γ-Secretase-regulated mechanisms similar to Notch signaling may play a role in signaling events, including APP signaling, which leads to Alzheimer's disease

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Abstract

Although γ-secretase was first identified as a protease that cleaves amyloid precursor protein (APP) within the transmembrane domain and produces Aβ peptides, which are thought to be pathogenic in Alzheimer's disease (AD), the physiological functions of γ-secretase have not been fully elucidated. In the canonical Notch signaling pathway, intramembrane cleavage by γ-secretase serves to release an intracellular domain of Notch that has activity in the nucleus through binding to transcription factors. Recently, it was demonstrated that many type 1 transmembrane proteins, including Notch, Delta, and APP, are substrates for γ-secretase, and the intracellular domains of these substrates are released from the cell membrane by γ-secretase. The common enzyme γ-secretase modulates proteolysis and the turnover of possible signaling molecules has led to the attractive hypothesis that mechanisms similar to Notch signaling contribute widely to proteolysis-regulated signaling pathways. It is likely that APP also has a signaling mechanism, although the physiological functions of APP have not been elucidated. Indeed, we have shown that the intracellular domain of APP (AICD) alters gene expression and induces neuron-specific apoptosis. These results suggest that APP signaling responds to the onset of AD. Here, we review the possibility of γ-secretase-regulated signaling, including APP signaling, which leads to AD.

Keywords: Alzheimer's disease, γ-secretase, amyloid precursor protein, Notch, signaling, the intracellular domain

Abbreviations

AD, Alzheimer’s disease; APP, amyloid precursor protein; AICD, the intracellular domain of APP; bHLH, basic helix-loop-helix; C. elegans, Caenorhabditis elegans; Dll, Delta-like protein; Dll1IC, the intracellular domain of Dll1; EGF, epidermal growth factor; GO, Gene Ontology; Hes, Hairy/Enhancer of split; JM, juxtamembrane; PS: presenilin; ICD, intracellular domain; NICD, the intracellular domain of Notch; RA, all-trans-retinoic acid; RIP, the regulated intramembrane proteolysis; TM, transmembran

Introduction

Both Notch receptors and their ligands are evolutionarily conserved single transmembrane spanning proteins (type 1 transmembrane proteins; the amino terminus is extracellular and the carboxy terminus is cytoplasmic) that mediate the fates of numerous cells in both invertebrates and vertebrates (Artavanis-Tsakonas et al., 1995; Lewis, 1998). Notch signaling mediates somitogenesis, differentiation of lymphoid cells, as well as neural development, and dysregulation of Notch signaling has been shown to lead to developmental defects or cancer in mammals (Bolos et al., 2007). For example, cells expressing the major ligand Delta inhibit the neural determination of neighboring Notch-expressing cells during neurogenesis in *Drosophila*. Similar inhibition of vertebrate neurogenesis has also been reported. Notch signaling has attracted interest because of its relation to Alzheimer's disease (AD). The *Caenorhabditis elegans* (*C. elegans*) gene sel-12, which appears to facilitate the reception of signaling mediated by lin-12 (*C. elegans* Notch), was identified by screening for a suppressor of lin-12 gain-of-function mutation (Levitan and Greenwald, 1995). As sel-12 is thought to be a counterpart of human presenilin (PS), which is a catalytic component of γ-secretase and has been implicated in AD, the Notch signaling pathway may have some relation to AD. Indeed, as described below, it has become clear that the Notch signaling pathway is controlled by γ-secretase-mediated proteolysis.

The Notch signaling pathway is quite unique in that it is controlled by proteolytic cleavage reactions (Artavanis-Tsakonas et al., 1999; Justice and Jan, 2002). In the canonical Notch signaling pathway, ligands bind to the extracellular domain of Notch on neighboring cells, and trigger sequential proteolytic cleavage. Finally, the intracellular domain (ICD) of Notch (NICD) is released from the cell membrane by γ-secretase and translocates to the nucleus to modulate gene expression through binding to transcription factors. Therefore, γ-secretase plays a central regulatory role in Notch signaling.

γ-Secretase was first identified as a protease that cleaves amyloid precursor protein (APP) within the transmembrane domain and produces Aβ peptides (Haass and Selkoe, 1993), which are thought to be pathogenic in AD (Hardy, 1997; Selkoe, 2001). However, the physiological functions of γ-secretase have not been determined (Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007). Recently, it was demonstrated that more than five dozens of type 1 transmembrane proteins are substrates for γ-secretase (McCarthy et al., 2009) and their ICDs are also released from the cell membrane. These observations that the common enzyme, γ-secretase, modulates proteolysis and the turnover of possible signaling

molecules have led to the attractive hypothesis that mechanisms similar to the Notch signaling pathway may contribute widely to γ-secretase-regulated signaling pathways (Koo and Kopan, 2004; Nakayama et al., 2008a). Indeed, it has been reported that the intracellular domain of Dll1 (Dll1IC) is also released from the cell membrane by γ-secretase and translocates to the nucleus (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003). Moreover, we have shown that Dll1IC binds to Smads, which are transcription factors for the TGF-β/Activin signaling pathway, and enhances transcription of specific genes leading to neuronal differentiation (Hiratochi et al., 2007).

Interestingly, it has also been reported that the intracellular domain of APP (AICD), which is released from the cell membrane by γ-secretase, translocates to the nucleus (Cupers et al., 2001; Gao and Pimplikar, 2001; Kimberly et al., 2001) and may function as a transcriptional regulator (Cao and Sudhof, 2001; Guenette, 2002). Several AICD-interacting proteins, which are thought to regulate AICD stability and cellular localization, have been identified (Zheng and Koo, 2006; Muller et al., 2008) and some models of APP signaling have also been proposed. For example, AICD recruits Fe65 protein and translocates into the nucleus where the AICD-Fe65-Tip60 ternary complex may control transcription of target genes (Slomnicki and Lesniak, 2008). As the apoptotic potential of AICD has been demonstrated (Zheng and Koo, 2006; Muller et al., 2008), it is likely that APP signaling induces cell death, which leads to AD.

To explore APP signaling, we established embryonic carcinoma P19 cell lines overexpressing AICD (Nakayama et al., 2008b). Although neurons were differentiated from these cell lines with all-*trans*-retinoic acid (RA) treatment, AICD expression induced neuron-specific apoptosis. The effects of AICD were restricted to neurons, with no observed effects on non-neural cells. Furthermore, we evaluated changes in gene expression induced by AICD through this process of neuron-specific cell death using DNA arrays (Ohkawara et al., in press). The results indicated that AICD induces dynamic changes in the gene expression profile. Therefore, it is likely that APP also has a signaling mechanism and that AICD may play a role in APP signaling, which leads to AD.

