gene targeting and homologous recombination using these cells were developed to study gene function [35]. The first genetically modified mouse was generated in 1989 using homologous recombination in ES cells [36].

Many properties of EG cells are similar to ES cells. In 1992, mouse EG cell lines were obtained from primordial germ cells [37]. Mouse ES cells were derived from blastocysts using somatic nuclei transfer into enucleated oocytes [38]. The first human ES cell [39] and EG cell [40] lines were derived in 1998 and 2001 respectively, using similar strategies to those that were employed in mice. The establishment of human cell lines using nuclear transfer has yet to be successful.

Markers of Pluripotency in Human and Mouse Cells

Despite the parallels and similarities between mouse and human pluripotent cell lines, they do exhibit some significant differences in culturing conditions and markers for characterization. Alkaline phosphatase was one of the first pluripotency markers identified. It is not unique to ES cells, however. Mouse and human EC cells, cells in the ICM in the mouse blastocyst, and cells of the ectoderm and primordial germ cells express alkaline phosphatase activity [41, 42]. Anti-SSEA1 monoclonal antibodies recognize a specific carbohydrate on the surface of the mouse EC cells and have been used to monitor EC cell differentiation (*vide supra*). However, anti-SSEA1 antibodies do not react with human ES cells. Interestingly, some cells differentiated from human ES cells do express anti-SSEA1 antigens. Other differences between mouse and human pluripotency markers involve the expression of the SSEA3 and SSEA4 antigens [43]. Both SSEA3 and SSEA4 are expressed on mouse embryos and on mouse EC cells that have been differentiated, whereas in human cells they are expressed on undifferentiated EC and ES cells [44]. Taken together these data suggest: that human ES cell lines are somehow more primitive than mouse ES cell lines, that current markers for pluripotency are inadequate, or that early development in the murine model system is not as similar to that in humans as was once thought. This latter possibility may explain the seemingly different differentiation and self-renewal properties exhibited between pluripotent cells of mouse and human origin (*vide infra*). Other markers that have gained popularity are TRA1-60 and TRA1-81. They seem to be able to identify human pluripotency in that they react with human EC and ES cells as well as with early human embryos [45].

Growth Conditions to Promote Self-Renewal in ES Cells

All with the ability to differentiate, stem cells also have the property of undifferentiated propagation, i.e. self-renewal. Progeny can be produced that possess all the differentiation ability of the parent cell. This self-renewal can take place through symmetric cell division where both daughter cells retain the same differentiation potency and self-renewal ability of the parent, or through asymmetric division where one of the two daughters is differentiated and has a limited capacity for cell division.

Being able to expand stem cell lines in culture is necessary for the easy laboratory production of cells for study and is essential for the advancement of the field. The first mouse ES cell line was derived by expanding blastocyst ICM on fibroblast feeder cells (either embryonic fibroblasts or STO cells) (*vide supra*) [11]. The presence of feeder cells is important for the *in vitro* growth and maintenance of the undifferentiated state as the cells are otherwise prone to spontaneous differentiation. The inclusion of LIF in the culture medium is also important for the inhibition of differentiation (*vide supra*) [46]. Recently, feeder cell-free conditions have been established that use LIF along with bone morphogenetic proteins (BMPs) to derive ES lines and to achieve self-renewal in culture [47].

Human ES cell lines were originally derived by culturing human blastocysts formed from *in vitro* fertilized eggs on mouse embryonic fibroblast feeder cells in medium conditioned by feeder cells [48]. In contrast to mouse ES cells and emphasizing the possible differences between mouse and human early development (*vide supra*), the feeder layer cannot be replaced by LIF and BMP alone [49, 50]. Therefore the critical factors that help to maintain the human ES cells in the pluripotent state remain unknown. Genetic and proteomic tools that provide insight into these processes may prove helpful to gain a better molecular understanding and to design better self-renewal culture conditions. Recently, RNAi has emerged as a powerful technology to perform loss-of-function studies in mammalian cells. This technology should be ideal for identifying and studying genes required for ES cell self-renewal and differentiation [51]. It is not inconceivable that high throughput studies involving small- or bio-molecules could complement these studies.

Human ES cells that were derived on mouse feeder cells may be compromised for clinical applications. Not only do they run the risk of cross-transfer of animal pathogens from the feeder layer, matrix, or conditioned medium, their use also results in the expression of animal antigens on the surfaces of the cells. Presumably these antigens would then be

carried through to any ES cell-derived tissue resulting in an immune response when used in the clinic. For example, it has been found that when grown in animal-derived conditions, human ES cells will metabolically incorporate the nonhuman sialic acid, Neu5Gc [52]. A solution to this problem could be to employ human feeder cells and culture conditions that are completely humanized. A new ES cell line has recently been derived using serum- and feeder cell-free conditions. Characterization of these cells confirms that they are pluripotent and would be safe for clinical applications in that they express the markers of pluripotency, can generate teratomas, differentiate into all three germ layers, and have the correct number of chromosomes exhibiting the proper phenotype [53]. All of the cell lines available to researchers in the U.S. who receive federal funds have been derived using the animalized conditions. Although humanized human ES cell lines have been established, their use is restricted and U.S. researchers are limited to the use of potentially contaminated cells. This may slow the progress of transitioning ES cell technology to actual therapies [6, 54].

Differentiation Conditions for ES Cells

Human ES cells will grow as compact, flattened colonies with distinct cell borders; express SSEA3, SSEA4, TRA1-60, TRA1-81, and alkaline phosphatase; and express SSEA1 upon differentiation (*vide supra*). When the human ES cells are removed from the feeder layer cells and grown in suspension, they begin the differentiation process, in a manner similar to that of mouse ES cells, by first forming groups of aggregated cells or embryoid bodies (EBs). Once EB formation occurs, the cells begin to follow the normal embryonic development and differentiate to form the three germ layers. EBs have been shown to express markers specific to neuronal, hematopoietic and cardiac lineages [55, 56], and EB formation is the first step in a number of differentiation procedures in culture. The aggregation event is dependent on a number of factors including culture conditions, cell density, time, stirring, and the ES cell line itself and therefore tends to be inconsistent [57]. The formation of EBs may not be necessary for the establishment of differentiation conditions, however. It has recently been demonstrated that it is possible to directly transition ES cells grown in a monolayer to neural stem cells with the application of appropriate growth factors [58].

Differentiation of ES cells can be partially directed by manipulation of the culture conditions and addition of exogenous factors to EBs. For example, mesodermal differentiation can be induced by the addition of activin-A and transforming growth factor–β1 (TGF-β1). Retinoic acid, epidermal growth factor (EGF), BMP-4 and basic fibroblast growth factor (bFGF) will elicit ectodermal and mesodermal differentiation. Heart muscle cell formation can be promoted by culturing with a mixture of hepatocyte growth factor, EGF, bFGF, and retinoic acid. It should be noted that none of these conditions can promote ES cells to differentiate exclusively, to a single cell type, nor are these conditions high yielding and often methods coupling cell sorting techniques with expansion are employed [55].

ADULT STEM CELLS

In the 1960's, it was shown that the transplantation of mouse bone marrow cells into irradiated mice gives rise to spleen colonies, each of which can be proven to arise from an individual cell (*vide infra*) [59]. This experiment suggested the existence of multipotent cells that exist in the bone marrow and gave birth to the study of adult stem cells.

Adult precursor cells are found in specific organs or tissue types. Like ES cells, they are defined by two distinct properties: the ability to self-renew, and the capacity for differentiation into mature cells that can perform specialized functions [60]. As an embryo develops, its cells gradually lose the power to form all cell types and become more limited in differentiation potential. However, clusters of adult stem cells remain in specific body tissues. These stem cells reside in niches or micro-environments in their respective tissues. They are subjected to extrinsic cues, in the form of secreted or surface immobilized factors present within the niche [61], which trigger differentiation or self-renewal in order to maintain homeostasis of the cell population and create vital stores for the replacement of cell populations in the event of tissue injury or degeneration.

The conventional distinction between embryonic and adult stem cells has been made in terms of their differentiation potential. ES cells are pluripotent and can differentiate into any cell type except extra-embryonic tissue. Adult stem cells, on the other hand, are multipotent. That is, they possess the capacity to give rise only to cell types of defined lineages.

