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NeuroD: The Predicted and the Surprising

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NeuroD (otherwise known as BETA2) is a basic helix-loop-helix (bHLH) transcription factor that is capable of converting embryonic epidermal cells into fully differentiated neurons in *Xenopus* embryos. In insulinoma cells, NeuroD can bind and activate the insulin promoter. When NeuroD is deleted in mice, the early differentiating pancreatic endocrine cells and a subset of the neurons in the central and peripheral nervous systems die, resulting in cellular deficits in the pancreatic islets, cerebellum, hippocampus and inner ear sensory ganglia. As a consequence, mice become diabetic and display neurological defects including ataxia and deafness. These gain-of-function and loss-of-function phenotypes suggest that NeuroD controls both common and distinct sets of molecules involved in cell survival and differentiation in different tissue types. In this review, we examine what is known about NeuroD and what remains to be answered. Understanding the primary function of NeuroD will be extremely valuable in the diagnosis and cure of the diseases that involve this transcription factor, which plays essential roles in the development and function of the pancreas and the nervous system.

Keywords: Ataxia; Basic Helix-Loop-Helix; BETA2; bHLH; Diabetes; Deafness; NeuroD; Neurogenesis.

Basic helix-loop-helix transcription factors

Basic helix-loop-helix (bHLH) transcription factors control determination and differentiation of cell fates in various tissue types during embryonic development. In particular, bHLH factors related to MyoD or NeuroD have been shown to be capable of converting embryonic cells into fully differentiated tissue types, such as skeletal muscle or neurons, respectively. Since the mid-1980's, much effort has been devoted to understanding how these bHLH transcription factors carry out their functions at the molecular and cellular levels.

In general, the tissue-specific bHLH transcription factors interact with ubiquitously expressed E proteins (which are also bHLH proteins). The negative regulatory bHLH proteins, such as Id proteins, can inhibit the formation of these active protein complexes by disrupting dimer formation between the tissue-specific bHLH proteins and E proteins (see Fig. 1). Thus, the activity of a bHLH protein is highly dependent on its protein-protein interactions.

NeuroD NeuroD was originally cloned from a yeast two-hybrid screen that was designed to identify new members of the bHLH transcription factor family. NeuroD is capable of converting skin precursor cells into neurons in *Xenopus* embryos (Lee *et al.*, 1995). NeuroD also promotes premature cell cycle exit and differentiation in neural precursor cells, indicating that it is primarily a differentiation factor (Lee *et al.*, 1995). Independently, the hamster ortholog of NeuroD, known as BETA2, was cloned in a yeast one-hybrid screen designed to identify the bHLH factor that dimerizes with E47 (a ubiquitous bHLH protein). BETA2 binds to the bHLH consensus E-box binding site within the insulin promoter, and activates transcription (Naya *et al.*, 1995).

Since the discovery of NeuroD/BETA2 (referred to as

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Abbreviations: βGK, β-glucokinase; AD, Alzheimer's Disease; bHLH, basic helix-loop-helix; EGL, external granule layer; HAP1, Huntingtin-associated protein 1; Htt, huntingtin; IGL, internal granule layer; IGRP, islet-specific glucose-6-phosphatase (G6Pase) catalytic-subunit-related protein; LacZ, β-galactosidase gene; MLK2, mixed-lineage kinase 2; MODY, maturity-onset diabetes of the young; NCAM, neural cell adhesion molecule; Ngn, neurogenin; SHP, small heterodimer partner; SUR, sulfonylurea receptor; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; VCG, vestibular-cochlear ganglia; X-ngnr-1, *Xenopus* neurogenin related factor-1.

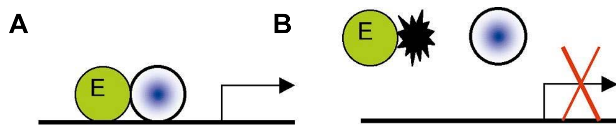

Fig. 1. bHLH protein interactions. **A.** Ubiquitously expressed E proteins ( ) interact with tissue-specific bHLH proteins ( ) to activate the downstream target genes. **B.** The inhibitory bHLH proteins ( ) disrupt the dimer formation, thereby inhibiting the transcriptional activity of bHLH proteins.

NeuroD hereafter) about 10 years ago, many groups of scientists have been studying the function of NeuroD in various systems, which has led to the identification of three other mammalian neuronal bHLH transcription factors that display close similarity to NeuroD. These transcription factors include NeuroD-related factor (NDRF)/ NeuroD2, mammalian atonal homolog 3 (MATH3)/ NeuroM, and MATH2/ NEX2 (see reviews by Kageyama *et al.*, 1997; Lee, 1997a; Sommer *et al.*, 1996). These transcription factors and NeuroD share a high degree of sequence similarity at the amino acid level throughout their entire length, but particularly within the bHLH domain and the 40 amino acid sequence immediately following the helix 2. In fact, the ortholog of NeuroD in *C. elegans* was discovered using this 40 amino acid sequence in a data base search (Lee *et al.*, 1995). However, the functional significance of this 40 amino acid sequence has not been determined.

The *neuroD* gene has been identified in several species including human, mouse, chicken, *Xenopus*, zebrafish, and *C. elegans*. In *Drosophila*, however, Atonal protein seems to represent the functional ortholog both of NeuroD and other mammalian Atonal-related proteins (Lee, 1997b). Analysis of the gain-of-function phenotypes in *Xenopus* and zebrafish, as well as the loss-of-function phenotypes in mice, zebrafish, and *C. elegans*, have demonstrated the critical roles that NeuroD plays in developing organisms.

In *C. elegans*, a reporter gene under the control of *C. elegans neuroD* promoter (*cnd-1*) indicated that *cnd-1* is expressed in the embryonic ventral cord motor neurons. A loss-of-function mutant, cnd-1 (ju29), results in a reduced number and poor differentiation of the ventral cord motor neurons (Hallam *et al.*, 2000).

In *Xenopus*, all primary neurons, neurogenic placodes, and retina strongly express the neuronal differentiation gene *XNeuroD* (Lee *et al.*, 1995; Schlosser *et al.*, 2000). In frogs, *X-ngnr-1* (*Xenopus* neurogenin related factor-1) and Xcoe2 (the *Xenopus* ortholog of *Drosophila* Collier and rodent early B-cell factor/olfactory-1) function upstream of NeuroD in promoting primary neurogenesis (Dubois *et al.*, 1998). Overexpression of any of these proteins in embryos or in animal caps results in ectopic neurogenesis, presumably through the action of NeuroD (Dubois *et al.*, 1998; Lee *et al.*, 1995; Ma *et al.*, 1996). The ectopically generated neurons display the circus movements (motility-correlated behavior). These neurons do not form functional synapses with myocytes nor respond to glutamate, yet they exhibit tetrodotoxin-sensitive action potentials, suggesting that they are either Rohon-Beard sensory neurons or poorly differentiated neurons despite their extensive neurite formation (Olson *et al.*, 1998). It is unclear whether neurons other than primary neurons are generated ectopically in the epidermis by overexpresion of NeuroD. Ectopic expression of NeuroD induces the neuronal fate in the entire cranial structure, indicating that all of the cranial neural crest cells are permissive to NeuroD function (Lee *et al.*, 1995).

Much effort has been put into understanding the role of NeuroD in mammals. The biochemical and molecular analyses performed in cultured cell lines have provided important insights into how NeuroD carries out its function at the molecular level. In addition, NeuroD seems to be expressed in several other types of neuroendocrine cells, including those in the stomach, gut, and adult lung (Ito *et al.*, 2000; Naya *et al.*, 1997). In this review, we will focus on reviewing what is known about NeuroD in the structures that exhibit a gross phenotype in NeuroD-null mice, namely the pancreas and the nervous system. Extensive study of the role of NeuroD in the enteroendocrine cells is not covered in this review, and the readers are referred to an excellent review recently written by Schonhoff *et al.* (2004). Lastly, mutations in NeuroD are linked to susceptibilities to Type 1 and Type 2 diabetes in humans. Our understanding of this intriguing factor, which is involved in diabetes, ataxia, deafness, and possibly learning/memory, will greatly enhance our ability to diagnose and treat several human conditions and diseases in the future.

