

Using Small Molecules to Great Effect in Stem Cell Differentiation

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Several recent reports, including two *Cell Stem Cell* papers (Zhu et al., 2009 [this issue]; Borowiak et al., 2009), screened small molecule libraries for compounds that promote embryonic stem cell differentiation. Their combined success helps bypass challenges associated with using natural protein factors and has revealed insights into controlling stem cell differentiation.

The ability to differentiate human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) into desired cell types is anticipated to facilitate research and ultimately provide therapies for damaged or diseased human tissues. The most successful protocols for differentiating stem cells rely upon the knowledge of extracellular signals and gene regulatory factors that govern the normal differentiation of cells in embryonic development. Typically, the signals that induce differentiation are mediated by intracellular pathways that involve enzymatic activities, such as for phosphorylation, acetylation, methylation, ubiquitylation, or for the reversal of such activities. These enzymatic functions result in changes in the expression or activity of regulatory (transcription) factors, which, in turn, govern the differentiation state of the cell. Because signal transduction pathways are often activated by extracellular protein ligands (growth factors), the field began by using such protein-based materials to elicit stem cell differentiation. However, as described below, growth factors face significant practical challenges prior to their use in deriving commercialized stem cell-based therapies, though these issues will not be crucial for the initial application of stem cell differentiation for research. Fortunately, given the enzymatic basis of cell differentiation, the use of synthetic small molecules as agonists and antagonists may surmount challenges with protein-based approaches. Two papers in recent issues of *Cell Stem Cell* (Zhu et al., 2009; Borowiak et al., 2009) illustrate high-throughput screening paradigms that discover small molecule alternatives to protein-based approaches to stem cell

differentiation. And interestingly, the small molecule candidates provide new insights into facilitating stem cell differentiation.

First, why bother to develop small molecule alternatives when natural protein effectors, refined by millions of years of evolutionary selection, already perform the job efficiently (see Table 1)? In fact, there are numerous challenges facing protein-based generation of ESC and iPSC derivatives for human therapies. The first is the task of producing high-quality, purified, bioactive growth factors from a natural source, such as via recombinant expression in *E. coli*, following an efficient process that will be consistent and reproducible for many years. Next, consider the logistics and expense required to scale protein production to yield sufficient amounts to generate a billion differentiated cells for a single liver or pancreatic β cell transplant, and multiply that value by the thousands or tens of thousands of doses required to make the process economically feasible for commercial ventures. For example, Activin A is a protein-signaling molecule used for one of the first steps of ESC differentiation to make endoderm, the progenitor of liver and pancreas cells. Recombinant Activin A is already expensive for differentiating millions of ESCs, much less billions or trillions. Furthermore, any commercial profit on stem cell-based therapies employing growth factors will be reduced by royalties due for product application licensing fees. Also, the production of the protein would have to be redesigned under the auspices of a "Good Manufacturing Process" (GMP) to allow the product to be used in humans (Krouac and Zandstra, 2008). Moreover, state of the art pancreatic and liver differ-

entiation protocols typically employ half a dozen different growth factor effectors, each of which faces the same hurdles listed above. The cost issues for differentiation factors are also above and beyond those required to maintain and expand ESC or iPSC populations and to process the differentiated derivatives for human therapeutic delivery (Ährlund-Richter et al., 2009). Clearly, to bring stem cells to market, so to speak, alternatives to protein effectors must be found.

Small chemical compounds, e.g., under 1000 Daltons, can be easier to manufacture than proteins, are often entirely synthetic, are more amenable to scale-up, and are simpler to manage in terms of intellectual property ownership, by virtue of their limited size and complexity of production. Given the remarkable diversity of protein surfaces and enzyme-active sites, in principle, it should be possible to discover small molecules that function specifically as agonists or antagonists of any intracellular signaling pathway. Furthermore, chemical library compositions can be tailored to favor cell-permeable compounds that are known to be relatively stable and not chemically reactive. The tricks are to obtain a chemical library with high structural diversity so that a large range of potential target specificities can be tested and to devise a high-throughput screen that reliably reports a specific developmental step, such as endoderm induction from ESCs. Such were the recent efforts of the Melton and Schreiber (Borowiak et al., 2009) and Schultz and Wu (Zhu et al., 2009) laboratories.

