

# Chemical Genetics: Adding to the Developmental Biology Toolbox

# Review

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Recent advances in cell and molecular biology have generated important tools to probe developmental questions. In addition, new genetic model systems such as *Danio rerio* now make large-scale vertebrate early developmental mutant screens feasible. Nonetheless, some developmental questions remain difficult to study because of the need for finer temporal, spatial, or tuneable control of gene function within a developmental system. New uses for old teratogens as well as novel chemical modulators of development have begun to fill this void.

## Interface of Chemistry and Developmental Biology: Chemical Genetics Concept

The use of small molecules to affect biological phenomena, also known as chemical genetics, has made a significant impact in diverse areas of biology (Crews and Splittgerber, 1999; Koh and Crews, 2002; Schreiber, 1998). The majority of these biologically active compounds are potent, specific inhibitors of known intracellular signaling pathways. However, the identification of small molecules with very specific biological properties, but unknown intracellular targets, can also reveal significant insights into various biological questions. The impact of biologically active small molecules has been greatest in the study of those processes that are lacking model systems easily investigated with genetics. In addition, the ability to control the timing of addition of these compounds to a system has afforded a greater temporal control of gene function than is possible with conventional genetic manipulations. Given that animal development is a sequence of highly regulated events requiring expression of key players at the right place, the right time, and the right levels, the use of small molecule “conditional alleles” has great potential for the modulation of early embryonic processes.

The intersection of biologically active small molecules and developmental biology has had an inauspicious history. The teratogenic potential of specific compounds found in dyes, foods, the environment, and pharmaceuticals was well recognized by the early twentieth century. Although small molecules have long been used to study embryonic development, traditional teratogenic research has generally provided little information about the molecular mechanisms underlying developmental processes. This is due in large part to the fact that, unlike genetic mutations, the molecular targets of these compounds are generally unknown. Here we review the recent identification of specific signaling modulators

and their use as new probes in the study of developmental biological questions (see Table 1). Although a chemical genetic approach offers great potential in facilitating developmental biological studies, the numbers of compounds that have an effect on embryonic development is small. Therefore, we will also discuss recent examples of high-throughput screens to identify new compounds that modulate embryonic development (Figure 2). Finally, on the basis of the current knowledge of cell culture techniques and cell marker expression profiling, it is now possible to probe the fundamental processes underlying cell differentiation and dedifferentiation in tissue culture systems with small molecules (see Table 2). Some of these examples will also be presented.

## Use of Signaling Pathway-Specific Probes to Dissect Developmental Processes

### Cyclopamine

One natural teratogen that has proven useful in the investigation of a key developmental pathway is the alkaloid cyclopamine isolated from the California corn lily (*Veratrum californicum*). Lambs born to ewes that had ingested this wildflower during pregnancy displayed multiple birth defects, most notably holoprosencephaly (HPE), characterized by an absence of midline facial structures and cyclopia (Keeler, 1978). The teratogenic effects of cyclopamine on domestic livestock have been known for decades, but only recently has the molecular mechanism of action of *Veratrum californicum* been reported to act via inhibition of the sonic hedgehog (Shh) pathway. Like cyclopamine-treated embryos, *Shh*<sup>-/-</sup> mouse embryos also display pronounced craniofacial defects (Chiang et al., 1996). This similar HPE phenotype led Beachy and colleagues to explore the potential inhibition of the Shh pathway by cyclopamine (Cooper et al., 1998). Using a chick embryo explant system, Beachy and colleagues demonstrated that cyclopamine blocked the Shh-mediated induction of floor plate cells in responsive tissue (neural plate ectoderm) expressing the Shh receptor Patched (Ptc).

Cyclopamine has been useful in the study of Shh signaling underlying organogenesis. Presumptive pancreatic tissue arising from gastrointestinal endoderm lacks Shh expression, unlike adjacent tissue that will give rise to the stomach and duodenum. This observation and the finding that forced expression of Shh in this tissue blocked normal pancreatic development (Apelqvist et al., 1997) led to the hypothesis that inhibition of Shh is critical for rendering gastrointestinal endoderm competent for pancreas development (Hebrok et al., 1998; Kim and Melton, 1998). Testing this hypothesis, Kim and Melton incubated chicken embryos with cyclopamine in ovo to inhibit Shh signaling. Thirty percent of embryos treated at stage 11 with 0.5–1.0 mg of cyclopamine displayed heterotopic pancreatic cells. Thus, the use of this specific Shh signaling inhibitor confirmed the importance of Shh signaling repression in pancreatic organogenesis.