NeuroD in the pancreas

NeuroD-null phenotype in the pancreas NeuroD has been shown to be important for the tissue-specific expression of the insulin gene and its regulation by physiological stimuli (Clark and Docherty, 1993; Clark *et al.*, 1993; Cordle *et al.*, 1991; German *et al.*, 1991; Karlsson *et al.*, 1987; 1989; Khoo *et al.*, 2003; Naya *et al.*, 1995; Sharma *et al.*, 1999; Whelan *et al.*, 1990). An *in vivo* study confirmed the importance of NeuroD in pancreatic development (Naya *et al.*, 1997). In the mouse, *neuroD* mRNA is first expressed in the pancreatic primordium at e9.5 and continues to be expressed in β cells throughout development. In postnatal mice, the activity of the reporter β-galactosidase gene (*lacZ*), which was inserted in place of *neuroD*, was detected in all mature β cells and 1−2% of α cells, but not in δ or PP cells.

NeuroD-null mice die within five days after birth due to severe hyperglycemia (Naya *et al.*, 1997). Examination

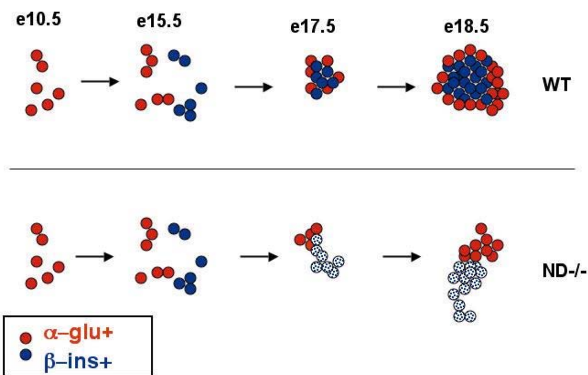

Fig. 2. At e10.5 in mice, glucagon-expressing α cells (marked in red) are prevalent in the developing pancreas. NeuroD is expressed in these α cells as well as in all the β cells (marked in blue) that are born later. In NeuroD-null mice, increased cell death and failure to form islets are evident in the pancreas, but not until e17.5. Circles with blue speckles indicate β cells that are poorly differentiated and/or dying.

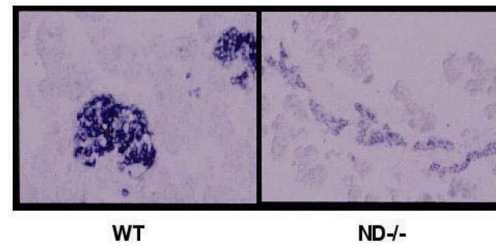

Fig. 3. Islets fail to form in NeuroD-null pancreas as detected by Chromagranin A immunostaining. Instead, the endocrine cells are found clustered along the ducts.

of the pancreas indicates that the endocrine cells fail to form islets. While expression of all four endocrine hormones has been detected in the NeuroD-null pancreas, the total pancreatic insulin level is only about 5% of the normal level (Naya *et al.*, 1997). Moreover, β cells undergo massive cell death starting around e17.5, at which time the failure to form islets becomes apparent in NeuroD-null mice (Fig. 2) (Naya *et al.*, 1997). The remaining β cells fail to produce a sufficient amount of insulin, thereby causing neonatal lethality of NeuroD-null pups within days after birth (Naya *et al.*, 1997). There is also a reduction in the number of α cells, although not as significantly as in β cells.

It is unclear why the earlier-born endocrine cells, which also express the *neuroD* mRNA, are not affected when NeuroD is absent (Naya *et al.*, 1997). It is possible that these cells are defective in function, even though this is not apparent at the level of simple immunohistological analysis.

As mentioned above, the islets fail to form in NeuroD-null pancreas. Normally, the islets consist of glucagon-expressing α cells surrounding a cluster of β cells. Instead, the α and β cells in NeuroD-null mice are clustered separately along the ductal structure (Fig. 3). The molecular basis of the failure in islet morphogenesis is currently unknown. Because both α and β cells are affected in NeuroD-null mice, it is difficult to determine whether the failure to form islets is due to defects in α or β cells, or both. Deletion of NeuroD in a cell type-specific manner in either α- or β-cells should resolve this issue.

Strikingly, when the NeuroD-heterozygous mice in the 129sv/J and C57/BL6 mixed genetic background are backcrossed to 129sv/J strain, as much as 60% of the NeuroD-null mice survive beyond the neonate stage (Huang *et al.*, 2002). While the epigenetic basis of this rescue is unknown, it was found that the β cell mass was significantly increased as early as postnatal day 4 and reached the wild-type level by two months of age (Huang *et al.*, 2002). Meanwhile, the α cell mass remained reduced in these genetically rescued mice, possibly due to an insufficient rate of proliferation after birth (Huang *et al.*, 2002). The increased β cell mass seems to be the result of β cell neogenesis from both the ductal structure and existing islets, as evidenced by the detection of an increased number of dividing cells in the islets of rescued mice (Huang *et al.*, 2002). Interestingly, it has been found that hyperglycemia in various diabetes models, including partial pancreatectomy (Px), induces de-differentiation of islets and a change in gene expression. Among the genes that are increased is c-Myc, which is another bHLH protein that can also bind to the E-box within the insulin promoter and inhibit NeuroD (Kaneto *et al.*, 2002; Laybutt *et al.*, 2002). This systemic response to hyperglycemia may explain the phenomenon observed in the genetically rescued NeuroD-null islets in which many dividing cells were detected only after birth when a hyperglycemic condition exists. Nevertheless, newly generated β cells remain poorly differentiated in the absence of NeuroD.

Despite the recovery in β cell mass, the islets do not achieve their normal architecture in the pancreas of these genetically rescued mice; a small number of α cells exists within the islets rather than in the periphery of the islets. The apparent α cell abnormality observed in these genetically rescued mice suggests that NeuroD functions not only in the development, but also in maintenance of α cells. It is also possible that α cell defects observed in these mice represent a secondary effect of having poorly differentiated β cells. Whether any aspect of the pancreatic phenotype is cell autonomous or non-cell autonomous needs to be addressed when a cell-type-specific deletion can be achieved using conditional knock-out mice.

NeuroD, calcium, and beta cell function The ability of

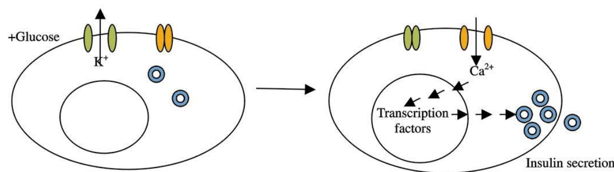

Fig. 4. Insulin secretion upon glucose stimulation. NeuroD has been implicated in gene regulation of SUR1, which forms a potassium channel with Kir6.2. Closure of this potassium channel upon glucose sensing will result in Ca^{2+} channel opening and an increase in the level of intracellular calcium.

NeuroD to activate the insulin promoter has been shown in many cell culture assays (Glick *et al.*, 2000; Naya *et al.*, 1997; Qiu *et al.*, 1998). Recently, NeuroD was also shown to bind and activate the promoter of sulfonylurea receptor (SUR1), which forms a potassium channel with Kir6.2 (Kim *et al*., 2002). This potassium channel closes in response to glucose, which results in activation of the Ca^{2+} channel and increases in intracellular Ca^{2+} levels (Fig. 4) (Kim *et al.*, 2002).

While it has not been established whether Ca^{2+} plays a role in β cell differentiation, increases in Ca^{2+} levels are clearly shown to be critical for mature β cells to activate transcription factors responsible for elevated insulin secretion upon glucose stimulation. Because NeuroD has been shown to play a major role in insulin activation upon glucose stimulation (Khoo *et al.*, 2003). NeuroD may be controlled at the transcriptional and post-transcriptional levels upon increase in the intracellular Ca^{2+} level. In addition, the *LacZ* reporter gene expression in NeuroD knock-in mice indicates that NeuroD continues to be expressed in mature β cells, which suggests that NeuroD may have a role in mature β cell function (Naya *et al.*, 1997). This role is also supported by the fact that several mutations of NeuroD have been linked to diabetes in humans.