This traditional concept has recently been undermined by studies showing a greater plasticity of adult stem cells, indicating that adult stem cells might have more pluripotent potential than was previously thought. There is increasing evidence that some adult stem cells, most notably bone marrow and neural stem cells, can transdifferentiate to produce cells across lineage barriers. For example, it has been shown that bone marrow stem cells have the potential to develop into muscle cells [62]. However, the concept of transdifferentiation itself has been challenged through experiments that show that when either haematopoietic or neural stem cells are fused to ES cells, the adult stem cells take on characteristics similar to those of ES cells [63, 64]. These data have led to the theory that stem cells do not solely follow a preordained autonomous differentiation program, but also respond to extrinsic differentiation cues from their environment. Hence, when an adult stem cell is placed in an environment other than its usual niche, it may be instructed by extrinsic factors to follow an atypical differentiation pathway. This suggests that these cells might be ripe for extensive manipulation in culture through the manipulation of "artificial differentiation niches" by the application of novel, synthetic molecules.

Hematopoietic stem cells (HSCs), which differentiate into blood lineages are the best-characterized of the many different adult stem cells. Others under intensive research include neural, mesenchymal, and epidermal stem cells.

Hematopoietic Stem Cells

HSCs are stem cells residing in the bone marrow, placenta or umbilical cord blood that can give rise to all blood cells of the myeloid and lymphoid lineages. Early evidence of the existence of HSCs in the bone marrow was demonstrated by experiments involving bone marrow transplantations into irradiated mice [65, 66] in the 1950's and 1960's.

However, the first mouse HSCs were not isolated from bone marrow until 1988 [67]. This isolation was accomplished by negatively selecting cells that did not express the surface markers that characterize differentiated hematolymphoid cells (B220 for B cells, Gr-1 for granulocytes, Mac-1 for myelomonocytic cells and CD4 and CD8 for T cells). The collective absence of these and other mature blood lineage markers, is described as "Lin$^-$". Other markers of HSCs include Thy-1$^{+/low}$, Sca-1$^+$, CD34$^{low/-}$, CD38$^+$ and c-kit$^+$. Such cell surface markers are essential to recognizing and isolating HSCs due to the fact that HSCs strongly resemble other blood cells morphologically.

Markers for human HSCs have also been successfully employed in their isolation, although these markers differ somewhat from those used for murine HSCs. A human HSC population was first isolated in 1992 by screening for Thy-1$^+$ Lin$^-$ CD34$^+$ bone marrow cells [68]. Other markers include CD59$^+$, CD38$^{low/-}$ and c-kit$^{low/-}$. There is still ongoing debate concerning the most definitive set of stem cell markers expressed on human HSCs [69, 70] and there are no known stem cell markers that can be used to obtain a pure population of HSCs. No doubt, more complete sets of markers need to be established for human HSCs.

Of all the adult stem cell lineages, the clearest understanding exists of HSCs, and they are the only adult stem cells that have been used in transplantation therapy. Despite this, problems still exist with culturing HSCs *ex vivo*. Proliferating or differentiating HSCs outside the body has proven difficult, thereby impeding steps to expand their therapeutic potential. It has been proposed that culturing problems are associated with the properties of the cells themselves as they inherently undergo asymmetric cell division to generate a multipotent daughter cell and a lineage progenitor that will be differentiated. This situation will result in the continuous generation of terminally differentiated cells [71].

Despite the challenges some inroads have been made in culturing these cells *ex vivo*. It has been demonstrated that the murine bone marrow stromal cell line (MSCs-*vide infra*), HESS-5, can sustain the proliferation of co-cultured human CD34$^+$CD38$^-$ cells with the addition of cytokines like interleukin-3 (IL-3), thrombopoietin (TPO) and flk-2/flt-3 ligand [72]. The cell-cell interaction of HSCs with bone marrow stromal cells is so crucial because the stromal cells serve a supportive role physiologically and provide soluble factors that could enhance stem cell proliferation [73, 74]. Thus, the co-culturing condition effectively reconstitutes the hematopoietic microenvironment *in vivo*. The requirement for large quantities of cytokines limits the practicality of these techniques and the dependence of non-humanized conditions could pose problems with mouse antigen expression similar to that seen with human ES cells (*vide supra*).

Mesenchymal Stem Cells

In the 1970's it was found that another class of stem cells, mesenchymal stem cells (MSCs), resides in the bone marrow, along with HSCs [75-77]. MSCs are also known as bone marrow stromal cells and have the potential to differentiate into a wide range of cell types, like chondroblasts (which form cartilage cells), adipocytes (which form fat cells), and osteoblasts (which form bone). MSCs are not exclusively located in the bone marrow. They can also be isolated from tissues like peripheral blood [78, 79], adipose tissue [80], synovial tissue [81], and heart tissue [82], among many others. It is currently unknown how MSCs isolated from different tissues differ.

The ability of MSCs to differentiate into many different cell types and support the growth of HSCs (*vide supra*) confers on them a high clinical importance. However, knowledge in this area is currently too limited to make medical use of MSCs. A major constraint is the lack of specific cell surface markers to identify and isolate MSCs. Other than identifying MSCs by recognizing their ability to form fibroblast colony forming units (F-CFU), some rather ambiguous markers of human bone marrow-derived MSCs exist: STRO-1, HOP-26 (CD63), CD49a and SB-10 (CD166) [83], SH2$^+$, SH3$^+$, CD29$^+$, CD44$^+$, CD71$^+$, CD90$^+$, CD106$^+$, CD120a$^+$, CD124$^+$, CD14$^-$, CD34$^-$, and CD45$^-$ [84]. Of this extensive list, STRO-1 is the most widely used although its expression gradually decreases in culture [85], and some bone marrow-derived MSCs are negative for STRO-1 [86]. As with HSCs, additional, more robust markers need to be established. Murine MSC markers differ somewhat from human markers: Sca-1$^+$, CD29$^+$, CD44$^+$, CD81$^+$, CD106$^+$, Nucleostemin$^+$, CD116$^-$, CD34$^-$, CD45$^-$, CD48$^-$, CD117$^-$, and CD135$^-$ [87].

Unlike HSCs, bone marrow-derived MSCs have been shown to possess a high rate of proliferation *in vitro* [88]. Studies have indicated that the fibroblast growth factor (FGF) family, notably FGF-2, is involved in maintaining the self-renewal ability of human MSCs [87]. Other growth factors shown to cause human MSC expansion include platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [89]. It has also been reported that dickkopf-1 (Dkk-1), an inhibitor of the Wnt signaling pathway, increases proliferation of MSCs [90].

Conditions required for controlled MSC differentiation are not completely established. Establishing these conditions is complicated by the many lineages that MSCs can follow. Culturing in the presence of added bone morphogenetic proteins (BMPs) has been shown to enhance osteoblast formation [91], whereas, differentiation of bone marrow-derived MSCs to chondroblasts is mediated by TGFβ [92]. Recent findings have also indicated that BMP-2 induces *in vitro* cartilage formation [93].

Neural Stem Cells

Until recently it was thought that neurogenesis occurs only during embryo formation and early childhood. Although this belief was questioned as early as the 1960s [94-96], it was not until the 1990s that it lost widespread acceptance when it was demonstrated that cells isolated from the adult rodent forebrain could generate new neurons and astrocytes [97]. Isolation of neural stem cells from the adult human brain was also carried out in 1999 [98].

Neural stem cells are multipotent adult stem cells that are able to differentiate into neurons, astrocytes (glial cells that form the blood-brain barrier), and oligodendrocytes (glial cells that myelinate neuronal axons). Neural stem cells can be isolated from the subventricular zone (SVZ) of the lateral ventricles, the subgranular zone (SGZ) of the hippocampus and also the spinal cord. There is also evidence that ependymal cells (cells that form the membrane surrounding the

brain and the spinal chord) from the adult brain and spinal chord are actually neural stem cells [99].

Because adult neurogenesis had not been widely recognized until recently, few markers have yet been identified for neural stem cells. Notch 1 has been commonly used as a neural stem cell marker. More specifically, Notch 1 expression identifies ependymal cells of which at least a portion represents neural stem cells [99]. Neural crest stem cells (NCSCs) were isolated by enriching for cells expressing the low affinity neurotrophin receptor, p75, but not a peripheral myelin protein, P_0 ($p75^+$ P_0^-) [100]. Although little work has been published on the culture and differentiation of neural stem cells, it has been established that their proliferation requires the addition of FGF-2 [101] and EGF [97]. The limited knowledge we have about neuronal stem cells must be greatly expanded before we can use them to aid diseases like Parkinson's and Alzheimer's.

Cardiac Stem Cells

Until the mid-1980's, the heart, like the brain (*vide supra*), was widely thought of as a static organ that does not undergo regeneration [102]. More recently, cardiac cell progenitors have been isolated, debunking this. These cells can generate all three differentiated cardiogenic lineages, endothelial, cardiac, and smooth muscle cells [103]. These stem cells were characterized as being $Isl1^+$, $Nkx2.5^+$, and $flk1^+$ cells, suggesting possible use of these markers in the future.

Cardiac stem cells could eventually play a role in tissue regeneration after heart muscle death due to heart attack. Research in this area is still in its infancy however, controlled conditions for cardiac stem cell proliferation and differentiation have yet to be firmly established [104].