In this review, we focus on the possibility that γ-secretase-regulated mechanisms similar to the Notch signaling pathway may play a role in signaling events involving type 1 transmembrane proteins. We also discuss the possibility that APP signaling induces dynamic changes in gene expression, which may be closely correlated with AICD-induced neuron-specific apoptosis, leading to AD.

Notch signaling pathway

The typical Notch gene encodes about a 300 kDa type 1 transmembrane protein (Wharton et al., 1985). The extracellular domain of *Drosophila* Notch and some vertebrate homologs contain 36 tandem epidermal growth factor (EGF)-like repeats. The 11th and 12th EGF-like repeats are necessary and sufficient for binding to its ligands (Rebay et al., 1991). NICD has six tandem ankyrin-like (CDC10) repeats, a nuclear localization signal, and a PEST sequence (Wharton et al., 1985). While *Drosophila* has only one Notch isoform, four Notch isoforms (Notch1 to 4) have been identified in mammals. TAN1 (Notch1), which was the first mammalian homolog of Notch to be identified, was cloned as a gene responsible for human T cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). Notch2 was also identified as an oncogene of cat thymic lymphoma (Rohn et al., 1996). Interestingly, CADASIL, in which the main symptom is cerebral vascular disorder, is caused by a mutation of the Notch3 gene (Joutel et al., 1996). In addition, Notch4 is a cellular counterpart of the oncogene of mouse mammary tumor virus (int3) (Sarkar et al., 1994).

Drosophila has two different ligands, Delta (Kopczynski et al., 1988) and Serrate (Fleming et al., 1990). However, two families of ligands, Delta-like protein (Dll 1, 3, and 4) (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Shutter et al., 2000) and Jagged (Jagged 1 and 2) (Lindsell et al., 1995; Shawber et al., 1996), have been identified in mammals. The extracellular domains of all of these ligands also contain EGF-like repeats. All of these ligands also have a cysteine-rich conservative motif called the DSL (Delta: Serrate: Lag-2) domain (Tax et al., 1994), which plays an essential role in binding to Notch (Henderson et al., 1997).

As shown in Fig. 1, in the canonical Notch signaling pathway, ligands bind to the extracellular domain of Notch on neighboring cells and trigger sequential proteolytic cleavage reactions. This process is called the regulated intramembrane proteolysis (RIP) mechanism (Brown et al., 2000). The RIP mechanism requires sequential cleavage steps to occur within the juxtamembrane (JM) and transmembrane (TM) domains, and these steps are carried out by metalloproteases such as ADAM protease and γ-secretase, respectively (Selkoe and Kopan, 2003). RIP serves to release NICD from the cell membrane and NICD translocates to the nucleus. In the nucleus, NICD has activity through binding to transcription factors. Members of the CSL family (CBF1/RBP-jκ in mammals, Su(H) in *Drosophila*,

and Lag-1 in *C. elegans*) are major downstream transcription factors of Notch signaling (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999). NICD binds to CSL transcription factors; six tandem ankyrin-like repeats lying in NICD are essential but not sufficient for binding to CSL transcriptional factors (Roehl et al., 1996). As NICD also binds to Mastermind-like proteins (MAML family in mammals) (Wu et al., 2000), the CSL-NICD-MAML complex is formed. As a result of forming these complexes, co-repressors are dispersed from CSL and co-activators such as P/CAF and P300 are recruited by these complexes (Wu et al., 2000; Wallberg et al., 2002). Through this process, the CSL complexes are converted from transcriptional repressors to activators. Finally, CSL complexes bind to the *cis*-acting DNA sequences of target genes and promote the transcriptional activity of these genes.

The best-established target genes for Notch signaling are Hes (Hairy/Enhancer of split in *Drosophila*) genes, which encode the basic helix-loop-helix (bHLH) transcription factors (Kageyama et al., 2007). Seven mammalian Hes, designated Hes1 to Hes7, have been identified to date, although the mouse does not have Hes4. Hes is known to act as a repressor for tissue-specific gene transcription (Kageyama et al., 2007). Hes1 and Hes5 have been shown to bind to their target DNA sequence, called the N box (CACNAG), by forming homodimers or heterodimers with Hey (Hes-related with YRPW motif) 1 or Hey2, and to recruit histone deacetylase (HDAC) activity by association with Groucho, resulting in transcriptional repression (Akazawa et al., 1992; Leimeister et al., 1999; Iso et al., 2001). Furthermore, they associate with ubiquitously expressed bHLH factors (E proteins) and prevent tissue-specific bHLH factors, such as Mash1, from forming functional complexes with E protein (Kageyama et al., 2007). In this manner, Notch represses the differentiation of cells to specific lineages. In addition, Delta expression is induced by proneural genes that encode bHLH transcriptional factors, while multiple POU-binding factors are also important for Delta expression (Nakayama et al., 2004). Thus, Notch signaling strongly inhibits Delta expression.

APP

APP was first identified as a cDNA cloned using a partial amino acid sequence of Aβ fragment from the amyloid plaque of AD brains (Kang et al., 1987). This cDNA encodes a type 1 membrane protein expressed in many tissues and that is concentrated in the synapses of neurons. In humans, the APP gene contains at least 18 exons in a total length of 240 kb (Yoshikai et al., 1990), and several alternative

splicing isoforms of APP have been observed ranging in length from 365 to 770 amino acids, differing mainly in the absence (APP-695 which is predominately expressed in neurons) or presence (APP-751 and APP-770) of a Kunitz protease inhibitor (KPI) domain located toward the N-terminus of the protein (Sisodia et al., 1993). Counterparts of this protein have been identified in other organisms, such as *Drosophila* (Rosen et al., 1989; Luo et al., 1990), *C. elegans* (Daigle and Li, 1993), and all mammals (Coulson et al., 2000). As mentioned below, APP undergoes sequential proteolytic cleavage reactions to yield the extracellular fragment, intracellular fragment (AICD), and Aβ fragment located in the membrane-spanning domain, which is thought to be the main cause of the onset of AD.

Although APP has central roles in AD (Hardy, 1997; Selkoe, 2001), the physiological functions of this protein have yet to be fully elucidated (Zheng and Koo, 2006). While it has been reported that APP acts as a cell adhesion molecule for cell-cell interaction (Soba et al., 2005), and as a neurotrophic and/or synaptogenic factor (Hung et al., 1992; Bibel et al., 2004; Leyssen et al., 2005), the hypothesis that APP is a cell-surface receptor is interesting from the signaling perspective. Several lines of evidence support this suggestion; *e.g.*, Aβ can bind to APP and thus may be a candidate ligand for APP (Lorenzo et al., 2000). It has also been reported that F-spondin (Ho and Sudhof, 2004) and Nogo-66 receptor (Park et al., 2006) could bind to the extracellular domain of APP and regulate Aβ production. In addition, the extracellular domain of APP can potentially interact in *trans* suggesting that APP molecules can bind to each other in a homophilic manner (Wang and Ha, 2004).