NeuroD mutation and diabetes in humans

Susceptibility to Type 1 diabetes mellitus (T1DM) Ala45Thr mutation: The *neuroD* gene in the human is located on chromosome 2q32, a region linked to Type I diabetes IDDM7 (D2S152). Ala45Thr is associated with a susceptibility to Type 1 diabetes mellitus (T1DM) in Danish (Hansen *et al.*, 2000), Czech (Cinek *et al.*, 2003), and Japanese populations (Iwata *et al.*, 1999; Mochizuki *et al.*, 2002; Yamada *et al.*, 2001). However, no such links were made in Caucasian descendants from several European countries, including the French population (Dupont *et al.*, 1999; Vella *et al.*, 2004). Meanwhile, no link has been established between Ala45Thr polymorphism in NeuroD and Type 2 diabetes mellitus (T2DM) (Hansen *et al.*, 2000; Malecki *et al.*, 2003).

Susceptibility to Type 2 diabetes mellitus (T2DM): Maturity-onset diabetes of the young (MODY) is an autosomal dominant form of early-onset type 2 diabetes mellitus (T2DM). Two mutations in NeuroD are linked to MODY6 in humans (Malecki *et al.*, 1999). One is an Arg 111 missense mutation within the DNA binding domain of NeuroD that abolishes its ability to bind DNA. This mutation is associated with T2DM in the heterozygous state. A second mutation that causes a C-terminal truncation of NeuroD leads to a more severe T2DM clinical phenotype. As described below, the C-terminus of NeuroD, which contains the transcriptional activation domain, is subject to post-tran-slational regulation. It is possible that this mutation produces a dominant negative form of NeuroD that causes the more severe phenotype.

Mutations of several other genes are also linked to MODY: HNF4A/MODY1 (Yamagata, 2004; Yamagata *et al*., 1996a), GCK/MODY2 (Vionnet *et al.*, 1992), TCF1/MODY3 (Yamagata *et al.*, 1996b), IPF1/MODY4 (Stoffers *et al.*, 1997), and TCF2/MODY5 (Horikawa *et al.*, 1997). Among these MODY genes, all except GCK/MODY2 are transcription factors that are also known to be involved in islet development.

The evidence that mutations in NeuroD are linked to both T1DM and T2DM speaks for the functional roles of NeuroD in mature islets. Notably, Yamada *et al.* (2001) have reported that there is a strong link between Ala45Thr mutation in the acute-onset, but not in slow-onset, type 1 diabetes, suggesting that NeuroD could also be involved in β cell regeneration.

NeuroD in the nervous system

Cerebellum and hippocampus NeuroD is highly expressed in developing neurons of the peripheral and central nervous systems, supporting its role as a neuronal differentiation factor. *NeuroD* mRNA expression is at its highest during differentiation in the cerebral cortex and spinal cord and decreases as the neurons mature. However, the structures such as the cerebellum and hippocampus, maintain robust levels of *neuroD* mRNA expression throughout adulthood in humans and mice, indicating that NeuroD may have a maintenance role in these tissues (Lee *et al.*, 2000a; Yokoyama *et al.*, 1996).

Because NeuroD-null mice die shortly after birth due to neonatal diabetes, two approaches have been taken to reveal the role of NeuroD in the nervous system. First, Miyata *et al.* (1999) used a transgenic rescue approach in which a myc-tagged NeuroD under the control of the insulin promoter is introduced to the NeuroD-null background so as to specifically rescue the pancreatic phenotype. The insulin promoter is used for this transgenic rescue because NeuroD-null mice were known to express at least 5% of insulin. Using this approach, the investigators

were able to obtain mice that survived beyond a few days after birth, some surviving over a year (Miyata *et al.*, 1999). These transgenically rescued mice were found to display severe neurological disorders including stumbling gaits (ataxia) and head circling. Upon examining the brain, Miyata *et al*. (1999) found severe cellular depletion in the brain; over 90% of the cerebellar granule cells were depleted due to cell death shortly after differentiation. In addition, there was a complete lack of dentate gyrus of the hippocampus (Miyata *et al.*, 1999). The second approach was rescuing NeuroD-null mice genetically. When NeuroD-null mice were crossed to MATH-2/NEX-1-null genetic background, some NeuroD-null mice were found to survive beyond the neonatal stage, resulting in genetic rescue (Schwab *et al.*, 2000). The neuronal phenotype found in the genetically rescued mice was very similar to that found in the transgenic rescue, namely depletion of the cerebellar granule cells and loss of the dentate gyrus of the hippocampus (Schwab *et al.*, 2000).

Detailed analysis of the hippocampal defect revealed that the formation and organization of early born cells in the dentate gyrus, such as Cajal-Retzius cells and radial glial cells, were normal. Moreover, the migration of dentate precursor cells from the ventricular zone to the dentate gyrus occured normally. However, once the granule cells reached the dentate gyrus, both the proliferation and differentiation were significantly perturbed, leading to massive cell death (Liu *et al.*, 2000a). In addition, it was determined that the granule cells of the dentate gyrus in mice lacking both NeuroD and MATH-2/NEX1 fail to mature, lack normal sodium currents and display poor dendritic arborization (Schwab *et al.*, 2000). These results indicate that NeuroD is critical not only for the survival of early differentiating neurons, but also for their differentiation. The cause-and-effect relationship between cell death and failure to differentiate has not been established. Based on available information, we cannot rule out the possibility that NeuroD has a role in neuronal survival that is independent of its role in differentiation.

In the cerebellum, neuronal death is observed most intensely within the inner layer of the external granule layer (EGL). Some granule cells migrated into the internal granule layer (IGL) and then died (as shown in Fig. 5), suggesting that the failure to morphologically differentiate or migrate may not be the only reason for cell death.

The EGL layer that contains the dividing population of granule cell precursors expresses another bHLH protein MATH-1. In mice lacking MATH-1, no granule cells form. Because the *neuroD* transcript is detected in the cell layer adjacent to the dividing precursor cells (Miyata *et al.*, 1999), it is likely that MATH-1 lies immediately upstream of NeuroD in transcriptional cascade. Surprisingly, overexpression of MATH-1 in EGL leads to aberrant and premature expression of NeuroD and Doublecortin (which are markers of IGL) in the EGL layer, resulting in premature

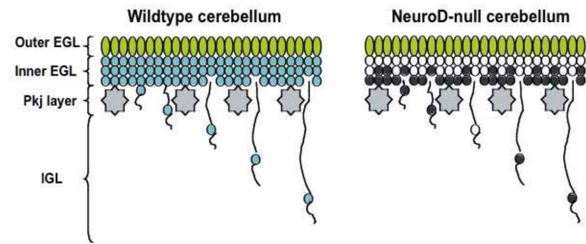

Fig. 5. Locations of NeuroD-expressing cells (in wild-type, in blue) and dying cells (in NeuroD-null mice, in black).

differentiation and a smaller cerebellum (Helms *et al.*, 2004). Whether expression of NeuroD alone in the EGL can cause premature differentiation of EGL cells has not been tested. Interestingly, *neuroD* is also expressed in the auditory hair cells that also depend on MATH-1 for commitment and proliferation. In contrast to the cerebellum, however, the survival of the inner ear hair cells is not affected in NeuroD-null mice (Kim *et al.*, 2001).

Inner ear In mice, the inner ear develops from the otic placode starting at embryonic day 9.5 (e9.5), and giving rise to inner ear sensory neurons and sensory hair cells (Brown *et al.*, 2003; Rubel and Fritzsch, 2002). While the auditory sensory neurons develop by e13.5, the auditory hair cells are not born until around e14 and only reach their maturity between postnatal days 12 and 14 (P12-P14) to form about 6600 hair cells (Corwin *et al.*, 1993; Kros, 1998).

Three tissue-specific bHLH transcription factors, Ngn1, Math1, and NeuroD, play critical roles in inner ear development (Fritzsch, 2003): Mice lacking Ngn1 lack all sensory neurons in the inner ear although many hair cells still develop (Ma *et al.*, 1998; 2000). Mice lacking Math1 lack all hair cells without much effect on the inner ear sensory neurons (Bermingham *et al.*, 1999). Ngn1 and Math1 affect the precursor stages of sensory neurons and sensory hair cells, respectively (Bermingham *et al.*, 1999; Ma *et al.*, 1998). Meanwhile, NeuroD is required for the survival and differentiation of the inner ear sensory neurons during later stages (Kim *et al.*, 2001; Liu *et al.*, 2000b).