Epidermal Stem Cells

The epidermis is mainly made up of keratinocytes, which populate different epidermal layers depending on their developmental stage. The keratinocyte stem cells are located at the deepest layer, the stratum basale, and eventually undergo terminal differentiation as they gradually move upwards to the outermost epidermal layer, the stratum corneum. The stem cells will form transit amplifying cells, keratinocytes with a more limited self-renewal potential, after about three rounds of division before further differentiation.

Human epidermal basal cells are enriched in β_1 integrins, prompting their use as human epidermal stem cell markers [105]. Murine keratinocyte stem cells can be isolated using their high expression of α_6 integrin and low expression of transferrin receptor CD71 ($\alpha_6^{bri}CD71^{dim}$) [106]. When grown in culture, epidermal stem cell populations expand rapidly but eventually lose their self-renewal potential. It has been shown that this self-renewal loss could be inhibited by the addition of EGF, but this treatment slows proliferation [107]. It has been shown that β_1 integrins and mitogen-activated protein kinase (MAPK) may work together in maintaining the self-renewal potential of epidermal stem cells and in preventing differentiation, suggesting an eventual dual-target for future studies [108].

Differentiation of epidermal stem cells involves the Notch-Delta signaling pathway. Cells with a high expression of Delta induce the differentiation of adjacent cells *via* Notch signaling but do not differentiate themselves [109]. Furthermore, it was reported that the activation of c-Myc in epidermal stem cells stimulates terminal differentiation and enhances movement of the stem cells to the transit amplifying region [110]. While not providing exact recipes for controlling differentiation, these data hint at possible fertile ground.

SIGNAL TRANSDUCTION IN STEM CELLS

The self-renewal and differentiation properties of stem cells are specifically and tightly regulated. This regulation derives from extrinsic niche-related factors, and is transduced by complex networks of signaling pathways. Though intense research has been undertaken, the mechanisms for signal transduction in mouse and human stem cells are still not very clear. Generally, it seems that self-renewal is maintained by repression of cell type-specific genes, activation of progenitor cell-specific genes, and regulation of cell cycle and cell death. Meanwhile, differentiation is initiated by activation of cell type-specific pathways, repression of self-renewal signals and regulation of the cell cycle [111]. Often, many pathways may be activated in concert, and tipping their delicate balance may result in radically different cell fate outcomes.

The Smad Pathway

In mammals, the family of Smad proteins includes Smad1, 2, 3, 5 and 8. All these proteins possess an SXS motif at their C-terminus that is phosphorylated by ligand-activated TGF-β receptor kinases, therefore they are sometimes referred to as R-Smads (Receptor activated Smads). The different Smad proteins are activated by specific factors. For example, Smad2 and Smad3 specifically act downstream of only the TGF-β family, while Smad1, 5 and 8 are activated by BMPs (Bone Morphogenetic Proteins) [112, 113]. Upon ligand stimulation, the cytoplasmic Smads are phosphorylated and then translocate to the nucleus to regulate the expression of target genes [114].

It has been demonstrated that the balance between R-Smad/TIF1γ- and R-Smad/Smad4-mediated TGF-β signaling in hematopoietic stem cells is critical for controlling cell fate decisions [115]. TIF1γ acts as a transcriptional partner of activated R-Smads in competition with Smad4. While the R-Smad/TIF1γcomplex stimulates erythrocyte differentiation, the R-Smad/Smad4 complex inhibits hematopoietic stem cell proliferation [116].

Stem Cell Factor

Stem cell factor (SCF, also known as MGF, KL and SLF) is an acidic glycoprotein secreted by stromal cells in the bone marrow niche. It is an important haematopoietic factor, which has a significant regulatory effect on normal haematopoietic cells, mast cells, and melanoma cells among others. Its receptor, c-Kit, is a transmembrane protein with tyrosine kinase activity. The specific interaction with SCF may induce the homodimerization of c-Kit and therefore initiate a phosphorylation cascade. Downstream SCF/c-Kit signaling is complicated in that several pathways are activated. Therefore, SCF/c-Kit has become the focus of much current research [117].

Jak/STAT Signaling Pathway

The protein tyrosine kinases in the Janus family (Jaks) are activated in a ligand dependent manner by members of the cytokine receptor superfamily [118]. The Jak family consists of four members, Jak1-4, which are activated specifically in response to various cytokines and subsequently activate the specific signal transducer and activator of transcription (STAT) protein family [119]. It has been demonstrated that SCF can induce Jak2 activation by c-Kit, thereby inducing cell proliferation, while Jak2-deficient mice exhibit embryonic lethality due to the absence of erythropoiesis (red blood cell formation). This phenotype is identical to that exhibited by c-Kit or SCF knock-outs, suggesting that Jak2 lies in the same signaling pathway [120].

Ras/Raf/MEK/ERK Signaling Pathway

The activation of the Ras pathway has been implicated in the cell proliferation activity exhibited by SCF/c-Kit. It has been found that the GTPase activating protein nuclear factor (NF1) is involved in regulating the SCF-triggering of the Ras pathway. It has also been found that Raf-1 is involved in the SCF-initiated phosphorylation of casein threonine kinase, the activity of which is increased dramatically with SCF induction. In addition, SCF can turn on the MAP kinase cascade [121].

The PI3K Pathway

In stimulating certain target cells, SCF and other cytokines can directly activate and increase the activity of phosphatidylinositol 3-kinase (PI3K) through c-Kit, Shc, Rac, Ras or Rho. PI3K is a heterodimer of a regulative subunit, p85 and a catalytic subunit, p110. There are three isomers of p110, α, β, and δ, with only the δ subunit expressed in hematopoietic cells. Interestingly, the tyrosine of c-Kit that is important for interacting with the p85 subunit of PI3K is distinct from the tyrosine that is activated upon autophosphorylation [122]. The activated PI3K can, in turn, activate downstream signaling factors such as p70 ribosomal S6 kinase (p70S6K), protein kinase B (PKB), and NF- κB [123]. When a mouse mutant of the key tyrosine is constructed, which effectively blocks PI3K activation by SCF, all male mice are infertile due to sperm cell disruption suggesting that the SCF/c-Kit signaling transduction pathway has a significant effect on spermatogenesis during development [124].

Negative Regulators of SCF Signaling

There are a number of negative regulators of SCF signaling. Among these are SHP1 and SHP2. SHP1 is a tyrosine phosphatase that is widely expressed in hematopoietic cells and causes the negative regulation of a variety of growth factors and cytokines that induce mitotic signaling. SHP-1 causes an intracellular dephosphorylation after the initial activation induced by the SCF/c-Kit interaction [125].

Leukemia Inhibitory Factor (LIF)

Leukemia inhibitory factor (LIF) is a member of the superfamily of interleukin 6 (IL-6)-type cytokines. All IL-6 type cytokines stimulate target cells through the shared membrane receptor protein gp130, which acts as a heterodimer in conjunction with a ligand-specific LIF receptor (LIFR) [126]. Activation of gp130 leads to the activation of JAK-1, JAK-2, and TYK-2 which subsequently activate specific STAT proteins.

LIF is critical for the self-renewal of mouse ES cells under feeder-free conditions. It has been known for some years that murine ES cells can be maintained in a completely undifferentiated state when they are treated with LIF [127].

It has also been shown that LIF can prevent the differentiation of MESCs and that this response is dependent upon the activation of STAT3. Interestingly, STAT3 activation through other means can also propagate the cells in the absence of LIF. LIF has further been shown to induce additional signals including the activation of ERKs (extracellular receptor kinases), which promote differentiation. Thus, the balance between the activation of STAT3 and the activation of ERK signals determines the fate of mouse ES cells [128].

Members of the LIF pathway show divergent expression profiles across species. It is observed that LIF signaling is critical for murine ES cells but has no function in human ES cells [129, 130]. This difference may exist partly due to the altered regulation of LIFR transcription. The promoter structure of the gene encoding the receptor is not conserved between human and mice [131].

The Wnt Pathway

In the past 20 years since its initial discovery [132], more than 2000 papers have been published about the Wnt signaling pathway due to the fact that it plays a central role in both disease and development. The majority of these reports concern the canonical Wnt pathway, which results in the stabilization of the transcriptional co-activator, β-catenin. Non-canonical Wnt pathways that can also affect cell development and differentiation involve the planar cell polarity (PCP) pathway and the Ca^{2+} pathway which are β-catenin independent.