It has been reported that AICD translocates to the nucleus and may function as a transcriptional regulator. Indeed, APP homologs show significant evolutionary sequence conservation in the intracellular domain (Nakayama et al., 2008b), which may reflect the functional importance of AICD. Moreover, AICD is thought to form complexes with nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60 (Cao and Sudhof, 2001). These complexes can bind to the *cis*-acting DNA sequence of the tetraspanin protein KAI1 gene and regulate transcriptional activity (Baek et al., 2002). In addition, the Aβ region of ~~this protein~~ APP is not well conserved across species.

γ-Secretase

Although γ-secretase was first identified as a protease that cleaves APP within the TM domain and produces Aβ peptides (Haass and Selkoe, 1993), which are thought to be pathogenic in AD, the

physiological functions of γ-secretase have not also been fully elucidated (Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007). The γ-secretase complex is composed of PS, Nicastrin, anterior pharynx defective-1 (Aph-1), and PS enhancer-2 protein (Pen-2) (Iwatsubo, 2004; Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007). PS is recognized as a catalytic component of the γ-secretase complex, and the two PSs, PS1 (Sherrington et al., 1995) and PS2 (Rogaev et al., 1995; Levy-Lahad et al., 1995), were identified by genetic linkage analyses as the genes responsible for several forms of early-onset familial AD. Nicastrin, a single-pass membrane protein, is thought to be important for recognizing the substrate proteins (Yu et al., 2000). Its extracellular domain resembles an aminopeptidase, but lacks catalytic residues, and can interact with the N-terminal stubs of γ-secretase substrates after ectodomain shedding (Shah et al., 2005). Thus, shedding of the type 1 transmembrane protein may be essential to produce a free N-terminus of the protein retained in the membrane that can then be recognized by Nicastrin. Aph-1 is thought to act as a scaffold during the assembly process of the γ-secretase complex and Pen-2 is thought to be a trigger for the proteolytic cleavage of PS to regulate PS activity (Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007).

γ-Secretase cleaves a diverse set of type 1 transmembrane proteins, which have shed their extracellular domains, in a sequence-independent manner (Struhl and Adachi, 2000). As reflected by the flexible sequence specificity of γ-secretase activity, more than five dozens of type 1 transmembrane proteins have been reported as substrates of γ-secretase (McCarthy et al., 2009). As shown in Table 1, these substrates also have diverse functions, such as cell fate determination (Notch, Delta, and Jagged), cell-to-cell adhesion (N-cadherin, E-cadherin and CD44), synaptic adhesion (Nectin-1α), ion conductance regulator (β2 subunit of the voltage-gated sodium channel), axon guidance and tumor suppression (DCC), neurotrophin receptor (P75NTR), and its homolog (NRADD), lipoprotein receptor (ApoER2), and growth factor-dependent receptor tyrosine kinase (ERBB4).

It has been reported that several γ-secretase substrates follow the RIP mechanism and their intracellular domains are released from the cell membrane (Koo and Kopan, 2004; Selkoe and Wolfe, 2007). As shown in Fig. 2, the process of sequential proteolytic cleavages of APP is quite similar to that of Notch and follows the RIP mechanism. Cleavage of APP by α-secretase (Esch et al., 1990) or β-secretase (Vassar et al., 1999) at the α-site or β-site, respectively, within the JM region results in the

shedding of nearly the entire extracellular domain and generates membrane-tethered α or β-carboxy terminal fragments (CTFs). Several zinc metalloproteinases, such as TACE/ADAM protease (Buxbaum et al., 1998; Lammich et al., 1999), and the aspartyl protease BACE2 can cleave APP at the α-site (Farzan et al., 2000), while BACE1 (β-site APP cleaving enzyme) cleaves at the β-site (Vassar et al., 1999). After shedding the extracellular domain, the remaining stub is further cleaved at least twice within the TM domain at γ- and ε-sites by γ-secretase, producing either p3 peptide (in combination with α-secretase) or Aβ (in combination with BACE1), respectively, and AICD (Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007). In addition, AICD was found to be a substrate of caspase and is cleaved at the group III caspase consensus sequence (16 aa from the membrane border within AICD).

Furthermore, it has also been shown that the process of sequential proteolytic cleavage of CD44, which is important for immune system function, is very similar to that of Notch and APP, as shown in Fig. 2 (Nagase et al., in press). In addition, ICD of this protein (CD44ICD) also translocates to the nucleus.

As mentioned above, very similar to Notch, several γ-secretase substrates follow the RIP mechanism and their ICDs are released from the cell membrane. Moreover, these ICDs translocate to the nucleus. Therefore, these observations that the common enzyme, γ-secretase, modulates proteolysis and the turnover of possible signaling molecules have led to the attractive hypothesis that mechanisms similar to the Notch signaling pathway may contribute widely to γ-secretase-regulated signaling pathways (Koo and Kopan, 2004; Nakayama et al., 2008a).

Indeed, several groups have shown that Dll1 is cleaved sequentially by proteases, probably including ADAM and γ-secretase, and the intracellular domain of Dll1 (Dll1IC) is released from the cell membrane and translocates to the nucleus (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003). We have also shown that Dll1IC binds to Smads, which are transcription factors for TGF-β/Activin signaling pathway, and enhances transcription of specific genes leading to neuronal differentiation (Hiratochi et al., 2007). Therefore, these results suggest that Dll1 also has a signaling mechanism similar to Notch signaling.

What is the function of γ-secretase?

Contrary to the hypothesis that γ-secretase regulates the signaling pathways of certain membrane

proteins, Kopan and Ilagan (2004) proposed that γ-secretase is a proteasome of the membrane. They presented clear grounds for the thesis that several intracellular domains of these substrates, such as intracellular domains of Syndecan-3, Nectin-1α, p75, and DCC, all of which are released by γ-secretase, are rapidly degraded. Furthermore, ectodomain shedding seems to be constitutive for some substrates; only intramembrane cleavages of Notch (Schroeter et al., 1998), Delta (Hiratochi et al., 2007), Syndecan-3 (Schulz et al., 2003), and ERBB4 (Ni et al., 2001) have been shown to be enhanced by ligand binding. As AICD is also rapidly degraded (Cupers et al., 2001; Edbauer et al., 2002), it is usually difficult to detect intracellular domains of these substrates by western blotting *in vivo*. In addition, they noted that considerable evidence supporting the signaling hypothesis has been obtained in overexpression assays that are not quite the same as physiological conditions. Based on these observations, they proposed the proteasome hypothesis that the primary function of γ-secretase is to facilitate the selective disposal of type 1 membrane proteins (Kopan and Ilagan, 2004).