The vestibular-cochlear ganglia (VCG) consist of the interneurons that relay the primary information from the sensory hair cells of the cochlear and vestibular sensory epithelium to the auditory nuclei in the brain. Mice lacking NeuroD exhibit a near-complete loss of cochlear ganglia (survival of fewer than 30 neurons out of several thousands of neurons) and a significant loss of vestibular ganglia (Kim *et al.*, 2001). The neuronal loss in the VCG is due to a perturbed delamination of the neuroblasts from the otic vesicle epithelium and significant apoptosis among those neurons that do delaminate to form the VCG (Kim *et al.*, 2001; Liu *et al.*, 2000b). The loss of these interneurons in VCG of NeuroD-null mice resembled the pheno-

type found in mice lacking neurotrophin receptors TrkC (which is critical for cochlear sensory neuronal survival) and TrkB (which is critical for vestibular sensory neuronal survival) (reviewed by Fritzsch *et al.*, 1997). In NeuroD-null mice, expression of both TrkC and TrkB was absent or significantly decreased, respectively, while their expression in the nearby trigeminal ganglia was normal (Kim *et al.*, 2001). Therefore, NeuroD may support the survival of newly differentiating neurons by activating molecules such as TrkB and TrkC.

The surviving vestibular ganglia displayed disorganized fiber projection onto the vestibular sensory epithelia, suggesting that NeuroD may be important for differentiation of these neurons or the pathfinding of their fibers, or both (Kim *et al.*, 2001). Interestingly, the sensory epithelium lacking innervation was found to contain the sensory hair cells in mice older than 9 months, indicating that the generation and maintenance of hair cells do not depend on innervation (Kim *et al.*, 2001). The *lacZ* reporter gene expression from the endogenous *neuroD* locus was detected in all inner hair cells, but only in some outer hair cells (Kim *et al.*, 2001). The significance of this expression pattern is unknown. Recently, Lawoko-Kerali *et al.* (2004) reported a lack of immunostaining in the inner ear hair cells using anti-NeuroD antibody. These contradictory results could be due to either aberrant reporter gene expression resulting from substitution of important regulatory sequences in the *neuroD* gene of the knock-out strains, or to the failure of the anti-NeuroD antibody to detect a specific form of the NeuroD protein in the sensory hair cells.

Retina bHLH proteins play important roles in cell fate determination in the mammalian retina (see review by Hatakeyama and Kageyama, 2004). In the mouse retina, *neuroD* is expressed in undetermined precursor cells, developing amacrine cells, developing photoreceptors and a subset of mature photoreceptors. In NeuroD-null retina, a decrease in amacrine cell differentiation is accompanied by a two-fold increase in bipolar interneurons, suggesting that NeuroD favors amacrine over bipolar interneuron differentiation *in vivo* (Morrow *et al.*, 1999). The converse gain-of-function experiment in which NeuroD is overexpressed in developing retinal cells supported this finding (Morrow *et al*., 1999). A more dramatic effect on amacrine cells is found in Math3/NeuroD-double-null mice that completely lack amacrine cells. In the Math3/NeuroD-double-null retina, the cells that failed to become amacrine cells adopted the ganglion and glial cell fate rather than the bipolar fate seen in NeuroD-null retina (Inoue *et al.*, 2002; Morrow *et al.*, 1999).

NeuroD also affects the differentiation and maintenance of photoreceptors. Yan and Wang (1998) found that overexpresion of NeuroD in developing retinal explant cultures increased rod photoreceptors rather than amacrine cells. However, when homeobox gene Pax6 or Six3 was co-expressed, NeuroD significantly increased amacrine cell differentiation in retinal explant cultures (Inoue *et al.*, 2002). One can speculate that the discrepancy in results obtained by different investigators could arise from the differences in their retinal explant cultures or genetic variation. Morrow *et al.* (1999) observed that the survival of a subset of rod photoreceptors was affected in the NeuroD-null retina. This observation is further substantiated by a more extensive analysis of genetically rescued NeuroD-null mice that displayed a gradual loss of photoreceptors until they were completely missing by 18 months due to cell death (Pennesi *et al.*, 2003). Double- and triple-bHLH mutant analysis revealed that a complex combination of bHLH proteins play roles in determining retinal cell fate in mice (Akagi *et al.*, 2004).

In chicks, both gain- and loss-of-function analyses indicate that NeuroD is exclusively involved in photoreceptor development. Forced expression of NeuroD in chick retinal neuroepithelium or retinal pigment epithelium using a retrovirus-mediated vector resulted in generation of cone photoreceptor cells without affecting other cell types (Yan and Wang, 1998; 2004). Meanwhile, knock-down of *neuroD* expression and function in embryonic chick retina significantly perturbed photoreceptor development without causing defects in other types of retinal cells (Yan and Wang, 2004).

The gain-of-function experiment in *Xenopus* retina revealed that overexpression of XneuroD promotes the differentiation of later-born cell types (such as amacrine cells), but not of early-born cell types (such as retinal ganglion cells) (Kanekar *et al*., 1997; Moore *et al*., 2002). Subseqnently, it was determined that only a certain layer within the *Xenopus* retina is susceptible to the activity of ectopically expressed NeuroD due to endogenous glycogen synthase kinase 3β (GSK3β) activity that negatively regulates NeuroD activity (Moore *et al.*, 2002, also see below). In light of this finding, it is possible that NeuroD gain-of-function experiments might depend heavily on the timing and the cell type used, because this protein is heavily subjected to a post-translational modification (see below).

Other neurons NeuroD is also expressed in other regions of the brain that do not exhibit gross cellular deficit in loss-of-function mutant mice. These include the neurons of the limbic system, hypothalamic and thalamic neurons, cerebral cortical neurons, olfactory/vomeronasal neurons, and hippocampal pyramidal neurons, to name a few. Although expression of NeuroD in some of these neurons is described extensively (Bedard and Parent, 2004; Cau *et al.*, 1997; Nibu *et al.*, 1999; 2001; Suzuki *et al.*, 2004a; 2004b; Yasui *et al.*, 2004), it is not discussed because of the lack of functional phenotype. The fact that we cannot discern a phenotype at the histological/immunochemical

level does not preclude functional phenotype in these neurons. Deletion of NeuroD in specific tissues using conditional knockout mice followed by both physiological and behavioral studies will reveal any functional defects that may be present in these neurons.

Cell lines Various cell lines express *neuroD* while being differentiated into neurons. For example, expression of *neuroD* is observed during differentiation of P19 teratocarcinoma cells, F11 human neuroblastoma cells, HC2S3 immortalized neuronal progenitor cells and in murine cerebral cortical neurons (Cho *et al.*, 2001; Itoh *et al.*, 1997; Katayama *et al.*, 1997; Kim *et al.*, 2004a; Liu *et al.*, 2004; Ohtsuka *et al.*, 1998). In the case of the human neuroblatoma cell line, the neurotrophin receptor TrkB (cognate receptor for BDNF neurotrophin) and the cyclin-dependent kinase inhibitor p21 (Cip1) are concomitantly expressed. These two genes contain E-box elements within their promoters and can be activated by NeuroD and E47 in the absence of retinoic acid treatment (Liu *et al.*, 2004). As mentioned above, TrkB receptor expression is found to be dramatically decreased in NeuroD-null sensory ganglia of the inner ear. Accordingly, BDNF can induce neuronal differentiation in cultured mouse neural stem cells, closely linking neuronal differentiation by bHLH proteins through BDNF/TrkB signaling (Ito and Furukawa, 2004).