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Table 1. Signaling Pathway Modulators

Chemical	Activity	Pathway	References
cyclopamine	antagonist (direct ligand of Smoothened)	sonic hedgehog (Shh) signaling	Cooper et al. (1998)
Hg-Ag 1.2	agonist (direct ligand of Smoothened)	sonic hedgehog (Shh) signaling	Frank-Kamenetsky et al. (2002)
disuphiram	inhibitor of the conversion of retinol to RA	retinoid acid (RA) signaling	Gould et al. (1998)
BMS493	antagonist of RA receptors	retinoid acid (RA) signaling	Dupe and Lumsden (2001)
DAPT	inhibitor of $\gamma$ -secretase	Notch signaling	Micchelli et al. (2003)
PTK787/ZK22258	antagonist of VEGF receptors	VEGF signaling	Chan et al. (2002)
SU5402	antagonist of FGF and VEGF receptors	FGF and VEGF signaling	Poss et al. (2000)

Several natural and synthetic compounds with known signal transduction modulatory activities have been used to explore questions in vertebrate embryogenesis.

Since the discovery of its critical role in mediating autopodal anterior-posterior patterning activity of the zone of polarizing activity (ZPA), much attention has focused on modulating Shh activity in various developmental systems. Urodele amphibians are unique among tetrapod vertebrates in their ability to regenerate lost limbs throughout their lives. Axolotls are a good system for studying limb pattern regeneration, but a genetic approach to this question in this system is not tractable. Fortunately, a chemical genetic approach is proving fruitful. Roy and Gardiner recently demonstrated that cyclopamine administration to regenerating axolotl limbs results in loss of digits (Roy and Gardiner, 2002). This chemically induced malformation phenocopies digit loss observed in Shh knockout mice, demonstrating the conservation of Shh signaling during limb regeneration in urodeles, as in primary limb development in other vertebrates.

Both loss-of-function and gain-of-function mutations are important in genetic studies of an individual gene. Likewise, the identification of small molecule antagonists as well as agonists can aid the functional dissection of signaling pathways. Toward this goal, in addition to cyclopamine's use as a Shh antagonist, several Hedgehog signaling agonists have been identified with a cell-based assay. Screening 140,000 synthetic compounds for small molecules capable of upregulating the Gli promoter, Frank-Kamenetsky and coworkers (Frank-Kamenetsky et al., 2002) identified an active chlorobenzothiopeptide-containing compound, which was further refined to yield the SHH agonist Hg-Ag 1.2. Studies showed that Hg-Ag 1.2 treatment could partially rescue the midline defects in *Shh*<sup>-/-</sup> embryos. Since SHH is an

apparent ligand for the membrane protein Patched (Ptc), which, in turn, inhibits another membrane protein, Smoothened (Smo), it was important to determine the level within this signaling pathway where Hg-Ag 1.2 acted. Chemical epistasis studies with cyclopamine indicated that Hg-Ag1.2 acts at the same level as cyclopamine, most likely as a direct ligand of Smo. Direct binding of this class of Shh agonists to the heptahelical bundle of Smo was independently demonstrated with a photoaffinity analog (Chen et al., 2002). Additional Shh antagonists have also been reported (Chen et al., 2002), all of which were shown to bind to Smo directly but affect its function differently. These different chemical alleles of Smo function may provide much needed insights into the mechanisms by which Ptc inhibits Smo, Hh relieves this inhibition, and/or Smo activates downstream signaling events.

#### **Retinoic Acid Receptor Pathway in Hindbrain Patterning**

Possible roles for the physiological morphogen all-trans-retinoic acid (RA) have been proposed for a number of years. While some of the proposed roles for RA have proved controversial, a growing consensus is emerging in recent years for the involvement of RA in hindbrain and spinal cord development. RA joins several other signaling molecules such as Wnt proteins and FGFs in possible involvement of CNS posteriorization. Vertebrate hindbrain anterior-posterior patterning is known to depend upon Hox gene expression in segmental domains. Retinoid deficiency has been known to result in misregulation of Hox gene expression in the caudal hindbrain of quail embryos. In addition, exogenous addition of RA induces ectopic Hox expression, resulting in

Table 2. Small Molecule Modulators of Cellular Differentiation

Chemical	Target	Activity	References
trichostatin A (TSA)	histone deacetylases	inhibits oligodendrocyte differentiation	Marin-Husstege et al. (2002)
5-aza-cytidine (5-azaC)	cytosine-5-DNA methyltransferase	promotes cardiomyocyte differentiation	Rangappa et al. (2003); Xu et al. (2002)
T0070907	peroxisome proliferator-activated receptor $\gamma$	inhibits adipocyte differentiation	Lee et al. (2002)
purmorphamine	unknown	promotes osteoblast differentiation	Wu et al. (2002)
TWS119	glycogen synthase kinase-3 $\beta$	induces neuronal differentiation in embryonic stem cells	Ding et al. (2003)
myoseverin	unknown	promotes dedifferentiation of myotubes into myoblast	Rosania et al. (2000)