While the proteasome hypothesis of γ-secretase is persuasive, the signaling pathways of some substrates, such as Notch (Artavanis-Tsakonas et al., 1999; Selkoe and Kopan, 2003; Koo and Kopan, 2004), have also been established. Therefore, it is possible that γ-secretases are not uniform complexes and different γ-secretase complexes exist in different combinations with components such as Aph1, Pen2 and/or presenilin isoforms, and these different complexes may have different cellular functions, such as signaling or degradation (Kopan and Ilagan, 2004). In addition, from the viewpoint of signaling mechanism, it is not clear whether a large proportion of the intracellular domains of these substrates that are released by γ-secretase are required for signaling events. Generally, substrates of γ-secretase such as APP are considerably more abundant proteins than transcription factors, which are usually rare molecules. Therefore, although a large proportion of the intracellular domains of these substrates are rapidly degraded, it seems like that a small amount of the remaining protein may fulfill its function with a small quantity of transcription factors. Thus, the majority of the intracellular domains of these substrates may be degraded and only a small proportion may play a role in signaling.

In relation to this issue, an interesting model has been proposed in which a specific stimulus triggers Fe65 binding to AICD to prevent AICD degradation by the proteasome (Buoso et al., 2010). AICD/Fe65 complexes, alone or with Tip60, translocate to the nucleus, where they control the expression of certain gene in association with Tip60. On the other hand, other different stimuli induce phosphorylation of

AICD, which strongly inhibits binding to Fe65 and translocation to the nucleus. Phosphorylated AICD left in the cytosol is rapidly degraded probably by insulin-degrading enzyme (IDE) (Edbauer et al., 2002), which is ubiquitously expressed, with its highest expression in the liver, testes, muscle, and brain. Indeed, it has been shown that when AICD are phosphorylated at Thr^{668} in APP-695 isoform, AICD cannot bind to Fe65 (Kimberly et al., 2005).

From the viewpoint of the signaling mechanism, it is likely that AICD has no effect in the normal brain, because almost all AICD is rapidly degraded. However, both AICD and Aβ are overproduced in the AD brain. Therefore, although the majority of AICD is degraded, it is possible that a small amount of the remaining AICD may fulfill its function and cause neuron-specific cell death in the AD brain.

AICD induces neuron-specific apoptosis

There is accumulating evidence in support of the concept that AICD and its interacting proteins contribute to AD through APP signaling. For example, transgenic mice overexpressing AICD/Fe65 showed abnormal activity of glycogen synthase kinase 3 beta (*Gsk3b* protein) (Ryan and Pimplikar, 2005), leading to hyperphosphorylation and aggregation of TAU, resulting in microtubule destabilization, and reduction of nuclear β-catenin levels causing a loss of cell-cell contact mechanisms that may contribute to neurotoxicity in AD. Subsequent neurodegeneration and working memory deficits were also observed in these transgenic mice (Ghosal et al., 2009). In other experiments, similar transgenic mice exhibited abnormal spiking events in their electroencephalograms and susceptibility to kainic acid-induced seizures independent of Aβ (Vogt et al., 2009). In addition, it was also reported the function of c-Abl kinase in the transcriptional regulation of AICD and demonstrated that c-Abl modulates AICD-dependent cellular responses, transcriptional induction, as well as apoptotic responses (Vazquez et al., 2009). Interestingly, elevated AICD levels have also been reported in AD brains (Ghosal et al., 2009). Therefore, it is likely that AICD-dependent differential gene expression, reflecting the involvement of APP signaling, induces cell death, which may lead to AD pathology.

To explore APP signaling, we established several AICD-overexpressing P19 cell lines (Nakayama et al., 2008b). Undifferentiated AICD-overexpressing P19 cells retained epithelial cell-like morphology as well as control cells, while the differentiated cells became round and showed a bipolar morphology with neurite extension. In addition, undifferentiated cells were negative for the neuronal marker Tuj1, while

almost all differentiated cells were Tuj1-positive. These results indicated that all of these cell lines could differentiate into neurons. Although neurons were differentiated from these cell lines after aggregation culture with all-*trans*-retinoic acid (RA) treatment, AICD expression induced neuron-specific cell death. Indeed, while neurons from control cells were healthy, almost all neurons from AICD-overexpressing P19 cells showed severe degeneration four days after induction of differentiation (Fig. 3). Furthermore, DNA fragmentation was detected, and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling (TUNEL)-positive cells were also Tuj1-positive neurons. Based on these results, we concluded that AICD can induce neuron-specific apoptosis (Nakayama et al., 2008b). The effects of AICD were restricted to neurons, with no observed effects on non-neural cells. Thus, although further studies are required, it is possible that AICD plays a role in APP signaling, which leads to AD.

AICD induces dynamic changes in the gene expression profile

If APP signaling occurs, AICD should alter expression of certain genes. To test this hypothesis and identify the genes involved in this process of neuron-specific cell death, we cultured both AICD-overexpressing P19 cells and control P19 cells and evaluated AICD-induced changes in gene expression at 3 time points during culture: the undifferentiated state, after 4 days of aggregation with RA (aggregated state), and 2 days after replating (differentiated state) (Ohkawara et al., in press). The levels of expression of 41,256 transcripts and transcript variants of more than 20,000 independent genes were monitored by DNA microarray analysis (Ohkawara et al., in press). As shown in Fig. 4, the expression of 277 genes showed up-regulation by more than 10-fold in the presence of AICD, estimated from the intensity of hybridization signals. Conversely, the expression of 341 genes showed down-regulation to less than one tenth of the original level (Fig. 4). Reverse transcription-polymerase chain reaction of 17 selected genes showed excellent agreement with the microarray results. These results served to validate those of DNA microarray analysis.

Several genes were strongly up-regulated in the presence of AICD (Table 2) (Ohkawara et al., in press). For example, protein tyrosine phosphatase receptor T (*Ptprt*) was undetectable at all sampling points in control P19 cells. However, AICD-overexpressing P19 cells showed strong expression of this gene at all sampling points: 906-fold, 204-fold, and 116-fold up-regulation, in undifferentiated,

aggregated, and differentiated states, respectively, estimated from the intensity of hybridization signals. The biological functions of these up-regulated genes are varied: *e.g.*, protein amino acid dephosphorylation (*Ptprt*), proteolysis (*Cpb1*), and cytoskeletal protein (*Myh1*).

In contrast to these up-regulated genes, AICD strongly inhibited the expression of several genes (Table 2) (Ohkawara et al., in press). For example, although hairy and enhancer of split 5 (*Hes5*) expression level was extremely low in undifferentiated control P19 cells, its expression was markedly increased through the process of neural differentiation: an increase of almost 300-fold estimated from the intensity of hybridization signals in the aggregated and differentiated states. However, this extreme induction through the differentiation process in control P19 cells could not be detected in AICD-overexpressing P19 cells (Table 2), indicating that AICD strongly inhibits this induction. Several genes, such as the sodium-dependent organic anion transporter (*Slc10a6*), nidogen 1 (*Nid1*), and an analog of Na^+-dependent glucose transporter 1 (*LOC213332*), were also strongly inhibited by AICD. Therefore, AICD mediates both up-regulation and down-regulation of many genes. Although further studies are required, it is likely that AICD plays an important role in APP signaling.