Borowiak et al. (2009) screened a small but diverse library of 4000 compounds for their ability to induce the expression of

Table 1. Differences between Small and Large Molecule Effectors Used for Stem Cell Differentiation

Effector Type	Molecular Size	Origin of Target Specificity	Source	Manufacture	Intellectual Property
Protein growth factor	20–100 kD	Millions of years of evolution	Biological (e.g., recombinant in <i>E. coli</i> , yeast, insect, or mammalian cells)	More possible contaminants and complicated GMP	Multiple licenses and royalties
Small molecule	<1 kD	Structural diversity of compound library	Usually chemical or secreted from microbes	Fewer contaminants and simpler GMP	Fewer licenses and royalties

a fluorescent Sox17-dsRed reporter in mouse ESCs. Sox17 is a marker and an effector of endoderm differentiation (Kanai-Azuma et al., 2002), and Activin A treatment activated the dsRed reporter in modified ESCs, as expected. Positive small molecule hits were defined as compounds that induced Sox17-dsRed fluorescence at three standard deviations above the levels elicited by a DMSO solvent control, independent of Activin A treatment. Secondary screens involved testing hits across different mouse ESC lines and for the capacity to generate homogeneous clusters of Sox-17-dsRed+ epithelial cells that were negative for extraembryonic cell markers (a typical contaminant of endoderm differentiation protocols). Two compounds, IDE1 and IDE2, functioned in the submicromolar range without observed toxicity, elicited nearly the same whole-genome mRNA expression profiles as seen in native endoderm cells from mouse embryos and induced endoderm-like cells from human ESCs.

IDE1 and IDE2 induce Smad2 phosphorylation, as observed in response to Activin A and the related Nodal protein, the latter being a natural inducer of endoderm (Conlon et al., 1994). Indeed, the IDE compounds induce *Nodal* mRNA; thus, it is possible that the IDEs primarily induce Nodal synthesis and secretion, leading indirectly to endoderm induction. Importantly, the endoderm-like cells induced by the IDEs can, when ectopically introduced, integrate in vivo into the endodermal epithelium of mouse embryos. Furthermore, the endoderm-like cells induced by the IDEs could initiate pancreatic differentiation when exposed to Indolactam V, a different small molecule discovered in a screen to promote pancreatic differentiation of human ESCs (Chen et al., 2009). Still, it remains to be determined whether ESC-derived endoderm and pancreatic progenitors induced by these molecules are programmed well enough to continue

to differentiate into functionally useful β cells.

Zhu et al. (2009) set out with a similar goal: to screen 20,000 compounds for their ability to induce mouse ESCs to express endogenous Sox17 and not an extraembryonic marker. However, these authors performed their screen in the presence of low concentrations of Activin A. One compound, stauprimide, promoted efficient induction of Sox17 in both mouse and human ESCs and also helped induce extensive endoderm differentiation markers in the mouse ESC. Early stauprimide treatment also enhanced subsequent differentiation of the resulting cells toward hepatic and pancreatic lineages when the treated progenitors were exposed to additional growth factors. However, further studies showed that stauprimide did not induce endoderm in the absence of Activin A. Rather, the drug enhanced the ability of the ESCs to generally embark on any differentiation program in response to particular cues; e.g., the program could also lower the differentiation threshold for neural or mesodermal lineages.

The plot thickened when stauprimide was found to bind NME2, a *c-Myc*-activating transcription factor that is highly expressed in ESCs. Indeed, the *c-Myc* transcription factor is an integral component of the self-renewal/pluripotency regulatory network of ESCs (Cartwright et al., 2005; Kim et al., 2008). Knockdown of NME2 or stauprimide treatment initially decreased *c-Myc* expression without impairing other pluripotency factors, though the other factors did diminish at later time points. Stauprimide appears to function, at least in part, by impairing the nuclear localization of NME2. Without nuclear NME2, *c-Myc* is downregulated, which may facilitate ESC exit from the pluripotent state and enable differentiation. Yet, the ability of *c-Myc*^{-/-} ESCs to remain pluripotent (Davis et al., 1993) and the capacity of other NME proteins to bind MUC1 and thereby impact ESC self-renewal (Hikita et al., 2008) suggest that

the effect of stauprimide on NME2 may extend beyond *c-Myc*.

Regardless of the precise mechanism, the work of Zhu et al. (2009) yields unanticipated insight into ways to increase the efficiency of stem cell differentiation. Taken together with the discovery of small molecules that specifically promote endoderm and pancreatic differentiation, the ESC and iPSC fields are entering a new phase in which the possibility of using stem cells for human therapies edges closer to feasibility.

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