Compounds known to influence cell fate determination have proven useful in the exploration of lineage commitment and dedifferentiation.

posterior rhombomere transformation (Grapin-Botton et al., 1998). The molecular basis for this RA-induced regulation was shown to act via the early neural enhancer (Early NE), a *cis*-regulatory element found in several Hox loci (Gould et al., 1998). Furthermore, transgenic studies showed that the Early NE faithfully reproduces the temporal and spatial anterior boundary of HoxB4 expression (Gould et al., 1998). Using a murine explant culture system, Krumlauf and colleagues demonstrated that paraxial mesoderm tissue (i.e., somites) provide a transient signal that induces Hoxb4 expression in the neural plate (Gould et al., 1998). This signal is blocked upon treatment with disuphram, which inhibits the conversion of retinol to RA, thus implicating RA in this signaling. Meanwhile, the somite-generated inductive signal could be mimicked both spatially and temporally by pulsatile addition of RA.

Given the wide differences of hindbrain phenotypes previously observed in various models of retinoic acid deficiency, Dupe and Lumsden investigated the temporal regulation of RA signaling in hindbrain patterning (Dupe and Lumsden, 2001). Using an inhibitor (Bristol-Myers Squibb compound 493) of all three retinoic acid receptors, RA signaling was blocked in a staged series of chick embryos at sequential points during development. The results indicated that RA is needed for the specification of rhombomere boundaries from stage 5 to 10+. Interestingly, this RA dependency is lost in an anterior to posterior progression as development progresses. Moreover, the authors were able to vary the penetrance of this phenotype by varying the concentration of antagonist administered, thereby reproducing the phenotype observed in animals displaying vitamin A deficiency (VAD). The application of various concentrations of antagonist also showed that proper patterning of posterior rhombomere boundaries requires progressively higher concentration of endogenous RA. This result strengthens the hypothesis that a complex retinoid gradient acts to pattern the posterior hindbrain.

#### ***γ*-secretase-Notch Pathway**

Many lines of evidence have suggested that the presenilin (PS)-dependent transmembrane-cleaving activity required for processing and releasing the intracellular domain of Notch receptor is similar to the *γ*-secretase proteolysis of the amyloid- $\beta$  (A $\beta$ ) precursor protein. Therefore, Micchelli and coworkers (Micchelli et al., 2003) recently tested whether A $\beta$ -lowering *γ*-secretase inhibitors designed for the treatment of Alzheimer's disease would phenocopy developmental defects in *Drosophila* Notch mutants. They found that the *γ*-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) can block Notch signaling both in the mammalian cell culture system and in flies. Meanwhile, the progeny of adult flies exposed to DAPT displayed an identical wing-notching phenotype to that of mutant Notch flies as well as other morphological defects consistent with perturbation of Notch signaling. This wing phenotype was also shown to be dependent on the time of compound administration. Notch is known to be required for proper wing development during the third larval instar. Only larvae transiently exposed to DAPT during the fourth day of development developed the Notch wing phenotype. These studies demonstrate that a small molecule manipulation of protein function

can substitute for genetic manipulations and potentially have the advantage of finer temporal control over protein function.

Nevertheless, DAPT was not able to fully elicit the defects observed in Notch genetic mutants. These other phenotypes, which include an increased number of bristles on the notum, defects in leg segmentation, and small eyes, were only seen at a low frequency upon DAPT treatment. In addition, some degree of lethality at high concentrations of DAPT were found, which may be related to the reduced Notch activity and the requirements of Notch for viability. However, other explanations for these findings may include a particular pharmacokinetic property of DAPT in flies, such as the uptake, metabolism, and distribution of the compound in different parts of the organism. The possibility that this compound may be associated with a toxicity not related to Notch signaling also exists. All of these factors are important concerns when a chemical genetic approach is employed. The incomplete inhibition of the function of a protein can be either beneficial or undesired. In the case of a protein that plays an important role in multiple developmental pathways, and, thus, a straight null mutation leads to pleiotropic effects, a chemical genetic approach equivalent to either a temporal or spatial conditional allele will be likely desirable.

The discovery that therapeutics designed to treat Alzheimer's disease can affect the Notch signaling pathway may also have an impact on the future drug design for amyloid-related diseases or some cancers associated with aberrant Notch activity. It is not yet known whether DAPT can inhibit Notch processing in the adult flies. However, it is conceivable that, while chemicals can substitute genetic mutations in developmental biology, the developing model organisms can also help identify potential pharmacological agents.