The canonical Wnt signaling pathway has emerged as a critical regulator of stem cells [133]. Wnt ligands interact with the cognate receptor complex, which is made up of the Frizzled receptor and the LDL receptor family member, Lrp5/6. β-catenin is a cytoplasmic protein, whose stability is regulated by a destruction complex consisting of axin, APC and GSK-β. In the absence of the Wnt signal, β-catenin is bound to the complex, which induces its phosphorylation and subsequent targeting for degradation. Upon Wnt activation, β-catenin translocates to the nucleus, where it binds the Lef/Tcf transcription factors and activates specific target genes [134].

In the non-canonical Wnt/Ca^{2+} signaling pathway [135, 136], Frizzled acts through heterotrimeric guanine nucleotide-binding proteins (G proteins) [137] to activate phosphodiesterase (PDE) [138], leading to increased concentrations of free intracellular calcium which decrease intracellular cGMP.

Notch Signaling Pathway

Notch controls a highly conserved pathway implicated in cell-cell communication, and has been shown to be involved in the regulation of cellular differentiation, proliferation and specification [139]. The Notch pathway is highly expressed in the precursor cells of the developing central nervous system [140].

Notch encodes transmembrane receptors that are cleaved upon ligand binding to release an intracellular domain (Nicd) that translocates to the nucleus and is directly involved in transcription. Four Notch receptors have been identified in mammals, Notch1-4, and five structurally related Notch ligands, Delta-like1, Delta-like3, Delta-like4, Jagged1 and Jagged2, have been found [141]. They undergo extracellular cleavage of the precursor protein to form heterodimers, which consist of a totally extracellular subunit and a transmembrane one, on the plasma membrane [142].

Studies using gene-modified animals have demonstrated the essential role of Notch in stem cell signaling. For example Notch1 [143] or Notch2 homozygous knock-out mice exhibit lethality near embryonic day 11. In Notch1-null mutant mouse embryos hematogenic endothelial cells are formed. They do not develop into HSCs, however, indicating that Notch1 has an indispensable role at the point of development right before HSCs are generated [144, 145]. Notch3-null and Notch4-null mice survive, but with abnormal vascular development [146-148]. And in the central nervous system during embryogenesis, Notch signaling has been shown to promote glial cell fate [149, 150].

COMBINATORIAL SCIENCE APPLIED TO STEM CELL MANIPULATION

In countless studies, it has been shown that high throughput screening and combinatorial methods provide powerful techniques to discover novel ways to manipulate cellular signal transduction pathways. A developing trend is to apply these techniques to more complex cellular outcomes. Recently, in a growing number of, but not yet widespread, studies combinatorial science has been applied to the manipulation of stem cells. Traditional small soluble ligand-based phenotypic screens have been performed, and new techniques using arrayed, immobilized molecules have been recently developed.

Small Molecule Screening Techniques Applied to Stem Cells

One of the first applications of combinatorial and high throughput screening techniques to the stem cell field was performed by Schultz and co-workers to discover small molecules that can control differentiation [151]. Certain skeletal muscle precursor cells, or myoblasts, differentiate by fusing into multinucleated assemblies called myotubes. The dividing and mononuclear myoblasts go into cell cycle arrest once differentiated. Some organisms have the ability to regenerate limbs. Commonly, as part of this process, the myotubes in the limb stump disassemble and dedifferentiate into individually dividing myoblasts, a defined precursor cell type that can perform undifferentiated self-renewal. Being able to induce similar dedifferentiation could aid in the development of regeneration therapies. With the goal of finding compounds with this ability, a library of several hundred 2,6,9-trisubstututed purines [152] was screened against myotubes that were formed from the mouse myoblast cell line, C2C12. Upon treatment with the library and manual morphological scoring, one molecule, myoseverin, appeared phenotypically to reverse differentiation. This hit molecule presents an isopropyl and two methoxyphenyl substitutents at the 2-, 6-, and 9-positions on the purine scaffold respectively. Application of myoseverin causes the elongated myotubes to reversibly break into spherical, adherent, mononuclear cells which were initially thought to be precursor myoblasts. Through immunostaining and microscopy it was determined that the compound's activity is a result of simple cytoskeletal disruption and therefore the perceived morphological change is due to the simple disruption of adherence between the cells in the myotube. A derivative with a biotin affinity tag at the 9-position allowed a pull-down-type experiment that demonstrated, *via* Western blot, that tubulin is a binding partner of myoseverin. Tubulin polymerization *in vitro* experiments supported this mechanism and provided a readout for SAR studies. Viability assays demonstrated that myoseverin is less toxic than other drugs that interact with microtubules. It was also determined, that the compound assists in reentry into the cell cycle and cell proliferation which follows a mechanism of tubulin disruption. Supporting this mode of action, an mRNA transcript profile indicated that it does not cause the upregulation of many genes associated with the cell cycle, or muscle-specific genes, but that expression of genes associated with extra-cellular matrix remodeling and growth factor signaling are induced which is inconsistent with the formation of myoblast precursor cells. This result suggests that myoseverin does not actually induce dedifferentiation *per se,* but that it more simply allows the mechanical clipping apart of myotubes into individual cells that are still at the myotube differentiation stage. These individualized cells can form myotubes again after the compound is removed. However, due to the fact that myoseverin causes muscle cells to proliferate and reenter the cell cycle it may still be useful in muscle regeneration applications.

Because it was determined that myoseverin was not dedifferentiating myotube cells to upstream myoblast precursor cells, a primary screen that was not dependent on myotube disruption was established [153]. As opposed to simply screening for phenotypes that match a dedifferentiated cell type, a screen was set up for multipotency by identifying alternative redifferentiated cell types that should arise if dedifferentiation had taken place. The logic of this is that if a differentiated cell type is identified that differs from the original, then the cells must have dedifferentiated and passed through a multipotent progenitor cell stage. In this study, a library of about 50,000 members was screened for the ability to transdifferentiate C2C12 myoblasts into osteoblasts (bone-forming cells) presumably through a multipotent mesenchymal progenitor cell type. The cells were incubated with the molecules as discrete preparations and then subjected to established osteogenic culturing conditions. The bone-specific marker alkaline phosphatase was used to identify the osteoblast lineage. Through this screen reversine, a disubstituted purine that projects both a benzylmorpholine and a cyclohexane from the 2- and 6-positions respectively, was obtained. This molecule caused a significant decrease in muscle cell markers during differentiation. Control experiments confirmed that no significant cell death was detected and that no transdifferentiation was observed. A secondary screen involved the exposure of the dedifferentiated progenitors to media known to cause adipogenic differentiation and assayed for characteristic adipocyte phenotype. Cells induced to dedifferentiate by reversine treatment were able to differentiate into adipocytes under these conditions. Together these data confirm that reversine acts as a true dedifferentiation agent.

Recently, it has been reported that reversine has been applied to studies involving primary dermal cells [154]. The authors of the report note that the C2C12 cell line is an immortalized cell line and possesses chromosomal abnormalities. They imply, therefore, that results based on C2C12 cells may be artifactual or not be applicable to clinical usage. Treatment of skin fibroblasts isolated from transgenic mice expressing green fluorescent protein (GFP) with reversine resulted in cells with altered morphology that expressed little to no HSP47, a fibroblast marker. Exposure of these cells to established conditions shown to produce either osteocytes or smooth muscle cells produced cells that expressed alkaline phosphatase or smooth muscle actin indicative of differentiation to the proper cell type. Myogenesis proved to be more difficult. Simple exposure of the reversine-exposed cells to skeletal muscle differentiation conditions was unsuccessful in producing muscle cells. However, co-culturing of these cells with C2C12 cells resulted in expression of myocyte markers (MHC, MyoD) in the reversine-treated cells and incorporation of GFP-expressing cells into myotubes. These results suggest that communication between the C2C12 myoblasts and the reversine-treated cells was occurring and that this communication is necessary for myogenesis. Similar differentiation studies with human dermal cells gave results similar to those obtained with the mouse-derived cells. Impressively, injection of reversine-dedifferentiated cells into damaged muscle resulted in uptake of the cells into the tissue (as indicated by GFP incorporation), suggesting that this method may be part of a future application in muscle regeneration.

To begin to establish the mechanism of reversine-induced, dermal cell transdifferentiation and the nature of the dedifferentiated cells, characterization of the reversine-treated cells was attempted. The cells were probed by flow cytometric analysis using hemopoietic (CD34, CD45) and mesenchymal stem cell markers (CD73, CD105). Neither set of these markers was detected. Interestingly, overall proliferation and cyclin B transcription was reduced in these cells. Furthermore, the reversine-treated cells exhibited a binuclear, tetraploid phenotype which was reversed upon differentiation. It was noted that polyploidy of some cell types is sometimes observed as part of normal development. These results, while not conclusive, largely suggest that the reversine-treated cells do not dedifferentiate fully to a stem cell, and that the formation of some sort of multinuclear progenitor could play a role. While there is still much work to be done to obtain a clear picture, this study suggests an unexpected and alternative mechanism for dedifferentiation.