Gene Ontology (GO) analysis was performed, and these up-regulated and down-regulated genes were classified according to GO terms at several hierarchical levels (Ohkawara et al., in press). Although a few genes were classified into GO terms related to cell death, many genes were classified into GO terms unrelated to cell death. Furthermore, we evaluated AICD-induced changes in expression of genes thought to be involved in cell death in AD; however, we found no significant changes in expression of these genes. Therefore, it is likely that AICD does not directly induce the expression of genes involved in cell death. Although further studies are required to resolve the matter, it is likely that extreme dynamic changes in the gene expression described here disturb homeostasis of certain neurons and give rise to neuron-specific cell death. In addition, we also evaluated AICD-induced changes in expression of several genes encoding AICD-interacting proteins, which are thought to regulate AICD stability, cellular localization, and transcriptional activity; however, no significant changes were found.

There are many reported discrepancies regarding AICD target genes and AD-related genes

Several candidate target genes expression of which is affected by AICD have been reported. However,

there is still controversy regarding putative target genes with different groups reporting significant or no significant changes in expression of certain target genes. Such conflicting conclusions are thought to be due to studies in different cell culture systems or animal models. Reflecting these problems, our array data also conflicted with those reported previously; we found no significant correlation between our array data and those reported previously.

Several reports of AD-specific DNA microarray data are available, and we compared our results with these data. For example, Blalock et al. divided AD patients into 3 classes on the basis of cognition level: incipient, moderate, and severe. They compared the hippocampal expression profiles of these classes with those of normal subjects and extracted genes with different expression patterns (Blalock et al., 2004). We compared our data with their results and found no significant correlations.

Liang et al. studied the expression profiles of 6 regions of the brain (entorhinal cortex, hippocampus, medial temporal gyrus, posterior cingulate, superior frontal gyrus, and primary visual cortex) and compared the profiles of AD brains with those of normal brains (Liang et al., 2008). We also compared our data with these results, and no significant correlations were found.

When we found no significant correlation between our results and those reported in previous studies of AD brains, we compared the brain expression data reported by Blalock et al. and Liang et al. to each other. Surprisingly, we found no correlation between these 2 sets of data. It is likely that the cause of this discrepancy is variable quality of RNA isolated from the AD brain, because AD neurons are dying, and it is technically difficult to isolate high-quality RNA from dying cells. Therefore, AICD-expressing P19 cells described in this paper may be a more useful model not only for the study of APP signaling but also for the cellular and molecular study of AD.

Is Aβ sufficient to clarify all aspects of the onset of AD?

Autosomal dominant mutations in and around the Aβ region of APP cause hereditary early-onset AD (Goate et al., 1991), probably as a result of acceleration of proteolytic processing. In humans, the APP gene is located on chromosome 21 (Kang et al., 1987), which is abnormally present in an extra copy in individuals with Down's syndrome (trisomy 21). Almost all people with Down's syndrome also develop AD by 40 years of age, probably due to the gene dosage (Lott and Head, 2005). In addition, both PS1 and PS2 are recognized as a catalytic component of the γ-secretase complex (Iwatsubo, 2004) and were

identified by genetic linkage analyses as the genes responsible for familial AD (Sherrington et al., 1995; Rogaev et al., 1995; Levy-Lahad et al., 1995). Therefore, both APP itself and its proteolytic process may also be responsible for the onset of AD.

The amyloid hypothesis is widely accepted as the mechanism of the onset of AD. The traditional amyloid hypothesis is that overproduced Aβ forms insoluble amyloid plaques, which are commonly observed in the AD brain and are believed to be the toxic form of APP responsible for neurodegeneration (Hardy and Selkoe, 2002).

However, several questions about this hypothesis have recently been raised. One of the most significant arguments against the amyloid hypothesis is the presence of high levels of Aβ deposition in non-demented elderly people (Terry et al., 1999), suggesting that Aβ amyloid plaques are not toxic. Indeed, transgenic mice that overproduce Aβ mimic the amyloid deposition seen in AD brains but do not show any neurodegeneration (McGowan et al., 2003), although it has been reported that the soluble form of Aβ oligomers are toxic (Klein et al., 2001; Selkoe, 2002). Furthermore, several anti-amyloid drugs have failed in phase III clinical trials (Abbot, 2008). Therefore, some researchers suggest that AD may be caused by an APP-derived protein, but not necessarily Aβ-amyloid (Schnabel, 2009). Indeed, both extracellular fragment and AICD are generated at the same time as Aβ is being generated. From this point of view, Nikolaev et al. reported important results indicating that APP is a ligand of Death receptor 6 (DR6), which mediates cell death and is expressed at high levels in the human brain regions most affected by AD. APP is cleaved by β-secretase, releasing extracellular domain (sAPPβ), which is further cleaved by an unknown mechanism to release 35 kDa N-terminal fragment (N-APP). This fragment binds DR6 to trigger degeneration through caspase 6 in axons and caspase 3 in cell bodies. Therefore, they suggested that N-APP may be involved in the onset of AD (Nikolaev et al., 2009).

As mentioned above, since AICD induces neuron-specific apoptosis, AICD may also be involved in the onset of AD. Indeed, Ghosal et al. reported AD-like pathological features in transgenic mice expressing AICD (Ghosal et al., 2009), although opposite conclusions have also been reported (Giliberto et al., 2010). Therefore, it is possible that AD is caused through multiple mechanisms by multiple APP-derived fragments, not only Aβ but also N-APP and AICD.

Conclusions

Although γ-secretase was first identified as a protease that cleaves APP within the transmembrane domain and produces Aβ peptides, which are thought to be pathogenic in AD, the physiological functions of γ-secretase have not been fully elucidated. As reviewed here, the Notch signaling pathway is mediated by γ-secretase: intramembrane cleavage of Notch by γ-secretase serves to release an intracellular domain that has activity in the nucleus through binding to transcription factors. Recently, it was demonstrated that many type 1 transmembrane proteins, including Notch, Delta, and APP, are substrates for γ-secretase and release intracellular domains of these proteins from cell membranes. These observations that the common enzyme, γ-secretase, modulates proteolysis and the turnover of possible signaling molecules led to the attractive hypothesis that mechanisms similar to the Notch signaling pathway may contribute widely to γ-secretase-regulated signaling pathways, including APP signaling, which leads to AD. Indeed, as reviewed here, we have shown that the AICD alters gene expression and induces neuron-specific apoptosis. In this article, we discussed the possibility that APP may play a role in signaling events and γ-secretase-regulated APP signaling may be responsible for the onset of AD.