#### ***Pharmacological Analyses of Zebrafish VEGF and FGF Signaling Pathways***

The use of *Danio rerio* for genetic analysis of embryonic development has several advantages over other genetic model systems. The fact that the early ex utero development is rapid and transparent greatly facilitates analysis of mutant embryonic phenotypes. In addition, *Danio rerio* offers the ability to study complex vertebrate developmental processes such as angiogenesis and organogenesis. Moreover, a reverse genetic manipulation of developmental processes is possible in zebrafish through injection of morpholino antisense oligonucleotides (MO) into the early embryo. However, MO injection results in an early and sustained inhibition of gene function and, thus, is less useful for those studies requiring fine temporal control.

The permeability of zebrafish embryos to small molecules offers the possibility of using a chemical genetic approach to study vertebrate developmental questions. This has best been demonstrated with potent and selective protein kinase inhibitors. For example, addition of a vascular endothelial growth factor receptor (VEGFR) inhibitor (PTK787/ZK222584) to developing zebrafish embryos blocks major blood vessel development, a phenotype also observed upon loss of VEGF ligand function induced by MO injection. Since PTK787/ZK222584 is a reversible competitor, the authors were also able to perform drug washout experiments that permitted a fine

dissection of VEGF's temporal regulation (Chan et al., 2002). Likewise, treatment of fish embryos with a broad-spectrum protein kinase C inhibitor, PKC412, phenocopied the unique body axis curvature observed in the zebrafish atypical PKC $\lambda$  mutant *heart and soul* (*has*) (Horne-Badovinac et al., 2001). In addition, in zebrafish fin regeneration studies, SU5402, a potent inhibitor of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) receptors has been shown to block blastema formation and *msx-1* expression without affecting the wound-healing site (Poss et al., 2000). These results are consistent with the known role of FGFs in limb regeneration and development (Dungan et al., 2002; Han et al., 2001; Niswander et al., 1994). A role for FGF in patterning of the central nervous system was also confirmed with SU5402. Upon exposure of developing zebrafish embryos to this kinase inhibitor, the telencephalon was induced properly; however, the subsequent development of the subpallial telencephalon was inhibited, indicating a need for FGF signaling in establishing telencephalon regionalization (Shinya et al., 2001). Given its specificity and cell permeability, this compound has found additional uses in the investigations of FGFs in various developmental processes, such as otic vesicle development, neural cell fate determination, and retinal ganglion cell development in different systems (Maroon et al., 2002; McCabe et al., 1999; Minokawa et al., 2001; Wilson et al., 2001).

Unfortunately, no model system is perfect. In the case of STI571, a potent inhibitor of both c-Abl and c-Kit kinases fails to phenocopy the *c-kit* genetic mutant, even though the zebrafish c-Kit homolog is quite conserved relative to mammalian c-kit (Chan et al., 2002; Parichy et al., 1999). In addition, we have found that bathing zebrafish embryos with TNP-470, a potent anti-angiogenesis inhibitor that is being used in clinical trials against cancers, results in embryo developmental defects only at a very low frequency. Although the drug binding pocket of MetAP-2 (methionine aminopeptidase-2), the cellular target of TNP-470 (Sin et al., 1997), is well conserved between human and zebrafish and the phenotypes elicited from TNP-470 treatment indeed correspond to MetAP-2 morpholino injection, the reason of such low penetrance from drug treatment is not yet known (J.-R.J.Y. and C.M.C., unpublished data). Therefore, in addition to concerns about lack of protein homology or signaling pathway conservation between different species, the factors such as drug uptake, metabolism, and distribution mentioned above should be considered when contemplating a chemical genetic approach to a developmental question.

#### Chemical Genetic Screens for Small Molecule Developmental Modulators

While the examples above illustrate a reverse genetic approach with small molecule inhibitors of known targets, the zebrafish model also affords the possibility of a forward genetic screen for compounds targeting unknown proteins. Figure 1 is a simplified scheme of a phenotypic chemical screening with zebrafish embryos. Because of the small size of zebrafish embryos, the whole screening procedure can be virtually automated with only small quantities of the chemicals. In addition,