NeuroD expression was detected in 12 medulloblastomas as well as in neuroblastoma and retinoblastoma samples/cells (Rostomily *et al.*, 1997). Considering that these tumors are derived from the regions of the brain that highly express NeuroD, it is tempting to speculate that NeuroD activity may be inhibited post-translationally in these tumor cells. As we have learned from recent work, GSK3β and ERK can alter the activity of NeuroD in certain cellular contexts (Dufton *et al.*, 2005; Khoo *et al.*, 2003; Moore *et al.*, 2002, also see below). Therefore, it will be important to determine whether the activated form of NeuroD can cause these tumor cells to differentiate. Meanwhile, none of the glial tumors or cell lines expressed *neuroD*, again confirming the neuronal specificity of NeuroD (Rostomily *et al.*, 1997). The studies of NeuroD in these cell lines have and will enhance our ability to dissect the molecular mechanisms of NeuroD including what the upstream and downstream targets are and how this protein is regulated post-transcriptionally and post-translationally. What we learn from these studies can be tested *in vivo* in developing organisms such as *Xenopus*, chicks, and mice.

Molecular studies of NeuroD

Upstream regulators of NeuroD As is the case during myogenic development, a cascade of bHLH transcription factor activation occurs during neuronal differentiation. Neurogenin (Ngn) 1, 2, and 3 were identified based on their sequence similarity to the *Drosophila* Atonal protein (Ma *et al.*, 1996). Epistatic analysis *in vivo* indicated that Neurogenin proteins function upstream of NeuroD (Ma *et al.*, 1996). In developing central and peripheral nervous systems of the mouse, expression of *ngn1*, *ngn2*, and *ngn3* is confined to the dividing cells, such as the neuronal precursors in the ventricular zone (Fode *et al.*, 1998; Ma *et al.*, 1996; 1998). Meanwhile, *ngn3* is the only member of this family that is expressed in the progenitors of pancreatic endocrine cells (Sommer *et al.*, 1996; Schwitzgebel *et al.*, 2000). Therefore, the temporal and spatial patterns of *ngn* expression suggested that they are expressed in the population of cells that give rise to the *neuroD*-expressing cells.

Gain-of-function studies in *Xenopus* embryos indicated that overexpression of mouse Neurogenin or *Xenopus* Neurogenin-related-1 (X-Ngnr-1) proteins activated the endogenous *XneuroD* gene, while the converse experiment of overexpressing XNeuroD did not activate the endogenous *X-ngnr-1* (Huang *et al.*, 2000; Ma *et al.*, 1996). These experiments unequivocally demonstrated that Ngn proteins function upstream of NeuroD during neuronal development in *Xenopus* embryos. In addition, overexpression of Ngn3 in stably transfected endocrine cell lines activated the endogenous levels of *neuroD* mRNA (Huang *et al.*, 2000). At the biochemical level, it has been also shown that Ngn3-E47 heterodimer can physically bind to the mouse *neuroD* promoter (Huang *et al.*, 2000). In *Xenopus*, *Xcoe*, which is expressed after *X-ngnr-1* but before *XneuroD*, may also activate *XneuroD*. Ectopic expression of Xcoe has been shown to result in activation of the endogenous *XneuroD* gene followed by ectopic neurogenesis (Dubois *et al.*, 1998).

Loss-of-function studies in mice confirmed the epistatic relationship between Ngn and NeuroD: mice lacking Ngn1 and Ngn2 develop distinct defects in the cranial sensory neurons and fail to express NeuroD, MATH3, and NSCL-1 (Fode *et al.*, 1998; Ma *et al.*, 1998). Ngn3-null mice failed to generate any pancreatic endocrine cells and died shortly after birth from neonatal diabetes (Gradwohl *et al.*, 2000). In these mice, the expression of Isl 1, Pax4, Pax6, and NeuroD was lost, placing Ngn3 upstream of these genes. Once the *neuroD* gene is activated by Ngns, its expression is likely to be maintained auto-regulation, or by cross-regulation by other transcription factors present in the cell.

Approximately 2.5 kb of sequences 5′ to the transcription start site of the human and mouse *neuroD* genes were characterized (Huang *et al.*, 2000; Miyachi *et al.*, 1999; Xu and Murphy, 1998). The deletion analysis revealed that the -420 to -171 sequences of the human *neuroD* promoter were necessary for expression in the neuroblastoma cell line IMR-32 and insulinoma cell line HIT-T15 (Miyachi *et al.*, 1999). Within this region, four E boxes, named E1 to E4 boxes (the distal one being E1), and two

GC boxes are present. Co-transfection of the NeuroD expression vector into IMR-32 cells enhanced the human *neuroD* promoter activity by about 4-fold, indicating that NeuroD can autoregulate its own promoter. The E1 and E4 boxes were associated with autoactivation by NeuroD, with the E1 box having a more significant effect. Although the E3 box is not involved in autoactivation, the mutation analysis revealed that the E3 box was important for the activation of *neuroD* transcription. The analyses of the mouse *neuroD* promoter showed that the sequences from -1000 to -230, which contain nine E boxes, can confer cell-type-specificity in insulinoma cell line HIT-T15 (Huang *et al*., 2000). Two proximal E-box sequences bound the Ngn3-E47 heterodimer and mediated the activation effect of Ngn3-E47, suggesting that they are functionally equivalent to the E3 box of the human *neuroD* promoter (Huang *et al.*, 2000).

Insulinoma-associated antigen-1 (IA-1) encoding a zinc-finger DNA-binding protein is expressed in fetal brain and pancreas as well as tumors of neuroendocrine origin (Breslin *et al.*, 2003; Rostomily *et al.*, 1997; Zhu *et al.*, 2002). *IA-1* gene expression is closely associated with the expression pattern of *neuroD*. EMSA analysis and the transient transfection studies showed that *IA-1* and *neuroD* are two downstream target genes modulated by *IA-1* (Breslin *et al.*, 2002). Since the IA-1 has repressor activity and the IA-1 binding site is in the -177/-166 region of the *neuroD* promoter, one can speculate that IA-1 may compete with Neurogenins to modulate *neuroD* gene expression. Recently, it was shown that the NeuroD-E47 heterodimer can also bind and transactivate the E-box element in the *IA-1* promoter (-426/-65 bp), which is sufficient to confer tissue-specific transcriptional activity (Breslin *et al.*, 2003). These results suggest that the *neuroD* and *IA-1* genes may autoregulate and cross-regulate each other *in vivo* to keep their expression levels in check.

Downstream targets of NeuroD The insulin gene is the first identified downstream target of NeuroD (Naya *et al.*, 1995). BETA-2, a hamster homolog of NeuroD, was cloned and characterized as a regulator of the insulin gene. It was shown that BETA2 heterodimerizes with the E47, and binds to the insulin E-box enhancer and transactivates it. Through deletion analysis, the C-terminal region of NeuroD was shown to be important for insulin E-box-stimulated transcription (Sharma *et al.*, 1999). Activation of the insulin promoter by NeuroD is potentiated by the p300/CREB binding protein (CBP) coactivator (Qiu *et al.*, 1998). Although the exact mechanism involved in p300/CBP-mediated transcription is unclear, it is believed to bridge the basal transcriptional apparatus and other key transcription factor(s), such as NeuroD, and promote a transcriptionally active state of the target genes through its histone acetyltransferase activity (Eckner, 1996; Shikama *et al.*, 1997). Recently, it was shown that NeuroD is specifically acetylated by p300-associated factor, PCAF, in β cells (Qiu *et al.*, 2004). Insulin gene transcription was decreased by blocking the acetylation of NeuroD, which resulted in a decrease in DNA binding and in activation potential in HIT-T15 insulinoma cell lines. These findings suggest that acetylation of NeuroD plays an important regulatory role in insulin gene regulation and perhaps in other functions of NeuroD.