Figure legends

Fig. 1

Notch signaling pathway. Notch proteins are expressed on the cell surface as the heterodimers after cleavage at the S1 site by furin. The binding of Notch to the ligand triggers sequential proteolytic cleavage of RIP. When Notch binds to the ligand, Notch is shaded at the S2 site in the juxtamembrane region by TACE or ADAM protease. Next, the remaining protein stub is further cleaved by γ-secretase at the S3 and S4 sites within the transmembrane domain and NICD is released from the membrane. Then, NICD translocates into the nucleus and binds to the CSL together with MAML. The resultant CSL-NICD-MAML complex succeeds in removing co-repressors from CSL transcription factor and recruits a co-activator, resulting in a conversion from repressor to activator. Finally, the complexes of CSL-NICD-MAML-Co activators promote transcription of the target genes.

Fig. 2

Similarities of the proteolytic processes among Notch, APP, and CD44. (A) In response to ligand binding, Notch undergoes shedding by metalloprotease cleavage at the S2 site within the JM domain. After shedding the extracellular domain, the remaining Notch stub is further cleaved by γ-secretase at S3 and S4 sites within the TM domain. As the result of this sequential proteolysis, NICD and Nβ fragment are produced. (B) Cleavage of APP by α-secretase or β-secretase at α-site or β-site respectively within the JM domain results in the shedding of nearly the entire extracellular domain and generates membrane-tethered α or β-carboxylterminal fragments (CTFs). Several zinc metalloproteinases and BACE2 can cleave APP at the α-site, while BACE1 cleaves at the β-site. After shedding the extracellular domain, the remaining stub is further cleaved at least twice within the TM domain at γ- and ε-site by γ-secretase, producing either p3 peptide (in combination with α-secretase) or Aβ (in combination with BACE1) respectively, and AICD. (C) Several stimulations, such as PKC activation and $Ca2^{+}$ influx, trigger ectodomain cleavage of CD44 by a metalloprotease at the site within JM domain, resulting in the secretion of soluble CD44 (sCD44). After shedding the extracellular domain, the remaining CD44 stub is further cleaved by γ-secretase at two sites within the TM domain. As a result of this sequential proteolysis, the CD44 ICD and CD44β, an Aβ-like peptide,

are produced.

Fig.3. Overexpression of AICD in P19 cells induced neuronal cell death. After aggregation culture with RA, AICD-overexpressing P19 (AICD/P19) and control P19 cells (pCDNA/P19) carrying vector alone were replated and cultured for the indicated periods on dishes and allowed to differentiate. Undifferentiated AICD/P19 cells retained epithelial cell-like morphology as well as control cells, while the differentiated cells became round and showed a bipolar morphology with neurite extension. Two days after replating (Day2), all cell lines grew well and neurons with long neurites appeared. Four days after replating (Day4), control cells still grew well as clusters and many neurons had differentiated from these cells. However, many AICD/P19 cells showed severe degeneration, becoming spherical with numerous vacuoles and detaching from the culture dishes.

Fig. 4. Venn diagrams showing the total numbers of genes up-regulated by more than 10-fold in the presence of AICD (A) and genes down-regulated to less than one-tenth of their original level (B) at 3 states of neural differentiation in P19 cells: undifferentiated, aggregated, and differentiated.

Table 1. Substrates for γ-secretase. PS, presenilin; ICD, intracellular domain; APLP, amyloid precursor-like protein; CBP, CREB (cAMP-responsive element binding protein)-binding protein; TRE, TPA (12-o-tetradecanoylphorbol 13-acetate)-responsive element; AP-1, activator protein-1; CASK, calmodulin-dependent serine kinase; Tyr, Tyrosinase.

Table 2. Expression levels of 7 up-regulated and 7 down-regulated genes, as well as 3 housekeeping genes. Relative expression levels (folds) were estimated from the intensity of hybridization signals. Housekeeping gene expression was unaltered in AICD-overexpressing P19 and control P19 cells at any state, suggesting that these genes may not be affected by AICD. These results also show that the observed differences in expression were not due to technical problems, such as uneven hybridization or poor RNA quality.

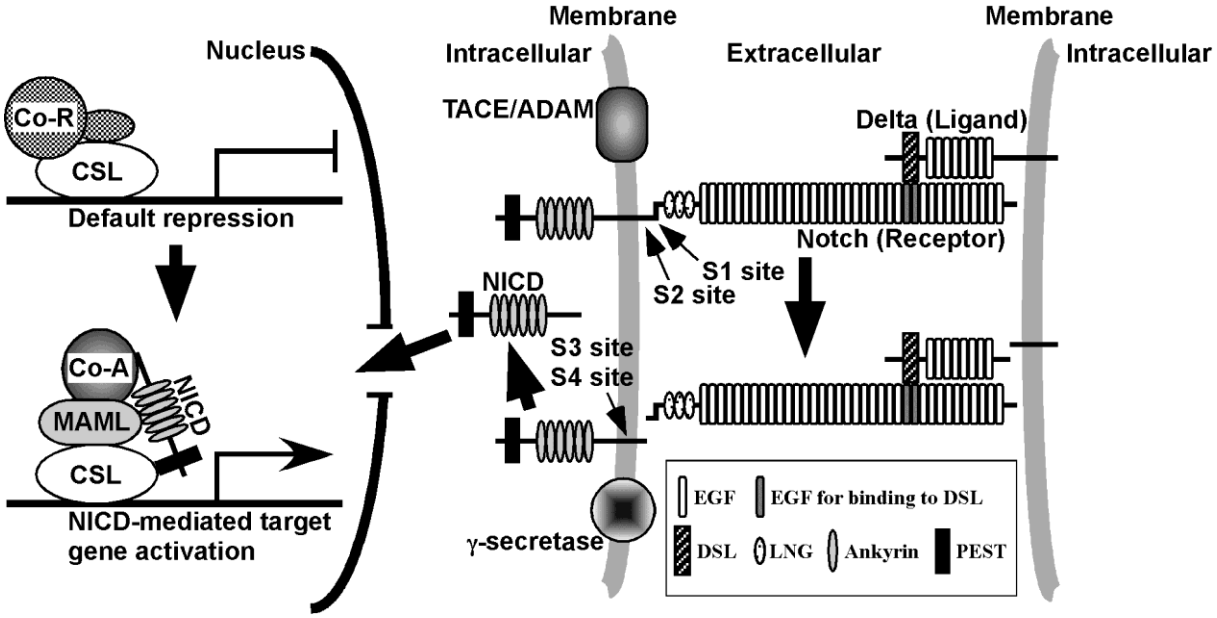
Membrane
Membrane
Nucleus
Intracellular
Extracellular
Intracellular
TACE/ADAM
Delta (Ligand)
Co-R
CSL
Default repression
Notch (Receptor)
S1 site
S2 site
NICD
S3 site
S4 site
Co-A
MAML
NICD
CSL
NICD-mediated target gene activation
γ-secretase
EGF
EGF for binding to DSL
DSL
LNG
Ankyrin
PEST