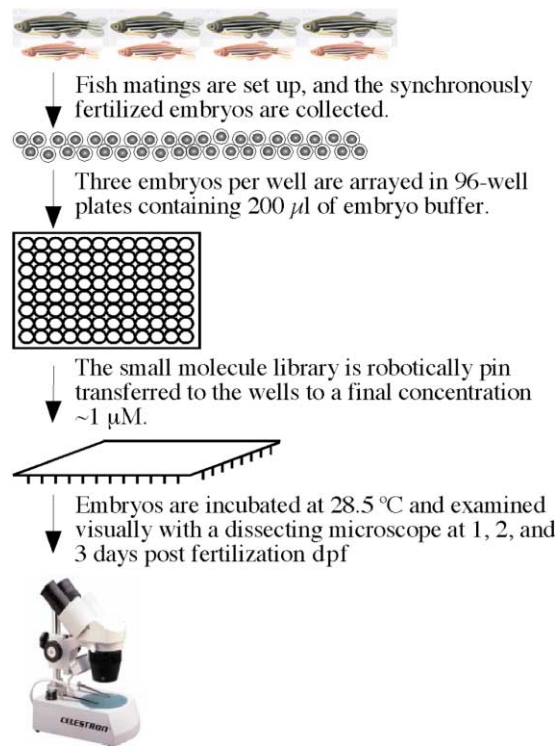


Figure 1. Zebrafish Small Molecule Developmental Screening Procedure (Peterson et al., 2000)

the procedure may be streamlined, theoretically, with more-specific readouts (i.e., with transgenic fish containing a reporter gene).

Screening for small molecule developmental modulators, Schreiber and colleagues identified compounds that modulate one of four systems that differ significantly between vertebrates and invertebrates, i.e., the central nervous system, the cardiovascular system, pigmentation, and the ear (Peterson et al., 2000). For example, one compound, 31J6 (Figure 2), was found to cause 2:1, instead of 1:1, atrium to ventricle contraction ratios. Interestingly, this phenotype is similar to a human cardiovascular condition called second-degree atrioventricular heart block. In addition, compound 31B4 (Figure 2) was found to block the production of pigment, but not the specification or migration of melanocytes. The structural similarity between 31B4 and phenylthiourea (PTU), a tyrosinase inhibitor commonly used in blocking pigment production, suggests that 31B4 is a potent inhibitor of tyrosinase. On the other hand, small molecule 33N14-treated fish appeared to lose their pigmentation because of a differentiation blockage from neural crest cells into melanocytes or because of proliferation inhibition (Figure 2). Further analysis of one compound (31N3) (Figure 2) that specifically affects ear development showed that treated embryos do not develop the small, bony otolith structure that is physically linked to hair cell bundles within the ear, despite the proper development of the otic vesicles. Since these structures respond to gravity and help maintain balance, 31N3-treated fish often swim on their sides or upside down. Again, the advantage of using small molecules as conditional alleles was exploited by adding or washing away 31N3 at

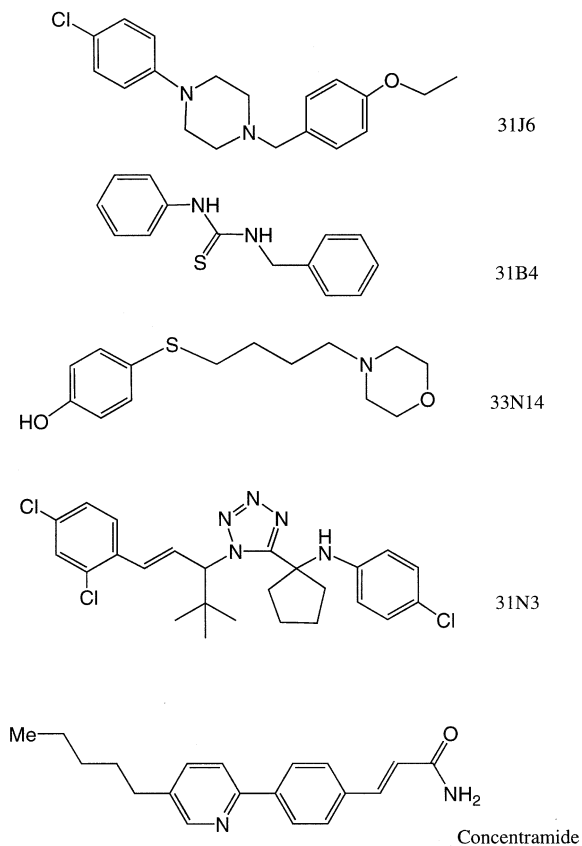


Figure 2. Small Molecules Affecting Zebrafish Development

Compounds identified from a chemical library on the basis of their ability to modulate heart, pigment, or ear development in zebrafish embryos (Peterson et al., 2000).

various time points during development. These studies showed that the period between 14 and 26 hr postfertilization (hpf) is a crucial window for otolith development. These results demonstrate the feasibility of a large-scale chemical genetic screen for small molecule modulators of developmental processes.