It has been demonstrated that Pax genes are essential for development of many organs, such as the eye, brain, kidney, thyroid gland, immune system and pancreas (Chalepakis *et al.*, 1993; Mansouri *et al.*, 1999; Noll, 1993; Walther *et al.*, 1991). Two members of the Pax gene family, Pax4 and Pax6, play important roles in islet differentiation (Dohrmann *et al.*, 2000; Smith *et al.*, 1999; Sosa-Pineda *et al.*, 1997; St-Onge *et al.*, 1997). Pax6, much like NeuroD, is expressed in both the developing nervous system and pancreas, albeit not always in the same cells (Callaerts *et al.*, 1997; Mansouri *et al.*, 1999). The absence of Pax6 leads to islet disorganization, indicating an involvement of Pax6 in the early stage of islet morphogenesis (St-Onge *et al.*, 1997). Recently, individual elements regulating Pax6 gene activity in the pancreas have been identified in a 1100 bp fragment that lies 4.6 kb upstream of exon 0 (Marsich *et al.*, 2003). It was shown that NeuroD-E47 heterodimers could bind specifically to three E-boxes in the 1100 bp element. In addition, the expression of NeuroD could induce activation of the Pax6 promoter in a non-endocrine cell line (NIH-3T3). Pax4 is also required for endocrine cell identities in the pancreas (Sosa-Pineda *et al.*, 1997, also, see the accompanying review). Pax4-knockout mice lack β- and δ-cells. Expression of Pax4 starts in pancreatic precursor cells, and then becomes restricted to β- and δ-cells before it is turned off after birth. A 0.9 kb promoter element located 2 kb upstream of the transcription start site has been shown to mediate the Pax4 expression pattern (Brink *et al.*, 2001; Xu and Murphy, 2000). It was shown that the A2 and E1 elements in the Pax4 promoter were necessary for embryonic Pax4 expression and that a 409-bp Pax4 promoter fragment containing the A2 and E1 element was sufficient to mediate strong pancreas-specific expression (Brink and Gruss, 2003). Even though the binding of NeuroD to the E element in the Pax4 promoter is not confirmed, it was suggested that Ngn3 or NeuroD may function as an upstream activator of the Pax4 gene. This hypothesis is supported by results showing that the expression of NeuroD, Pax6, and Pax4 was lost in the Ngn3-deficient mice (Gradwohl *et al.*, 2000) and that the expression of both Ngn3 and HNF1alpha are necessary to activate the Pax4 gene in the fibroblast cell line NIH-3T3 (Smith *et al.*, 2003). It remains to be determined whether NeuroD can directly bind and activate the Pax4 promoter.

The sulfonylurea receptor 1 (SUR1), islet-specific glucose-6-phosphatase (G6Pase) catalytic-subunit-related pro-

tein (IGRP), and β-glucokinase (βGK) were revealed as the target genes of NeuroD in β cells (Kim *et al.*, 2002; Martin *et al.*, 2003; Moates *et al.*, 2003). SUR1 is a regulator of ATP-sensitive K+ channels (K_{ATP}) (Inagaki *et al.*, 1995). In pancreatic β cells, increased glucose level results in the elevation of the ATP:ADP ratio, which causes closure of the K_{ATP} channels, the consequence of which leads to opening of voltage-dependent Ca^{2+} channels. As a result, an influx of Ca^{2+} ions into the cell signals a cascade of molecules to induce insulin production and secretion (Efrat *et al.*, 1994). Expression of NeuroD enhanced the promoter activity of the mouse SUR1 gene in cooperation with E47 in HIT insulinoma cells (Kim *et al.*, 2002). NeuroD bound specifically to the E3 element located at -141, and a mutation of this E3 eliminated the stimulatory effect of NeuroD on the SUR1 promoter.

IGRP1 is a homolog of the catalytic subunit of G6Pase, the enzyme that catalyzes the final step of the gluconeogenic pathway (Arden *et al.*, 1999; Martin *et al.*, 2001). Although the functional role of IGRP in β cells remains unclear, a recent report identified IGRP as an autoantigen in a mouse model of Type I diabetes (Lieberman *et al.*, 2003). The cis-elements responsible for islet-specific expression of the IGRP gene have been defined (Ebert *et al.*, 1999). This sequence, which consists of 306 bp upstream of the transcription start site, is sufficient to drive the expression of reporter genes in a β-cell-specific manner in both islet-derived cell lines and pancreatic islets of transgenic mice (Frigeri *et al.*, 2004). Two conserved E-Box motifs within this 306-bp region bound to NeuroD and Upstream stimulatory factor (USF), contributing to IGRP promoter activity (Martin *et al.*, 2003).

β-glucokinase (βGK) catalyzes the rate-limiting step of glucose-induced insulin release in mature β cells (Matschinsky, 1996). Experiments performed in transgenic mice have shown that tissue-specific-expression of the βGK promoter is mediated by cis-acting elements found between -280 and +14 bp (Jetton *et al.*, 1994). The E-box element at position -221/-216 was shown to be necessary for stimulation of the βGK promoter in β cells and enteroendocrine cells (Moates *et al.*, 2003). Gel shift assay and chromatin immunoprecipitation analysis showed the binding of NeuroD to the βGK promoter.

The expression of Secretin, a peptide hormone, is mainly restricted to the S-type enteroendocrine cells in the small intestine and in the colon (Wheeler *et al.*, 1992). Analysis of the *secretin* gene promoter identified an enhancer between -174/-53 region 5′ of the transcription start site. This enhancer was necessary and sufficient for high levels of reporter gene expression only in secretin-expressing cell lines. An E-box at -130 was required for full promoter activity, and NeuroD was shown to bind to this E-box as a heterodimer with E12/E47 (Mutoh *et al.*, 1997). The *secretin* gene seems to be absolutely dependent on the presence of NeuroD for expression *in vivo*, as evidenced by the absence of secretin-expressing enteroendocrine cells in NeuroD-null mice (Naya *et al.*, 1997). In the pituitary gland, it was shown that NeuroD is expressed in corticotroph cells, where it activates the proopimelanocortin gene transcription (Poulin *et al.*, 1997).

The direct downstream targets of NeuroD in the nervous system are not as well characterized despite the extensive expression of NeuroD in the developing CNS and PNS. Xebf3, a member of the Ebf/Olf-1 family of HLH transcription factors, and XBrn3d, a POU-homeodomain transcription factor, have been described as the downstream target genes of XNeuroD during neuronal differentiation (Hutcheson and Vetter, 2001; Pozzoli *et al.*, 2001). Xebf3 was expressed in the three strips of primary neurons in embryos at stage e15.5 and in the branchial arches, olfactory placode, neural tube, retina, and otic vesicle at later stages (Pozzoli *et al.*, 2001). The expression pattern of *Xebf3* partially overlaps with that of *XneuroD* (Lee *et al.*, 1995; Pozzoli *et al.*, 2001). When overexpressed, Xebf3 induced ectopic neurons at the neural plate stage. XNeuroD activated expression of *Xebf3* both in whole embryos and in animal caps in the absence of protein synthesis (Pozzoli *et al.*, 2001). These data indicate that Xebf3 is a regulator of primary neurogenesis in *Xenopus*, acting downstream of XNeuroD. However, Xebf3 is not likely to be the unique factor functioning downstream of XNeuroD. Overexpression of Xebf3 showed a more restricted pattern of ectopic neurons, mainly confined to the posterior part of the embryo, compared to that induced by overexpression of XNeuroD.

The Brn3 POU subfamily genes have been involved in the differentiation of a wide range of sensory neurons and expressed in retinal ganglion cells (Xiang *et al.*, 1993). In dissociated chick retinal precursors, overexpression of Brn3 can activate expression of several retinal ganglion cell markers (Liu *et al.*, 2000c). Xath5 and XNeuroD could directly activate XBrn3d expression both in *Xenopus* embryos and animal caps, indicating that Xbrn3d functionally lies downstream of both Xath5 and NeuroD for sensory neuron differentiation in *Xenopus* (Hutcheson and Vetter, 2001).

In Ngn-1-null mice, NeuroD expression and NSCL-1 expression were completely lost in the otic and trigeminal placodes at e9.0 (Ma *et al.*, 1998). In addition, the expression of NeuroD precedes NSCL-1 expression in this region. Analysis of sensory neurons that strongly express NSCL-1 revealed that the spatiotemporal expression of neuronal differentiation factors, such as NeuroD and SCG-10, was not altered in developing cranial ganglia of NSCL-1 knockout mice (Kruger and Braun, 2002). These data suggest that NeuroD may regulate the expression of NSCL-1 during the development of sensory ganglia. However, Bao *et al.* (2000) have reported contradictory results indicating that NSCL functions upstream of both XNgnr-1 and XNeuroD in *Xenopus* embryos. Therefore,

where NSCL fits in the transcriptional cascade in neurogenesis needs further clarification. It is possible that such a discrepancy is due to the differences in the regulatory mechanisms governing neurogenesis in mouse versus *Xenopus*. A case in point is in the developing chick retina, where NeuroD and NSCL-1 are clearly not expressed in the same cells (Li *et al.*, 1999). This observation argues for a parallel, rather than a linear transcriptional cascade (Ngn1→NeuroD→NSCL-1), in retinal neurogenesis in the chick. It has been also reported that NeuroD can activate adenylate kinase 1 and NeuroD2 transcription in PC12 cells and trophoblast cells, respectively (Noma *et al.*, 1999; Westerman *et al.*, 2004).