Fig. 1

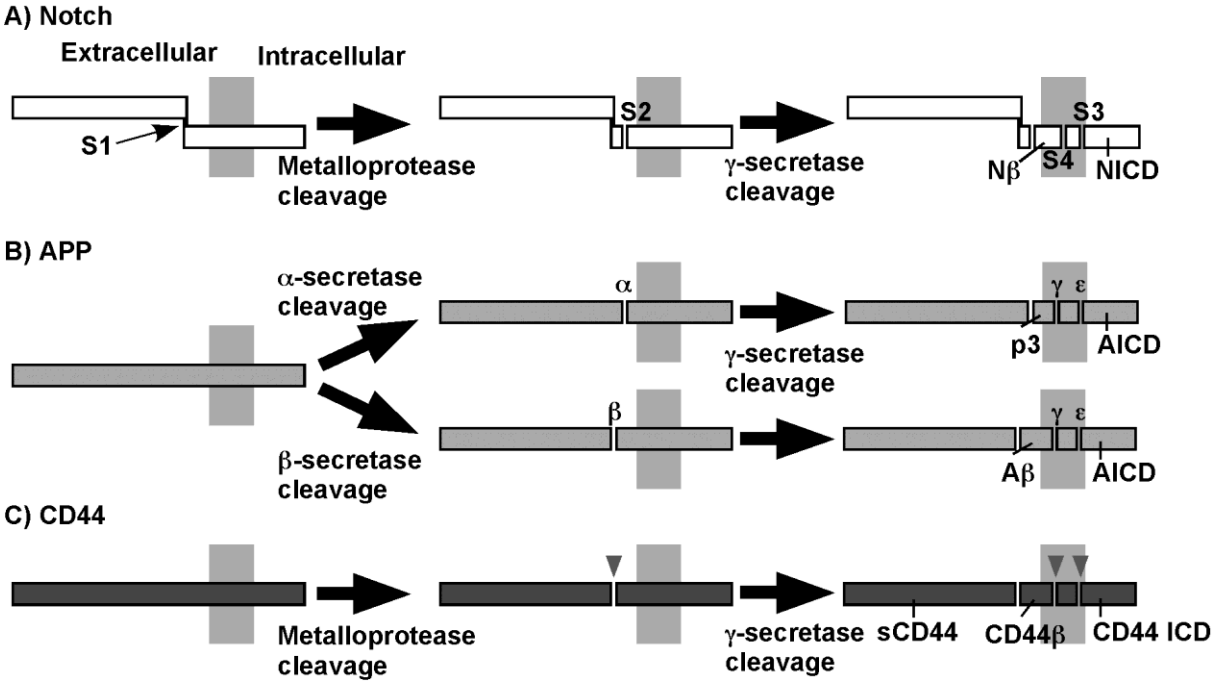
A) Notch
Extracellular
Intracellular
S1
Metalloprotease cleavage
S2
γ-secretase cleavage
S3
Nβ
S4
NICD
B) APP
α-secretase cleavage
α
γ ε
γ-secretase cleavage
p3
AICD
β
β-secretase cleavage
γ ε
Aβ
AICD
C) CD44
Metalloprotease cleavage
γ-secretase cleavage
sCD44
CD44β
CD44 ICD

Fig. 2

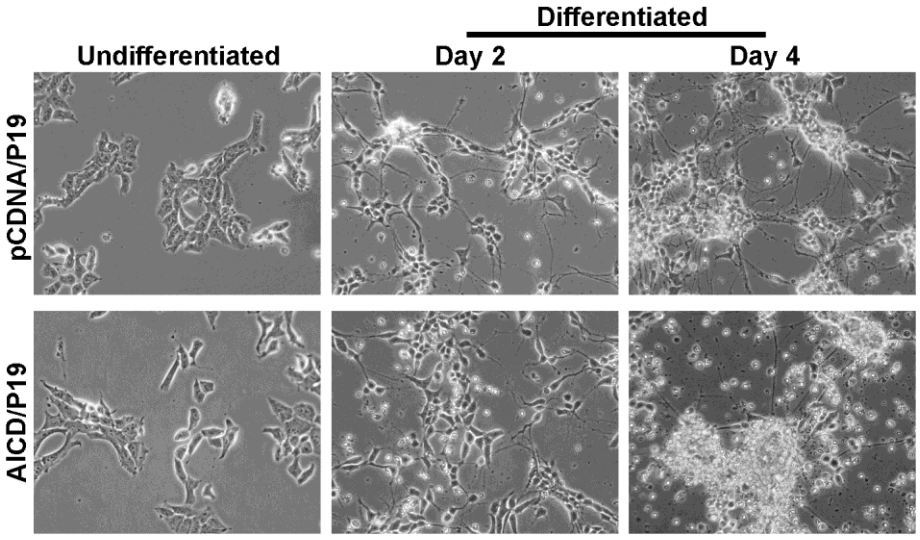
Differentiated
Undifferentiated
Day 2
Day 4
pCDNA/P19
AICD/P19