Several small molecules identified from this screening appear to phenocopy other genetic mutants. For instance, the heart phenotype caused by 31J6 treatment phenocopies *breakdance* mutants. In addition, the ear defects caused by 31N3 phenocopy *keinstein* and *empty ear* mutants. It is possible that these compounds target proteins involved in the same signaling pathways as perturbed by genetic lesion in these mutant fish strains. Epistasis studies between genetic mutants and chemical-treated mutants can also provide useful information regarding the drug target and the signaling pathways. However, it is also possible that independent pathways are needed for a particular developmental process. Another compound that arose from such a forward chemical approach has provided a new tool in the study of cardiac organogenesis. As mentioned above, the mutant *heart and soul* is a loss-of-function of PKC $\lambda$ . In addition to the pronounced body axis curvature, *has* mutant fish also display heart developmental defects. *Has* fish have disrupted epithelial cell-cell interactions

in many tissues, manifested in the heart by the formation of the ventricle within the atrium (Peterson et al., 2001). Interestingly, Peterson et al. identified the biaryl compound they named *concentramide* (Figure 2), which also induces ventricle formation within the atrium upon nanomolar treatment of zebrafish embryos. Moreover, *concentramide* induces this concentric heart chamber phenotype without widespread disruption of the epithelia, thus strongly suggesting that at least two separate pathways can regulate the polarity of cardiac tube assembly during atrial and ventricular cell fate determination.

The above example illustrates that chemical screens may complement genetic screens. When a genetic mutation results in early lethality or pleiotropic effects or when mutations are masked by redundancy, a chemical genetic approach may provide an alternative solution. Theoretically, compounds that only partially inhibit protein functions can be identified. For example, TNP-470 that targets MetAP-2 as mentioned above only inhibits the proteolytic activity of MetAP-2, but it does not block the role of MetAP-2 in protein translation (Griffith et al., 1997). Meanwhile, chemicals that can block multiple functional isoforms at once, such as various kinase inhibitors, can be informative about the function of a gene family. Although many of these results can be achieved with traditional genetic screens, chemical screens offer several distinct advantages: they are relatively inexpensive, do not require long-term breeding, and offer the possibility of scaling up and automation. The chemical genetic approach can also be used in combination with genetic mutants in genetic enhancer or suppressor screens. For example, the *gridlock* zebrafish mutant develops a blockade in the anterior blood flow that resembles a human congenital cardiovascular malformation. In both cases, coarctation of the aorta occurs, and survival is dependent on development of the collateral vessels and reconstituting the blood flow. A chemical suppressor screen has been used to find small molecules that can revert the defects caused by the mutation in the *gridlock* gene (in *Congenital Heart Defects: Small Molecules Provide Answers* by T.A. Peterson, R.T. Peterson, and M.C. Fishman, presented at the 2003 Annual Meeting of the American Association of Anatomists in San Diego, CA.). Such molecules may not only help elucidate the etiology of this developmental malformation, but may also directly benefit the treatment of human patients.

One major concern of a chemical genetic approach is the target specificity of any small molecule biological modulator. Unlike the identification of a genetic mutation where the completion of various genome projects has greatly accelerated the cloning process, the identification and validation of the target(s) of a small molecule are sometimes more difficult. Occasionally, a candidate gene approach may arise from epistasis studies, transcriptome studies, or studies of the downstream effectors. In addition, target(s) may be identified through rigorous biochemical purification. Before the target(s) are identified and validated, it is imperative that the specificity and the reproducibility of the phenotypes suggest that the compound indeed has specific target(s). For example, the lack of any additional developmental malformation upon treatment of fish with concentrations three orders of magnitude higher than

needed to induce the phenotype demonstrated specificity of concentramide for the cardiac organogenesis. In addition, the effect of structural modifications on the compound's efficacy may be another indication of its specificity. For example, failure of structure-activity relationship (SAR) studies to identify structural elements on the compound that correlate with biological activity might suggest that more nonspecific properties of the compound (i.e., amphipathicity and hydrophobicity) are responsible for the observed phenotype. Most importantly, since developmental screens use embryos to measure the effects of the compounds, reproducibility between individual embryos and even embryos from different strain backgrounds ensures the significance of the drug effects. For instance, all the compounds identified from the screens mentioned above have been tested in three different zebrafish strains, and they induced similar phenotypes in all backgrounds, with the exception of 31N3.