It is worthwhile pointing out that few of these genes have been confirmed to be the genuine downstream targets of NeuroD in NeuroD-null mice (by demonstrating decreased expression or absence of expression). The difficulty comes partly from the fact that there may be other compensatory mechanisms to activate these genes in knock-out mice. Because of the tissue- and stage-specific nature of NeuroD function, it is also possible that not all of these candidate genes are controlled by NeuroD at the same time. More careful and detailed gain- and loss-of-function analyses will provide the relevance of these downstream target genes (and undoubtedly other unknown target genes) in NeuroD function.

Proteins that interact with NeuroD Apart from the well-known E proteins that hetero-dimerize with NeuroD, several other proteins that can regulate the NeuroD activity by protein-protein interaction have been identified. Huntingtin-associated protein 1 (HAP1) and mixed-lineage kinase 2 (MLK2), both of which are known to interact with huntingtin (Htt), were identified by their interaction with NeuroD in a yeast two-hybrid screen and in the N2A neuroblastoma cell line (Marcora *et al.*, 2003). MLK2 can phosphorylate NeuroD and stimulate its activity, while Htt and HAP1 facilitated activation of NeuroD by MLK2, as assayed by ectopic neurogenesis in *Xenopus* embryos. Because Htt had no effect on NeuroD by itself but enhanced NeuroD activity in the presence of HAP1 and MLK2, it was suggested that Htt, together with HAP1, may function in the cytoplasm as a scaffold for activation of NeuroD by MLK2 (Marcora *et al.*, 2003).

Other groups have identified NeuroD as a partner of a homeobox gene Six3 (Tessmar *et al.*, 2002) and of the protein called small heterodimer partner (SHP) (Kim *et al.*, 2004b) by a yeast two-hybrid screen. Six3 plays a critical role in the development of the eye and forebrain structures (Loosli *et al.*, 1999; Wallis *et al.*, 1999). In *Xenopus*, NeuroD and Six3 were co-expressed during cell fate determination and differentiation in the retina (Tessmar *et al.*, 2002). XSix3 could interact with bHLH proteins XATH3 and XATH5 as well as XNeuroD. However, the functional relevance of these protein-protein interactions in eye development has not been determined. SHP, an atypical orphan nuclear receptor, lacks a conventional DNA binding domain. When co-expressed with various nuclear receptors, it repressed the transcriptional activity of these receptors, acting much like the negative regulators in the bHLH paradigm (Johansson *et al.*, 2000; Lee *et al.*, 2000b; Seol *et al.*, 1996). *In vitro* and *in vivo* protein interaction studies showed that SHP interacts with NeuroD, but not with its heterodimer partner E47 (Kim *et al.*, 2004b). SHP can repress NeuroD-mediated reporter activity and the p300-enhanced transcriptional activity of NeuroD by interfering with coactivator p300 that binds to NeuroD (Kim *et al.*, 2004b).

Cyclin D has been shown to repress NeuroD activity by indirect interaction with NeuroD (Ratineau *et al.*, 2002). The D-type cyclins have been implicated as negative regulators of transcriptional activity of a number of proteins including the myogenic bHLH proteins, myogenin and MyoD (Skapek *et al.*, 1995), v-Myb (Ganter *et al.*, 1998), and Sp1 (Adnane *et al.*, 1999). Recent data demonstrate that Cyclin D1 functions as a transcriptional repressor of NeuroD. *In vitro* binding assays showed that Cyclin D1 does not interact directly with NeuroD, but is recruited instead to the NeuroD-containing complexes through direct binding to p300 (Ratineau *et al.*, 2002). In the intestine, Cyclin D1 expression is restricted to proliferating cells in the intestinal crypts, whereas the secretin-expressing cells are found only in the villi (Chandrasekaran *et al.*, 1996). Thus, Cyclin D1 repression of NeuroD may contribute to the temporal and spatial differentiation of secretin-expressing enteroendocrine cells.

Id proteins, originally identified as a family of dominant inhibitors of bHLH activity, resemble bHLH transcription factors but lack the basic DNA binding domain (Benezra *et al.*, 1990). They bind to and sequester general bHLH proteins, preventing the heterodimerization between the ubiquitously expressed E proteins and tissue-specific bHLH proteins, such as Ngns and NeuroD. Indeed, Id2, Id3, and Id4 were able to inhibit NeuroD activity in *Xenopus* embryos injected with *neuroD* mRNA (Liu and Harland, 2003). It has also been reported that coexpression of Id2 and NeuroD inhibits the stimulatory activity of NeuroD in HIT and HeLa cells (Ghil *et al.*, 2002). Although biochemical evidence is lacking for NeuroD-Id interaction, it is likely that these proteins interact *in vivo*.

Post-translational regulation of NeuroD To understand how NeuroD regulates downstream target genes that ultimately control cell survival and differentiation of various cell types, it is important to determine how NeuroD activity is regulated in different cellular contexts. The importance of NeuroD phosphorylation in the control of its transcriptional activity during neurogenesis is emerging. Evidence from *Xenopus* injection experiments suggests that phosphorylation of NeuroD may be inhibitory and that GSK3β

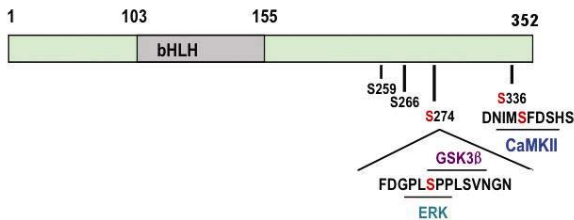

Fig. 6. Schematic diagram of NeuroD protein. The bHLH region (grey box) contains an ERK consensus docking domain. In both *Xenopus* and mouse NeuroD, serine 274 is found within an ERK consensus phosphorylation site and a GSK3β consensus phosphorylation site.

may be involved in NeuroD phosphorylation (Marcus *et al.*, 1998; Moore *et al.*, 2002). When overexpressed in *Xenopus*, GSK3β inhibits the formation of *N-tubulin* positive neuronal precursors. Conversely, a dominant-negative kinase dead form of GSK3β expands this population (Marcus *et al.*, 1998). Furthermore, co-injection of *GSK3β* and *NeuroD* completely inhibits NeuroD-induced ectopic neurogenesis (Marcus *et al*., 1998; Moore *et al.*, 2002). Moore *et al.* (2002) have recently demonstrated that serine to alanine mutation of the GSK3β phosphorylation consensus site at serine 274 (S274) of NeuroD relieved the repression of its neurogenic activity by GSK3β in *Xenopus* retinal neurons.

Interestingly, serine 274 of NeuroD is flanked by a GSK3β consensus phosphorylation sequence, as well as a consensus phosphorylation sequence for ERK (Fig. 6). Indeed, it has been shown that serine 162, which is in the conserved 40 amino acid domain following helix 2, and serines 259, 266 and 274, which are in the transactivation domain of NeuroD, can be phosphorylated by ERK2 (Khoo *et al.*, 2003). Interestingly, phosphorylation of NeuroD at serines 259, 266 and/or 274 appears to have opposite effects on activity in different types of cells or tissues. When these serines are mutated to alanines, which cannot be phosphorylated, mouse NeuroD is less able to induce transcriptional activation of the insulin gene in INS-1 insulinoma cells following glucose stimulation (Khoo *et al.*, 2003). In contrast, *Xenopus* NeuroD containing the same serine to alanine mutations has an increased ability to induce ectopic neurogenesis in *Xenopus* embryos (Dufton *et al.*, 2005). It is possible that phosphorylation of S274 could be under control of these two kinases, which may mediate opposite effects such that phosphorylation of S274 by GSKβ is inhibitory to NeuroD activity in neuronal cells, while phosphorylation of the same residue by ERK is stimulatory to NeuroD activity in β cells.