Fig. 3

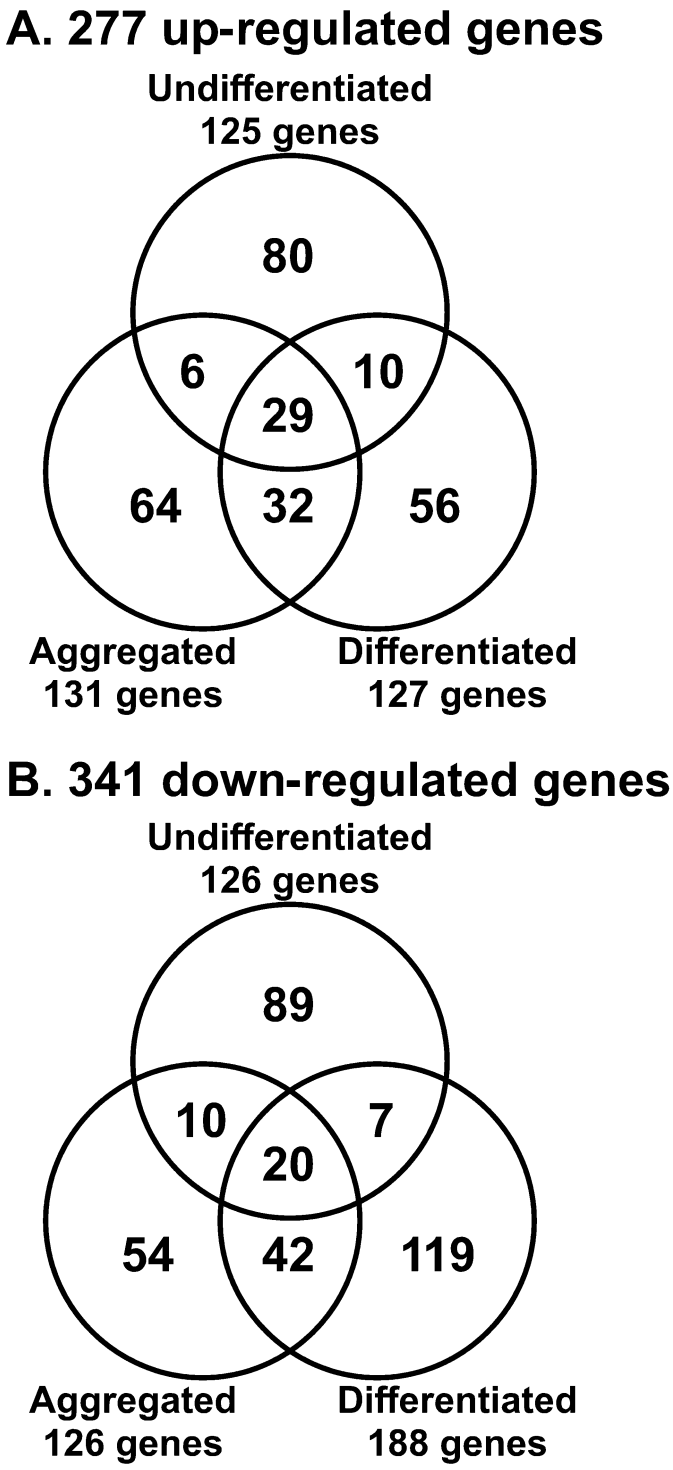
A. 277 up-regulated genes
Undifferentiated
125 genes
80
6
10
29
64
32
56
Aggregated
131 genes
Differentiated
127 genes
B. 341 down-regulated genes
Undifferentiated
126 genes
89
10
7
20
54
42
119
Aggregated
126 genes
Differentiated
188 genes

Fig. 4

Table 1

Substrate	Function	PS or ICD function
ApoER2	Lipoprotein receptor, neuronal migration	Activate nuclear reporter
APP	Precursor to Aβ, adhesion, trophic properties, axonal transport?	Aβ generation, release of ICD, complex with Fe65/TIP60, Cell death?
APLP1/2	Cell adhesion?	Form complex with Fe65 and TIP60
E-cadherin	Cell adhesion	Promote disassembly of adhesion comprex
N-cadherin	Cell adhesion	Promote CBP degradation
CD43	Signal transduction	Signaling molecule?
CD44	Cell adhesion	Activate TRE-mediated nuclear transcription
CSF1 receptor	Protein tyrosine kinase	Unknown
CXCL16 & CX3CL1	Membrane chemokine ligands	Unknown
DCC	Axon guidance, tumor suppressor	Activate nuclear reporter
Delta	Notch ligand	Transcription regulation
ERBB4	Receptor tyrosine kinase	Regulate heregulin-induced growth inhibition
HLA-A2	MHC class I molecule	Unknown
IFN-αR2	Subunit of type I IFN-α receptor	Transcription regulation
IL-1RI	Cytokine receptor	Unknown
IL-1RII	Cytokine receptor	Unknown
Jagged	Notch ligand	Modulate AP-1 mediated transcription
LRP	Scavenger and signaling receptor	Activate nuclear reporter
Na channel β-subunit	Cell adhesion, an auxiliary subunit of voltage-gated Na channel.	Alter cell adhesion and migration
Nectin-1α	Adherens junction, synapse receptor	Remodeling of cell junctions?
Notch1-4	Signaling receptor	Transcription regulation
NRADD	Apoptosis in neuronal cells	Modulate glycosylation/matutaion of NRADD
P75NTR	Neurotrophin co-receptor, dependence receptor	Modulate p75-TrkA complex? Nuclear singaling?
γ-protocadherin	Cell adhesion, neuronal differentiation	Regulation of gene transcription?
Syndecan-3	Cell surface proteoglycan co-receptor	Regulation of membrane-targeting of CASK
β-catenin	Transduce Wnt signals, stabilize adherens junctions	Facilitate phosphorylation
Telencephalin	Cell adhesion	Turnover of telencephalin
Tyrosinase, Tyrosinase-related protein 1/2	Pigment synthesis	Intracellular transport of Post-Golgi Tyr-containing vesicles

Table 2

	Gene Symbol	Gene Name	Function	Relative Expression Levels (fold)		
				Undifferentiated	Aggregated	Differentiated
Non-regulated (house keeping)	***Actb***	β-actin	cytoskeleton protein	**-1.2**	**1.2**	**1**
	Sdha	succinate dehydrogenase subunit A	electron transporter in the TCA cycle and respiratory chain	**-1.1**	**-1.6**	**-1.2**
	Eef1a1	eukaryotic translation elongation factor-1 alpha 1	essential component for the elongation phase during protein translation	**1**	**-1.1**	**1.2**
Up-regulated	***Ptprt***	protein tyrosine phosphatase receptor T	protein tyrosine phosphatase that regulates STAT3 activity	**906**	**204**	**116**
	Cpb1	carboxypeptidase B1	hydrolysis of C-terminal end of basic amino acid peptide bond	**16**	**296**	**222**
	Nr2e1	tailless homolog	transcription factor that is essential for neural stem cell proliferation and self-renewal	**5.8**	**244**	**54**
	Myh1	myosin heavy chain 1	one of the component of motor protein myosin	**-4.2**	**259**	**-1.1**
	Dnahc7c	axonemal dynein heavy chain	essential for motility of cilia and flagella	**133**	**41**	**43**
	Alkbh3	alkylation repair homolog 3	AlkB enzyme that repairs methylation damage in DNA and RNA	**69**	**80**	**43**
	Ctgf	connective tissue growth factor	skeletogenesis/vasculogenesis by modulating BMP, Wnt, and IGF-I signals	**90**	**54**	**40**
Down-regulated	***Hes5***	hairy and enhancer of split 5	transcription factor that inhibits neurogenesis	**-8.7**	**-3039**	**-2515**
	Slc10a6	sodium-dependent organic anion transporter	transport of sulfoconjugated steroid hormones and bile acids	**-145**	**-785**	**-1212**
	Nid1	nidogen-1	extracellular matrix linker protein	**-304**	**-165**	**-507**
	LOC213332	analog of Na+ dependent glucose transporter 1	putative glucose transporter	**-232**	**-325**	**-306**
	Dtx1	Deltex1	regulator of Notch signaling pathway	**-30**	**-85**	**-691**
	Rbp4	retinol-binding protein 4	retinol transporter from the liver to extrahepatic tissues	**-525**	**-100**	**-24**
	Col3a1	collagen type III alpha 1	extracellular matrix protein	**4.1**	**-29**	**-234**