A very recent paper [155] sheds further light on the mechanism of reversine activity. Again using C2C12 cells, media components were removed to isolate individual signal transduction pathways. Removal of insulin resulted in the nullification of reversine activity. Because insulin acts through the PI3K pathway, it was hypothesized that this pathway is necessary for reversine-mediated dedifferentiation. Treatment with a PI3K inhibitor confirmed this hypothesis by desensitizing cells to reversine. Affinity pull-down experiments using reversine immobilized to an insoluble matrix through the 6-position found three binding targets. FAK (focal adhesion kinase), MEK1 (mitogen activated extracellular signal regulated kinase), and NMMII (nonmuscle myosin II heavy chain) were identified by Western blot and shown to be direct binding partners. Upon overexpression, only the latter two reversed reversine activity. Biochemical enzyme inhibition assays demonstrated that reversine had activity against both enzymes. An siRNA knockdown of NMMII in conjunction with chemical inhibition of MEK resulted in cells with similar transdifferentiation abilities to reversine. However, blocking the activities of these enzymes alone did not reproduce the phenotype suggesting that they need to be knocked down together. NMMII is a cytoskeletal protein that plays a role in the transmission of force from the extracellular matrix, through focal adhesions, and into the cell. This process has previously been demonstrated to influence cell fate and differentiation outcomes [156, 157]. Additionally, NMMII is involved in cell cycle regulation and inhibitors of this protein block the G2/M transition. Supporting a mechanism involving a reversine interaction with NMMII, treatment with the compound causes accumulation of cells at the G2/M checkpoint. The other pulled-down protein, MEK1 is part of the evolutionally conserved MEK/ERK pathway which has been implicated in many cellular processes including those involved in differentiation and stem cell proliferation. One of its specific roles is in the regulation of histone acetylation by phosphorylating histone acyltransferase. Supporting a mechanism where reversine also interacts with MEK1, treatment with the compound causes a decrease in histone H3 acylation. In addition, inhibitors of histone deacetylase block reversine activity. Taken together these data suggest that reversine's activity is due to direct binding and inhibition of both NMMII and MEK in concert.

A model system for osteoblast differentiation is the embryonic fibroblast cell line, C3H10T1/2. These cells are mesodermal precursors and have been shown to differentiate into mineralizing osteoblasts much like mesenchymal stem cells. Upon induction with the growth factor BMP-4, the resulting osteoblasts express alkaline phosphatase which catalyzes the hydrolysis of pyrophosphates to phosphates and therefore plays an essential role in bone mineralization. The production of alkaline phosphatase activity can act as an output in screens for osteoblast differentiation due to the availability of colorigenic substrates. An alkaline phosphatase assay was used to screen a library of more than 45,000 members for the induction of differentiation of C3H10T1/2 cells into osteoblasts. This library was based on a diversity of heterocyclic scaffolds including purines, pyrimidines, quinazolines, pyrazines, phthalazines, pyridazines, and quinoxalines [158]. Of this large library, only one compound was discovered to induce differentiation [159]. This compound, a 2,6,9-trisubstituted purine, which presents cyclohexane, naphthaline, and morpholine substitutents around the heterocyclic scaffold, was dubbed purmorphamine. Purmorphamine was demonstrated to induce the production of alkaline phosphatase in, and the implied differentiation of, the cells in a dose- and time-dependent manner. This activity of the molecule operates synergistically with BMP-4 suggesting that purmorphamine does not act as a functional mimic of BMP-4. Supporting the assumption that purmorphamine induces differentiation of the cells, the cells treated with the molecule change from the spindle shape of a typical fibroblast to a more rounded morphology resembling osteoblasts. A secondary screen involved the construction of a reporter cell line which links luciferase activity to the expression of Cbfa1/Runx2 which is a transcription factor specific to bone

formation and has been shown to be essential for skeletal mineralization and osteogenesis [160, 161]. Purmorphamine induced the expression of Cbfa1/Runx2 in this cell line further supporting the previous results. Interestingly, purmorphamine was able to transdifferentiate subtly an adipose precursor cell line to an osteoblast-like cell lineage. Also, transdifferentiation was observed for a skeletal muscle progenitor cell line but, in this case, although Cbfa1/Runx2 was expressed, some morphological features of muscle cells remained, either confirming that Cbfa1/Runx2 is not sufficient for the transdifferentiation of these cells, or that purmorphamine cannot differentiate these cells completely to an osteoblast lineage [162].

Microarray studies have indicated that, unlike signaling induced by BMP-4 which activates the SMAD proteins, purmorphamine acts to agonize the hedgehog pathway. This was confirmed by the use of hedgehog antagonists which obliterate purmorphamine activity [163]. Through genetic-type studies it was determined that purmorphamine operates at the level of the seven transmembrane receptor smoothened (Smo) which activates a series of transcription factors which in turn target key hedgehog genes. Further studies involving displacement of fluorescent antagonists that make direct interactions with Smo demonstrate that purmorphamine agonizes the hedgehog pathway by binding directly to Smo [164]. These results not only establish a mechanism, but also suggest potential targets for future small molecule effector development. Because purmorphamine's Smo agonist activity is unique, it has been used to understand the signaling mechanism of Smo itself. It has recently been shown that Smo has two distinct signaling activities localized in two separate domains of the protein [165].

Small-molecule neurogenesis promoters have also been discovered through the high throughput screening of heterocycle libraries [166]. A primary screen of a large (>10,000 members) library was screened against the pluripotent murine EC cell line, P19, which was engineered to express firefly luceriferase upon activation of a neuron-specific gene, Tα1 tubulin. If a hit molecule is detected that can induce differentiation of the cells to a neuronal lineage, the Tα1 tubulin promoter is activated, and the resulting luciferase expression can be read out by its luminescence. The initial screen was carried out using cellular monolayers ensuring that hits could be established that bypass the need to form EBs to achieve differentiation. Secondary screens used immunochemistry to demonstrate the expression level of βIII-tubulin/TuJ1, another neuronal marker, and microscopy to confirm the development of neuronal morphologies which consist of rounded bodies surrounded by extensive cellular processes. Through these screens, the compound TWS119, which projects phenolic and analenic functionality from the respective 4- and 6-positions of a pyrrolopyrimidine scaffold, was discovered. Most of the cells treated with TWS119 immunostained positively for other neuronal markers such as nestin, neurofilament-M, Map2a, Map2b, NeuN, synapsisn and also the glutamate production. The compound has also been shown to work with mouse ES cells, demonstrating that its activity is not restricted to EC cells. To determine the target of TWS119, the molecule was linked to a solid support through the 6-position of the core heterocycle. Gel electrophoresis and subsequent mass spectroscopic analysis determined that TWS119 binds GSK-3β, a serine/threonine kinase. This interaction was further confirmed by Western blot, and the disassociation constant was determined using a surface plasmon resonance biosensor (~130nM). It has been shown previously that upon Wnt activation, GSK-3β is inhibited from phosphorylating β-catenin. The phosphorylation of β-catenin activates it for ubiquitin-mediated proteolysis. In the absence of GSK-3β-induced degradation, β-catenin migrates to the nucleus to regulate transcription through interaction with TCF/LEF. Using an engineered P19-based cell line transformed with a construct containing multiple TCF/LEF DNA binding sites upstream to a luciferase reporter gene, it was determined that addition of TWS119 can induce β-catenin downstream signaling. These results taken together suggest that the compound's binding to GSK-3β inhibits the protein's direct interaction with β-catenin, thereby turning on downstream signaling events.

This same murine EC cell line from the previous study, P19, was also used in a primary screen to discover small molecules that can differentiate pluripotent cells into cardiomyocytes from a heterocycle library of ~100,000 without the requirement of forming EBs [167]. A luciferase reporter construct was developed using the promoter of atrial natriuretic factor, a downstream gene product of heart muscle cell-specific transcription factors. The resulting hits were subjected to a secondary screen against murine ES cells. The most active hit molecule was cardiogenol C which projects para-methoxyaniline and 2-hydroxylamino groups from the C-2 and C-4 of a pyrimidine scaffold respectively. The compound-differentiated cells exhibited a cardiomyocyte phenotype in that they expressed the markers myosin heavy chain, MEF2, Mkx2.5, GATA-4, and also began to beat. An impressive 50-90% of the cells differentiated, a marked improvement over traditional differentiation techniques where only 5% of the cells formed heart muscle cells.