### **Probing the Cellular Basis of Development with Small Molecules**

#### ***Histone Acetylation***

While small molecules have only recently begun to be of use to developmental biologists at the macroscopic level, biologically active compounds have for years provided insights at the cellular level into developmental processes such as cell fate determination. Epigenetic control of chromatin remodeling has been shown in recent years to play an important role in cellular differentiation. For instance, histone acetylation on lysine residues is associated with chromatin regions of transcriptional activity, while deacetylation, through the action of histone deacetylases (HDACs), is associated with transcriptional repression (Kiermaier and Eilers, 1997). Much has been learned about this process through the development and use of specific HDAC inhibitors such as trichostatin A (TSA) and trapoxin (Yoshida et al., 1995). For example, the use of cell cycle inhibitors had shown that inhibition of proliferation was necessary, but not sufficient, for the differentiation of neuronal precursor cells into oligodendrocytes (Tang et al., 1999; Tikoo et al., 1998). Given the significant level of chromatin remodeling that accompanies cellular differentiation, Marin-Husstege and colleagues hypothesized that histone acetylation played a role in oligodendrocyte differentiation (Marin-Husstege et al., 2002). Using synchronized primary neonatal rat cortical progenitors induced to differentiate into oligodendrocytes, the authors showed that there is a temporal window during which histone deacetylation is correlated with the acquisition of a branched morphology and myelin gene expression. The importance of histone deacetylation was demonstrated with the HDAC inhibitor TSA, which blocked oligodendrocyte differentiation. TSA-treated progenitors were able to exit from the cell cycle but did not progress to oligodendrocytes. Interestingly, the authors showed that TSA blocked differentiation only when administered during a specific temporal window. The ability of HDAC inhibitors to inhibit oligodendrocyte differentiation is cell lineage dependent, since TSA did not affect these precursor cells' ability to differentiate into astrocytes. These results suggest that transcriptional repression is a crucial event during oligodendrocyte lineage progression.

#### ***DNA Methylation***

DNA methylation is another example of epigenetic control of gene expression that is believed to play a role in cell lineage commitment during development. Cytosine methylation of CpG dinucleotides is the most common form of DNA methylation in vertebrates. Cytosine-5-DNA methyltransferase (MTase) is responsible for both the establishment and maintenance of this stable, heritable DNA modification, which is believed to increase gene silencing via the recruitment of transcriptional repressors (Newell-Price et al., 2000).

The role of DNA methylation during the cell differentiation process has been widely demonstrated through the use of 5-aza-cytidine (5-azaC), a nucleotide analog that induces DNA hypomethylation. For example, it has been shown that the adult mesenchymal stem cells isolated from fatty tissue can be differentiated into cardiomyocytes through 5-azaC treatment (Rangappa et al., 2003). The transformed cells not only express myosin heavy chain,  $\alpha$ -actinin, and troponin-I, but they also form a ball-like structure and start to contract spontaneously in culture. In another study, Carpenter and colleagues also found that 5-aza-deoxycytidine, but not DMSO or retinoic acid, can enhance the differentiation of cardiomyocytes from human embryonic stem cells (Xu et al., 2002). Therefore, these results strongly suggest that DNA demethylation is involved in the cardiomyocyte cell lineage commitment.

#### ***Transcription Factor Agonists and Antagonists***

While small molecule inhibitors of DNA methylation and histone deacetylation have global transcriptional effects, chemical agonists and antagonists of selected transcription factors offer the ability to influence gene expression with finer resolution. One such transcription factor is the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). A member of the nuclear hormone receptor superfamily of ligand-activated transcription factors, PPAR $\gamma$  has been the focus of pharmaceutical attention, given its role in adipocyte differentiation. PPAR $\gamma$  is the molecular target of the thiazolidinedione class of antidiabetic compounds, including rosiglitazone, troglitazone, and pioglitazone. These PPAR $\gamma$  agonists increase insulin sensitivity and reduce glycemia, lipidemia, and insulinemia in patients with type 2 diabetes (Dubois et al., 2002). Recently, a selective nonthiazolidinedione PPAR $\gamma$  ligand, T0070907, has been described (Lee et al., 2002). This small molecule antagonist was shown to compete for rosiglitazone binding via covalent modification of PPAR $\gamma$ . Moreover, T0070907 blocks the cell differentiation of the adipogenic cell line 3T3-L1 in response to insulin or PPAR $\gamma$  agonists such as rosiglitazone. This increasing palette of PPAR $\gamma$  small molecule modulators provides powerful tools for dissecting the molecular events underlying adipogenesis.