The molecular mechanisms that underlie the inhibitory vs. stimulatory effects of NeuroD phosphorylation may be quite different. By studying the glucose responsiveness of MIN6 beta cells, Petersen *et al.* (2002) found that phosphorylation of S274 activates NeuroD by changing its subcellular localization from the cytoplasm to the nucleus, and that this process is dependent on the MEK-ERK signaling pathway. On the other hand, *Xenopus* NeuroD with the S259A, S266A and/or S274A mutations accumulates to a much greater extent than wild-type NeuroD in the ectopic neurogenesis assay (Dufton *et al.*, 2005). These two types of changes are consistent with the decreased activity of mutant mouse NeuroD in insulinoma cells and the increased activity of mutant *Xenopus* NeuroD in ectopic neurogenesis. Taken together, these data suggest that the consequences of NeuroD phosphorylation at the molecular and functional levels are highly context-dependent.

CaMKII-mediated phosphorylation of NeuroD at serine 336 is another physiologically important modification of NeuroD. In cerebellar granule cells, neuronal activity induces the CaMKII-mediated phosphorylation of NeuroD at S336, and this event is necessary for the resulting dendritogenesis in these neurons (Gaudillière *et al.*, 2004). This function is activity dependent, but is separable from the transcriptional activity of NeuroD, suggesting that NeuroD is regulated in various ways to perform different functions. This finding also demonstrates that NeuroD plays an important role in mature neurons that is distinct from its role as a survival factor in differentiating cells. As we embark on dissecting the molecular mechanisms of NeuroD, it is critical to understand how NeuroD activity can be differentially modulated in different cellular contexts, and what effects this might have on cell physiology and target gene specificity.

NeuroD in adult neurogenesis

The hippocampus of the adult mammalian brain contains multipotent neural stem cells, especially in the dentate gyrus. These adult stem cells can self-renew and differentiate into neurons and glial cells, such as astrocytes and oligodendrocytes. This capability may be crucial for the ability of the adult hippocampus to store new memories, clear out old memories or repair itself from damage, such as that produced by the Aβ peptide that plays a key role in the development of Alzheimer's Disease (AD) (Deisseroth *et al.*, 2004; Jin *et al.*, 2004). Hence, there is great interest in understanding the molecular mechanisms that control the behavior and fate specification of these cells. As mentioned above, NeuroD plays a critical role in the survival and differentiation of the dentate gyrus of the hippocampus during development (Miyata *et al.*, 1999). Because of this reason, a number of studies have investigated whether it plays a similar role in adult neural stem cells.

NeuroD is expressed during the differentiation of adult neural stem cells to dentate gyrus granule cells. For example, when cultured FGF-2 responsive neural stem cells from adult rat hippocampus were treated with retinoic acid, NeuroD was immediately upregulated, concurrent with exit from the cell cycle and differentiation of the cells

into immature neurons that also expressed TrkA,B,C and p75NGFR (Takahashi *et al.*, 1999). Similarly, when adult rat hippocampal stem cells were treated with soluble neural cell adhesion molecule (NCAM) to induce differentiation to the neuronal lineage, Ngn1, NeuroD and other markers of immature neurons were upregulated (Shin *et al.*, 2002). A study of cells newly born *in vivo* in the adult rat hippocampus (marked by incorporation of BrdU) showed that within one day, the BrdU-labeled cells included NeuroD-positive immature neurons and GFAP-positive glial cells, implicating NeuroD in the differentiation of the newly generated cells (Seki, 2002a). It has not yet been determined whether adult neurogenesis will be affected in the absence of NeuroD.

Neurogenesis, which is ongoing in the adult mammalian hippocampus, can be modulated by factors such as aging, epilepsy, ischemia and Alzheimer's disease (Elliott *et al.*, 2001; Jin *et al.*, 2004; Kawai *et al.*, 2004; Pleasure *et al.*, 2000; Uittenbogaard and Chiaramello 2000). Although there is still much uncertainty about how neurogenesis is regulated by these various conditions, NeuroD seems to play a role in the process. For example, although there are fewer immature neurons that express NeuroD, PSA-NCAM and CRMP-4 in the dentate gyrus of old rodents versus young rodents, the qualitative relationship between these markers of neuronal differentiation remains the same, suggesting that only a quantitative change in regulation has occurred (Seki, 2000b). When rats were subjected to global ischemia or NMDA receptor blockade, both of these treatments induced newborn cells in the adult rat hippocampus to express NeuroD transiently as they differentiated into mature neurons (Kawai *et al.*, 2004; Okuyama *et al.*, 2004). Treatment of adult hippocampal progenitor cells with valproic acid, an inhibitor of histone deacetylase, induced neuronal differentiation while suppressing the glial fate, and this process was dependent on increased expression of NeuroD, suggesting that changes in histone acetylation may underlie the change in expression of NeuroD during adult hippocampal neurogenesis (Hsieh *et al.*, 2004). Similarly, there is increased expression of NeuroD and other markers of neuronal differentiation, such as doublecortin, PSA-NCAM and TUC-4, in the hippocampus of AD brain versus normal human brain (Jin *et al.*, 2004). Finally, recent data indicate that excitatory stimuli *per se* can potently induce neurogenesis in the adult hippocampus, such that NeuroD expression is increased leading to the neuronal fate, while expression of the glial fate genes Hes1 and Id2 is repressed (Deisseroth *et al.*, 2004). This coupling of excitation and neurogenesis in the adult hippocampus could be an important mechanism for both the clearance of old memories and the storage of new memories, which are key activities in the hippocampus.

Summary

What began as a search for new bHLH proteins in differentiated embryonic stem cell tumors and in insulinoma cells led to the discovery of NeuroD and its hamster ortholog BETA2, respectively (Lee *et al.*, 1995; Naya *et al.*, 1995). Since then, we have learned much about NeuroD from various experimental systems. Both gain- and loss-of-function studies have revealed many expected and unexpected results. Among the expected results were the defects in differentiation and survival (due to failure to differentiate) of neurons and pancreatic endocrine cells. However, the specificity and extent of the phenotype was surprising. It was quite stunning not to see an obvious phenotype in many other neurons that seem to express high levels of NeuroD (e.g., olfactory neurons), while those that were affected by the absence of NeuroD were, in many cases, completely eliminated. What would be the molecular basis for such specificity of the phenotype? NeuroD was also one of the first neuronal transcription factors to have been shown to control pancreatic endocrine cell development and survival. While a pancreatic phenotype was also expected based on the work published by Naya *et al.* (1995), its variable nature in different genetic backgrounds was surprising. What would be the epigenetic factors that change the viability of NeuroD-null mice? We are again surprised at the complexity of the regulation of NeuroD activity at the protein level. In particular, modification of the same serine residues in the carboxy-terminus can have opposite effects on the function of NeuroD (Dufton *et al.*, 2005). Such context-dependent regulation of NeuroD adds another layer of complexity in the study of NeuroD. We have just begun to understand that NeuroD may also be interacting with proteins that can associate with Huntingtin protein (Marcora *et al.*, 2003) to form a higher order protein complex. Could NeuroD be mediating the role of Huntingtin protein during development? Is NeuroD involved in the manifestation of Huntington's disease? Is it also required during neurogenesis in the adult brain?

Many questions still remain unanswered, partly due to the lack of a decent anti-NeuroD antibody that detects all endogenously expressed NeuroD. The difficulty of obtaining a reliable anti-NeuroD antibody could be attributed to the extensive modification that NeuroD is subjected to (as described in this review). It is also desirable to generate a *neuroD* conditional knock-out strain of mice to address the temporal and spatial requirement for NeuroD during development. It seems clear that NeuroD has functions in early differentiating cells as well as in mature cells. What is evident from the complete NeuroD-null-mice is the earliest phenotype, namely its role as a survival factor in the affected tissues. Stage- and tissue-specific deletion of NeuroD will allow one to address the later roles of NeuroD in developing mice. It will also enable one to address the role of NeuroD in the hippocampal formation and its function through physiological and behavioral studies.

As evidenced by its association with many disease con-

ditions, such as diabetes, ataxia, deafness, and possibly learning and memory function, NeuroD is an important molecule whose thorough study is warranted. We predict that future research on NeuroD will continue to reveal surprises about how it acts and how it is regulated, and that such knowledge will aid in our ability to understand and treat these diseases.

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