High throughput screening has also been used to find a compound that induces differentiation to mature neurons from neural progenitor cells [168]. A 50,000 compound heterocycle library was screened against HCN rat hippocampal primary neuronal progenitor cells. In any neuronal differentiation endeavor, a possible pitfall is the differentiation into glial cell types. For example, the small molecule nuclear receptor ligand, retinoic acid, differentiates neuronal progenitor cells into both neurons and cells of a glial lineage. With this in mind, the primary screen used a microscopy/phenotype-based assay using dual immunostaining for β-tubulinIII, a neuronal marker, and glial fibrillary acidic protein, which is an astroglial marker using monolayer screening. After the primary screen it was determined that the resulting hits were all 4-aminothiazoles. A directed library was therefore synthesized and screened based on this core. The most active molecule, neuropathiazol, was then used for long-term culturing studies. Treatment with the compound resulted in the downregulation of the neural progenitor marker, Sox2, and upregulation of the neuronal markers NeuroD1, neurofilament H, and Map2, indicating that the cells differentiated to mature neurons. Further work demonstrated that cells cultured in LIF, BMP, and serum, which are astroglial selective differentiation conditions, could be inhibited from differentiation through treatment with neuropathiazol. Retinoic acid did not share this activity. These data demonstrate that neuropathiazol is a more selective differentiation agent than retinoic acid and that it pushes

cells to a more mature and defined state. Moreover, the mechanism of neuropathiazol may be inhibition of glial formation rather than direction toward a neuronal lineage. This suggests a possible activity to be targeted in the development of future compounds.

High throughput screening has also been used to find small molecules that promote self-renewal of ES cells lines [169]. A heterocycle library made up of 50,000 members was screened against a mouse ES cell line engineered with an Oct4-GFP reporter. These cells were derived from the OG2 murine ES cell line which does not self-renew in feeder-free conditions. However, the screen was conducted in the absence of feeder cells to ensure that hit molecules could induce feeder-free self-renewal. A 3,4-dihydropyrimido[4,5-d]pyrimidine named SC1, was found to have the greatest activity. Cells treated with SC1 proliferated and stained positive for SSEA-1, Oct4, and alkaline phosphatase, markers for pluripotency even after repeated passaging. They possess typical ES cell morphology and were shown, by RT PCR, to express Oct4, Nanog, and Sox2. The SC1-treated cells could form EBs and could be differentiated into neuronal, cardiac muscle, and endodermal lineages as evidenced by expression of the appropriate markers (βII tubulin/NeuroD1, myosin heavy chain/NKX2.5, and Sox17/FOXA2 respectively). The fact that these three cell types belong to the three developmental germ layers suggests that the SC1-treated cells retain pluripotency. As additional confirmation, the cells were used to generate chimeric mice. In an effort to trace down the mechanism of SC1's activity, the usual pathways of controlling self-renewal were investigated. LIF activates STAT3 phosphorylation at Tyr705, however Western blot indicates that SC1 does not. In addition, knocking down STAT3 or the treatment with the JAK2 inhibitor, AG490, also has no effect on SC1 activity. Together these suggest that SC1 acts in a manner independent of the JAK/STAT pathway. Id1 expression is linked to the BMP pathway. Investigations using RT PCR indicate that SC1 treatment has no effect on Id1 expression, suggesting that SC1 activity is also independent of the BMP pathway. Moreover, SC1 has no effect on the expression of GSK-3β, implying that it does not interact with the Wnt pathway. Further expression studies shed some light on the mechanism by indicating that SC1 activates Nanog expression by inhibiting the phosphorylation of p53. To find the direct binding target, the pyrazole N1 of the compound was linked to an affinity matrix which was able to pull out two proteins from cell lysates. These two proteins were identified as ERK1 and RasGAP (Ras GTP binding protein) by Western blot. These interactions were confirmed to be significant by the demonstration that SC1 can inhibit LIF-stimulated ERK phosphorylation and increases the expression levels of Ras most likely through the inhibition of RasGAP. Overexpression of ERK 1 or RasGAP results in differentiation which can be partially rescued by SC1. Knockdowns of RasGAP with RNAi in conjunction with treatment with an inhibitor of ERK1 phosphorylation results in cells with a similar phenotype to SC1-treated cells, further confirming this dual target mechanism. ERK has been implicated in various differentiation outcomes and Ras has been shown to be involved in both differentiation and proliferation. Self-renewal can be considered to be the combination of increased proliferation and inhibition of both differentiation and cell death. The fact that a molecule with self-renewal activity hits both these targets emphasizes the possible need for a dual phenotype for manipulation of complex cell functions. These results not only suggest a novel and possibly useful mechanism for the development of future stem cell self-renewal activators, but they also imply that it might be wise to screen for multiple, simultaneous molecular targets, or to perform phenotypic screens with cocktails of single-activity molecules.

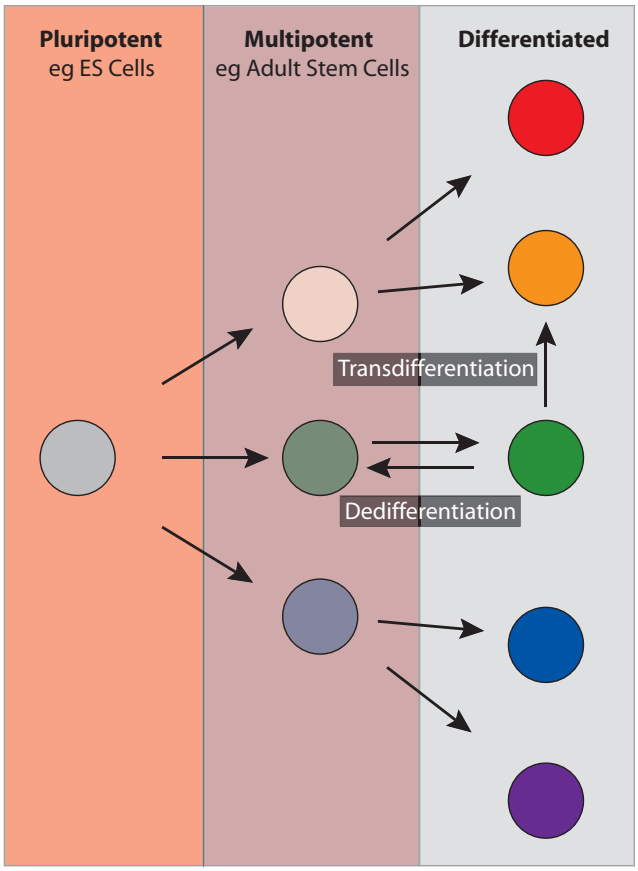

Fig. (1). The loss of differentiation power as the lineage of cells becomes defined.

A very recent report demonstrates how high throughput screens of small molecules can be used to find differentiation agents of skin cells [170]. Keratinocyte cells are progenitor cells that make up the epidermal skin layer. Primary normal human epidermal keratinocytes were transfected with a lucerifase reporter of infolucrin, a keratinocyte differentiation marker. A library of 13,000 compounds composed of known drugs, natural products, and heterocycles with known and suspected activity against kinases, was screened against this cell line as part of a primary screen for differentiation. A pyrrolopyrimidine was found that causes the cells to change morphology to resemble flattened, differentiated skin cells. This compound elicited production of the early differentiation markers keratin 1 and 10 in the first 48 hours, and the markers for late-stage differentiation, IVL, TGM, loricrin, and flilligrin within 96 hours. A global transcriptional analysis indicated that treatment with this compound alters the p38 MAPK signaling pathway. Additionally, integrin signaling is downregulated. The molecule was immobilized to an affinity matrix, and TANK-binding kinase 1, NIM-related kinase9, casein kinase2 α-subunit, and casein kinase2 α'-

subunit were pulled out of cell lysates. The compound inhibits the casein kinase's ability to bind ATP, however they have not been previously demonstrated to play a role in keratinocyte differentiation. Moreover, RNAi knock-down experiments of these kinases only affect expression of some of the keratinocyte markers and therefore don't completely reproduce the phenotype induced by treatment with the small molecule. This suggests that if interaction with these proteins is significant it is not the sole mechanism of activity. While this is a first step toward the establishment of a small molecule that can differentiate keratinocytes, and suggests a possible novel target, much work needs to be done until a complete mechanistic picture can be established.

Inspection of the set of soluble small molecules discovered by high throughput screening to influence differentiation outcomes of stem cells (Fig. **2**) demonstrates a commonality of structure in that all the hit compounds are based on nitrogen-containing heterocycles. Although, these are undoubtedly privileged structures, only a relatively small corner of structure space has been yet explored as few research groups have undertaken screens of this nature. It is highly likely that other structural classes of compounds remain to be discovered, and screening small molecule libraries based on other structural classes for complex cellular activities should prove to be a fertile endeavor.