#### ***Chemical Screens for Cellular Differentiation and Dedifferentiation***

In the recent years, cell-based chemical screens for biological modulators have been used in several disciplines. For example, because of the clinical potential of autologous cell replacement, Schultz and colleagues screened for small molecules capable of inducing differentiation of multipotent mesenchymal progenitor cells into osteoblast lineage. From a heterocycle combinatorial chemical library (Ding et al., 2002), they identified a

compound, purmorphamine, that can induce the expression of osteogenesis marker alkaline phosphatase and transcription factor *Cbfa1/Runx2* (Wu et al., 2002). Furthermore, the combination of purmorphamine and BMP-4 can induce the transdifferentiation of preadipocytes and myoblasts to osteoblasts synergistically. Thus, purmorphamine can serve as a chemical probe to study the molecular mechanism underlying bone development. Another screen set out to understand the process of cell fate determination in embryonic stem (ES) cells. In this case, they discovered TWS119, a 4,6-disubstituted pyrrolopyrimidine that could induce neuronal differentiation in pluripotent murine embryonic carcinoma (EC) cells and ES cells. Interestingly, they found that transient, but not prolonged, incubation of this small molecule resulted in a higher percentage of neurons. This finding suggests that early differentiation signals must be shut off during later stages of maturation. Furthermore, TWS119 has been shown to bind glycogen synthase kinase-3 $\beta$  with TWS119-linked agarose affinity matrices. These data, together with other reports, have implicated Wnt signaling in neuronal induction from ES cells (Aubert et al., 2002; Tang et al., 2002).

In addition to probing the molecular basis of differentiation, small molecules are also beginning to play a role in the investigation of cellular dedifferentiation. Screening for compounds that influence dedifferentiation, Schultz and colleagues again identified a small molecule capable of inducing a morphological reversion of differentiated myotubes from a multinucleate to mononucleate myoblast-like morphology (Rosania et al., 2000). Dubbed myoseverin for its myotube fragmentation activity, this 2,6,9-trisubstituted purine was shown to induce microtubule disassembly without affecting other cytoskeletal elements. However, other small molecule microtubule modulators such as Taxol had no morphological reversal activity on myotubes. In vitro assays also demonstrated myoseverin's ability to inhibit microtubule polymerization. Recent studies have shown that myotubes transiently treated with myoseverin and then subsequently incubated with growth factors can proliferate and are capable of undergoing myotube differentiation again (Perez et al., 2002). Interestingly, the authors report that this cycle of myotube differentiation/dedifferentiation could be repeated up to ten times with no observable cellular damage. Using myoseverin to toggle between myoblast differentiation states, the authors examined the expression of 6000 genes in dedifferentiated muscle cells. Not surprisingly, several muscle-specific differentiation markers, such as *Myf5*, *MyoD*, and myosin heavy chain, were downregulated, while proliferation-associated proteins, such as cyclin A and CDK2, were induced in the dedifferentiated state. While muscle dedifferentiation has long been known to be possible in urodele amphibians capable of limb regeneration, the identification of myoseverin demonstrates the capacity of cultured mammalian myotubes to undergo regeneration.

### Concluding Remarks

The advantages of using small molecules as in vivo surrogates for genetic developmental mutations are many. For example, a chemical genetic approach is relatively inexpensive and requires no long-term breeding.

In addition, a small molecule approach offers the ability to control protein function in developmental model systems such as the chick and *Xenopus laevis*, in which genetic approaches are cumbersome. One of the greatest advantages provided by small molecule developmental modulators is the immediate temporal control and precise dosage of protein function. This control allows one to identify the critical timing necessary for gene function and developmental events, often in the presence of multiple functional isoforms of a gene.

Chemical genetic approaches also have limitations. While they are highly compatible with some developmental model systems, they are not as useful in other systems because of the challenges of compound administration (e.g., in utero in the mouse and across the *C. elegans* cuticle). In addition, unlike genetic mutations, it is sometimes a challenge to determine whether a small molecule indeed has a specific target, which renders pleiotropic effects, or whether it simply possesses non-specific toxicity.

In recent years, several advances have allowed for a greater use of small molecules in developmental biology. The number of chemical substances known to have an effect on embryonic development has been small, which has limited the widespread use of this chemical genetic approach. In addition, the lack of cellular target identification of these biologically active compounds has hampered their use and study. While target identification remains a significant obstacle, many efforts have been made, and a systematic approach is starting to form toward this end (King, 1999). Additionally, advances in chemical library synthesis and the availability of commercial small molecule libraries have made high-throughput small molecule developmental screens feasible. Moreover, small molecule probes have been increasingly useful to study cell differentiation and dedifferentiation in cell culture systems. The future of the intersection between chemical biology and developmental biology will undoubtedly lie in additional screens for small molecule developmental modulators as well as the identification of new inhibitors of given signaling pathways. As these compounds are identified and become readily available, the palette of tools available to the developmental biologist for manipulating gene function will certainly grow.

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