Array-Based Screening Techniques Applied to Stem Cells

While screening soluble, small-molecule libraries has been shown to be effective in discovering agents for progenitor cell self-renewal and differentiation, an alternative strategy has been explored within a parallel chronology. Based on the knowledge that cell signaling is the result of a combination of pathways resulting from cellular interactions with soluble factors, the extracellular matrix, and other cells, array-based strategies have been developed to explore how various immobilized materials including small peptides, proteins, and polymers presented on a surface can induce cellular differentiation or self-renewal. Although much work in the materials field has been applied to stem cells [171, 172], high throughput approaches remain rare. Additionally, high throughput small molecule screening research, which relies on previously established technology, is much more mature than its array-based counterpart. Surface-based arraying of materials for controlling stem cell fate, on the other hand, has required initial technological development and proof of principle studies. However, one of the potential advantages of arraying molecules is that it can increase the throughput of a screen. Arraying technology can provide features down to the cell colony size limit. The practical throughput of a soluble small-molecule screen is limited by the size of a standard 384-well plate whereas arraying technology can realistically

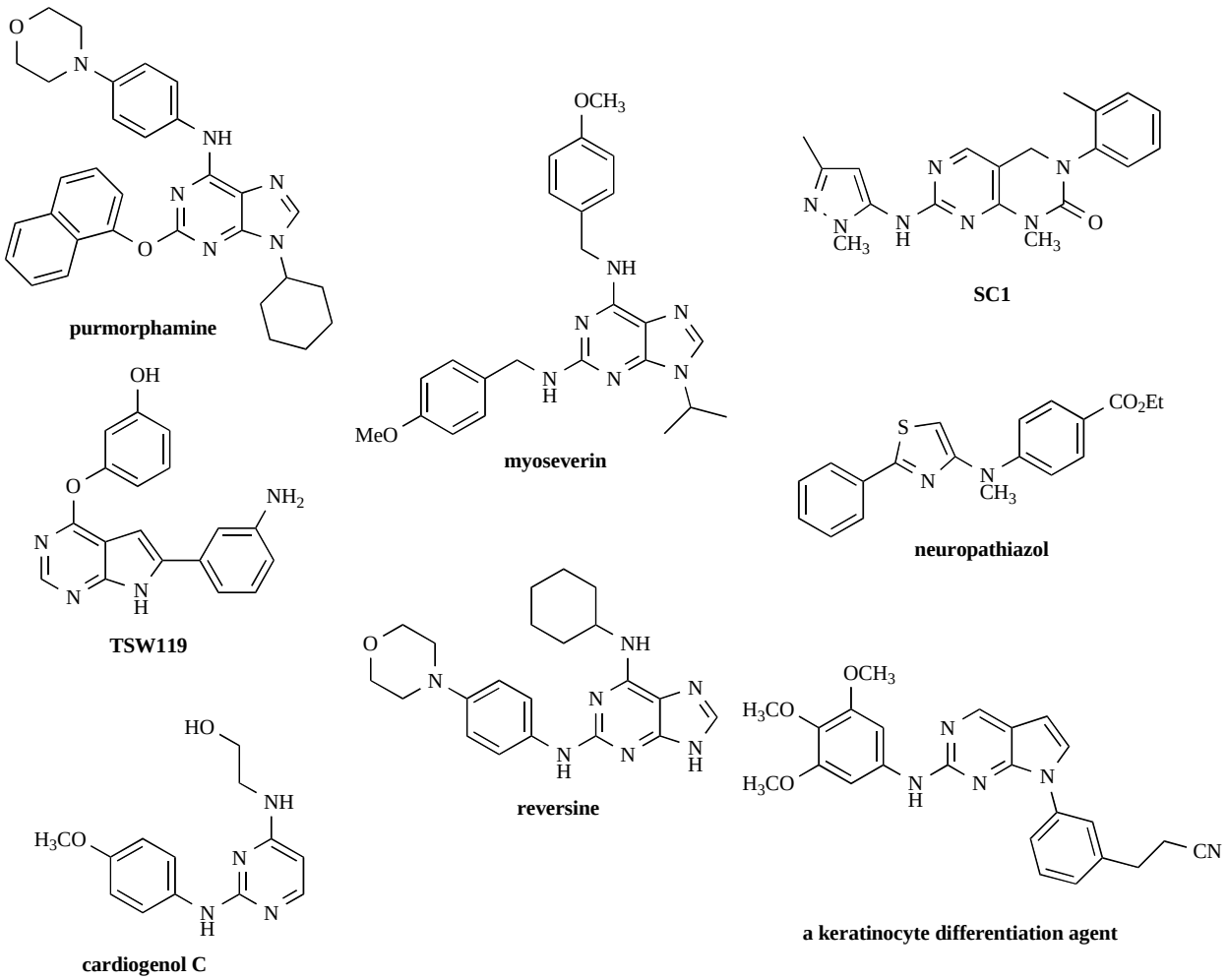

Fig. (2). Hits from small molecule screens for activities involving stem cell function.

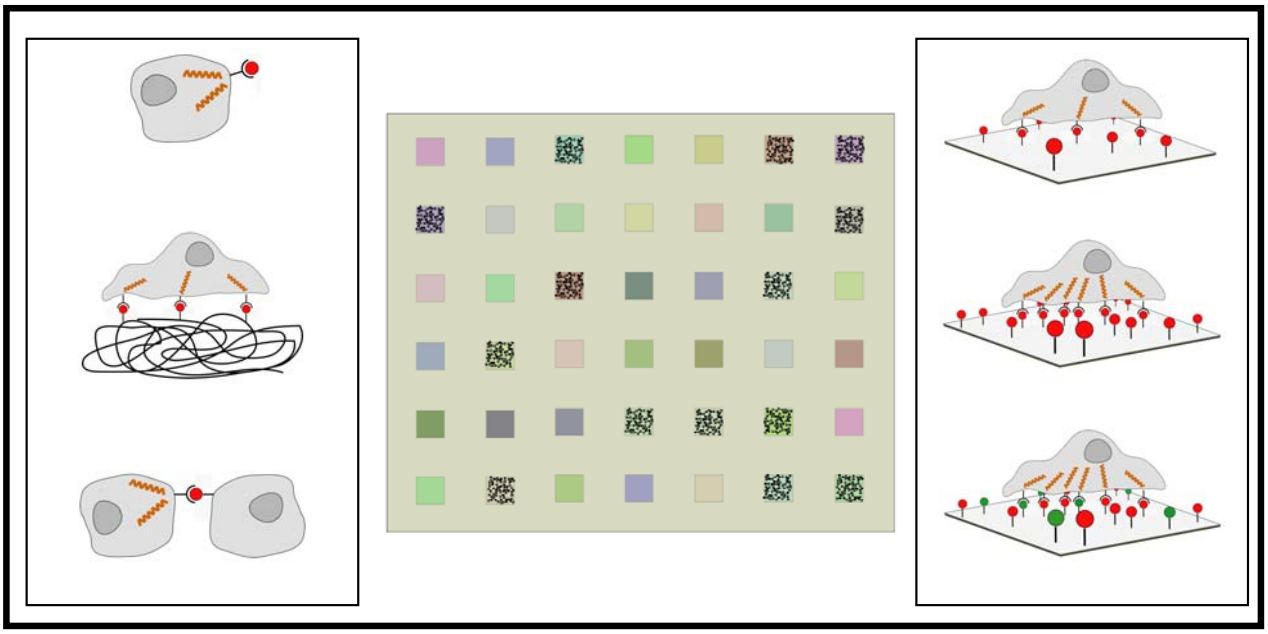

Fig. (3). Cells receive signals from the niche in which they reside by binding to soluble factors, the extracellular matrix, and other cells (left). Array-based strategies to screen libraries of natural and synthetic ligands that induce these signals (center) could discover new preparations for the manipulation of stem cells. Controlled presentation of these ligands could also allow the screening for density and synergistic effects (right).

screen 1,500 samples on a standard microscope slide, thereby increasing throughput while decreasing the amount of compound required to conduct the screen. Moreover, small molecule and array-based formats could be combined into one technology. Screening small molecule libraries simultaneously with an array-based, surface-presented library might prove more productive even than a very diverse library. It is conceivable that a "library *vs* library" strategy might help discover pairs of molecules whose activities act in concert to manipulate complex cellular phenotypes (*vide supra*).

In one of the first array-based screens for stem cells, a library of close to 600 different polymers was arrayed on a surface to determine how they affect stem cell growth, attachment, and differentiation [173]. These polyacrylate polymers were combinations of 25 different acrylate monomers and were polymerized on the chip itself. Although only 600 polymers were screened, the authors of the study note that up to 1,700 could conceivably be screened using a standard 25mm x 75mm microscope slide format. About twenty of these chips can be fabricated in one day. For cell arraying techniques, the area in between the array features must prevent cell attachment, otherwise the spatial information, and hence the utility, of arraying different materials on different places of the chip would be lost. Polyether and hydroxide-presenting materials have been widely used to prevent cell attachment. In this study, poly(hydroxyehtyl methacrylate) was employed as a background for the polymer arrays. To perform this screen, the human embryonic stem cell line, H9, was grown under standard EB forming conditions for six days, after which the cells were plated on the array and treated with retinoic acid for another six days. The cells were stained for cytokeratin 7, a filament protein found in epithelium and vimentin, itself a filament protein expressed in mesenchymal cells. Many of the polymers bound the cells, and permitted cell division to various degrees. A majority of the cells grown on the polymers differentiated into epithelial cells. Further studies with C2C12 myoblasts showed differences between attachment profiles for the two cell types. In a follow-up paper, polymers were screened against additional cell types [174]. In this case, ~1000 mixtures, or blends, of 24 different polyesters, which were synthesized through off-chip polymerization, were screened for attachment of human mesenchymal stem cells, differentiation of neural stem cells, and dedifferentiation of bovine chondrocytes. The majority of the polymers permitted the attachment of mesenchymal stem cells, dedifferentiation of the chondrocytes as exhibited by positive collagen I staining, and differentiation of the neuronal stem cells to astrocytes as exhibited by staining for glial fibrillary acidic protein. Due to the less defined nature of these polymers and polymer blends, the exact biological mechanism of these materials is unknown, and much work is left to be done to unravel the complete story or to establish the utility of these studies.

In an approach based more on established cell biology experience, 32 combinations of five extracellular matrix proteins were screened for their ability to control the fate of cells related to liver function [175]. The proteins collagen I, collagen III, collagen IV, laminin, and fibronectin were arrayed, using standard DNA-chip spotting techniques, on a polyacrylamide hydrogel chip in different combinations. The spot size was ~120um which can hold about twenty cells. Initially, mature primary rat hepatocytes were plated on the array and the protein combinations were assessed by their ability to induce liver-specific function as identified by albumin immunostaining. The array was also screened against

murine embryonic stem cells engineered to produce β-galactosidase upon the expression of the liver-specific gene ankrd17, in order to determine the ability of the various ECM mixtures to differentiate the cells to a hepatic lineage. Mixtures containing collagen IV were shown to be important for providing hepatocyte function. A powerful analysis technique was able to determine additional meaning from the data and demonstrated that fibronectin, laminin, and collagen III also play a role. The importance of presenting these proteins as mixtures was exemplified by the fact that collagen I, collagen III, and laminin together provide positive activity but alone exhibit negative effects. Conversely, non-additivity is demonstrated by collagen IV and fibronection which are negative in combination but positive alone. For stem cell differentiation, collagen I, collagen III, laminin, and fibronectin in combination had the highest activity with fibronectin and collagen I playing the largest roles. These results suggest multiple signaling pathways are being affected, again emphasizing the potential wisdom in screening for multiple and complementary targets and activities (*vide supra*).

Recently, small libraries of peptides based on extracellular matrix proteins have been displayed using an array format for the purpose of establishing more chemically defined conditions to proliferate human ES cells [176]. These arrays were formed by assembling monolayers of alkane thiols (ATs) on gold-coated glass chips. These monolayers were used because they provide reproducibly flat and uniform surfaces. The fabrication of these arrays is initiated by the formation of perfluorous self-assembled monolayers (SAMs). This surface acts as the background between the elements of the array. Areas within this background were removed by photolithography, the patterning of which solely defines the shape and size of the individual spots (*vide infra*), and individual ATs were spotted to fill in the photoetched monolayers. These ATs were pre-attached to the peptides in the library and were also mixed with other ATs to explore not only the effect of the array elements themselves but also the effect of mixtures and epitope density. Because the library members were pre-attached to the ATs, orientation effects of the library molecules could also be probed with this technique. The flourous monolayer was used as the background due to its dual cytophobic and solvophobic properties [177]. The cytophobicity prevents cells from binding, and the solvophobicity facilitates the beading of most solvents. The beading of solvents provides flexibility in the type of molecules that can be arrayed and ensures that the size and shape of the array spot can be controlled by the photolithography step and not be influenced by the individual properties of the solvents used or the irregularities of the spotting process. This allows the total control and uniformity of colony shape and size. Moreover, the beading self-corrects the spotting process by aligning the solvent droplet on the area of removed SAM. The arrays employed 500μm x 500μm squares. This array element size was larger than the smallest possible size available through fabrication technology. The spot size lower limit is restricted by the fact that current human embryonic stem cell culturing conditions require colony formation. The authors of this study note that as clonal culturing techniques develop, the possible spot size will decrease and the throughput of this technology will increase. However, the current format still allows the screening of hundreds of compounds in a square inch. Eighteen different peptides based on active elements of large extracellular matrix proteins were screened for attachment and self-renewal of the human embryonic stem cell lines H9 and H1. The peptides were assessed by their ability to induce the production of Oct4 and alkaline phosphatase activity, both markers of pluripotency. The hit peptide KGRGDS was subjected to a secondary screen using flow cytometry and the pluripotency markers Oct4 and SSEA-4. The mechanism of action was explored by the employ of various cancer cell lines which have been previously shown to express different levels of the α_v integrin. The expression level of the integrin correlated to the cell adherence at different densities of the peptide, suggesting that the peptide's activity is due to interaction with this protein. Hits obtained through this technology were shown to be easily expanded from the microscale to the macroscale. Hit peptides were appended to lipids that readily self-assemble into hydrogel-forming organic nanotubes. These lipids were used to coat tissue culture flasks which in turn were used to propagate the stem cells. The success of these hydrogels showed that the successful library members could be transferred rationally from the microarrays directly to a technology that could be adopted with standard cell biological procedures. Presumably a similar strategy could be used discover new conditions for other stem cells and for other differentiation outcomes.

In another recent paper [178], various combinations of growth factors, extracellular matrix proteins, and cell-adherent polymers commonly used in tissue culturing were screened in an array format for their ability to differentiate or promote undifferentiated proliferation. A SAM array composed of carboxylic acid-presenting ATs amongst a background of hydrocarbon ATs was fabricated by first activating the acids as succinate esters. The combinations of extracellular matrix proteins (collagen I, collagen IV, laminin, fibronectin), artificial cell-attachment polymers (polyethylimine and polylysine-two cationic polymers and pronectin-an engineered protein made up of repeats of the cell-binding region of fibronectin and the β-sheet stabilizing portion of silk fibroin), and growth factors (epidermal growth factor (EGF), fibroblast growth factor (FGF-2), nerve growth factor (NGF), neutrophin-3 (NT-3), cilliary neurotrophic factor (CNTF)) were linked to the array through amide bond formation with random orientations. The goal of this study was to discover synergies between these materials. After attachment of the proteins, the background was blocked with albumin to make it cytophobic. Primary rat neuronal stem cells were plated on the array and stained for nestin, a marker for undifferentiated neuronal stem cells. To screen for differentiation, either β-tubulin III, a marker for mature neurons, or GFAP, a marker for astrocyte glial cell lineages, was used. It was determined that neuronal stem cell proliferation was promoted equally well by all adherence polymers and extracellular matrix proteins if they were immobilized with EGF. Differentiation to astroglial lineages was promoted most strongly by either fibronectin or pronectin in combination with CNTF, a growth factor that modulates the STAT pathway by signaling through the LIF receptor and gp130. Furthermore, neuronal differentiation was favored by combinations using fibronectin, pronectin, or polyethyleneimine along with NGF, or NT-3. The authors note that due to thick growth of cells, microscopy-based assay techniques can gen-

erate false negative results, emphasizing that these types of screens should use low cell densities or employ high throughput flow cytometric screens. These results demonstrate that crosstalk between cell attachment- and growth factor-induced pathways play an important role in self-renewal or differentiation. More generally this work again reinforces the potential utility of screening either for synergies due to combinations of active molecules, or for molecules with multiple activities to find effectors of complex cellular processes.

CONCLUSION

The screening of soluble and arrayed compounds can provide tools to help advance stem cell research by establishing new conditions for differentiation and self-renewal or by providing new avenues for expanding the understanding of the fundamentals of mammalian development. Similar studies may also allow for the establishment of niches that the cell would not receive naturally to push them to generate cells that they normally would not [179]. These artificial niches along with recent advances in trans- and dedifferentiation, might allow the application of new techniques to help circumvent the political constraints that still restrict the embryonic stem cell field [180, 181]. This review has attempted to describe recent advances in the application of combinatorial sciences to stem cells while providing an historical context and an explanation of the state of the art of the field itself. It is hoped that researchers can use this review as a resource to predict fertile protein or signal transduction targets, to anticipate amenable cell types and conditions, or to design new scaffolds based on previously determined privileged structures in the development of new high throughput screens.

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