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ADAM10 Mediates *N*-Cadherin Ectodomain Shedding During Retinal Ganglion Cell Differentiation in Primary Cultured Retinal Cells From the Developing Chick Retina

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ABSTRACT

Here, we examined the role of ADAM10 during retinal cell differentiation in retinal sections and in vitro cultures of developing chick retinal cells from embryonic Day 6 (ED6). Immunohistochemistry showed that ADAM10 is abundantly expressed in the inner zone of neuroblastic layer at ED5, and it becomes more highly expressed in the ganglion cell layer at ED7 and ED9. Western blotting confirmed that ADAM10 was expressed as an inactive pro-form that was processed to a shorter, active form in control cultured cells, but in cultures treated with an ADAM10 inhibitor (GI254023X) and ADAM10-specific siRNA, the level of mature ADAM10 decreased. Phase-contrast microscopy showed that long neurite extensions were present in untreated cultures 24 h after plating, whereas cultures treated with GI254023X showed significant decreases in neurite extension. Immunofluorescence staining revealed that there were far fewer differentiated ganglion cells in ADAM10 siRNA and GI254023X-treated cultures compared to controls, whereas the photoreceptor cells were unaltered. The Pax6 protein was more strongly detected in the differentiated ganglion cells of control cultures compared to ADAM10 siRNA and GI254023X-treated cultures. *N*-cadherin ectodomain shedding was apparent in control cultures after 24 h, when ganglion cell differentiation was observed, but ADAM10 siRNA and GI254023X treatment inhibited these processes. In contrast, *N*-cadherin staining was strongly detected in photoreceptor cells regardless of ADAM10 siRNA and GI254023X treatment. Taken together, these data indicate that the inhibition of ADAM10 can inhibit Pax6 expression and *N*-cadherin ectodomain shedding in retinal cells, possibly affecting neurite outgrowth and ganglion cell differentiation. J. Cell. Biochem. 114: 942–954, 2013.

KEY WORDS: ADAM10; RETINAL GANGLION CELL (RGC); *N*-CADHERIN; DIFFERENTIATION; Pax6; RETINAL DEVELOPMENT

During retinal development, the seven retinal cells types (six neuronal cells and one glial cell type) differentiate sequentially from the same population of retinal neuroblasts [Adler, 2000; Mu and Klein, 2004]. In retinas from chick embryos between ED3 and ED8 Tuj-1-positive cells are located exclusively in the post-mitotic ganglion cells, the major neuronal cell type generating at these ages [Watanabe et al., 1991; Snow and Robson, 1994; Pimentel et al., 2000; Kim et al., 2012]. Neuronal microtubules have unique stability properties achieved through developmental regulation [Fanarraga et al., 1999]. Class III β-tubulin (Tuj-1) is regarded as a neuron-specific marker of newly generated cells. It contributes to microtubule stability in neuronal cell bodies and axons, and plays a

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role in axonal transport. The ganglion and photoreceptor cell types are specified at a similar time in development, but the specific factors that regulate the timing of retinal differentiation are not fully understood [Schulte and Bumsted-O'Brien, 2008; Ferreiro-Galve et al., 2010].

The cadherins are a family of Ca^{2+}-dependent intercellular adhesion molecules that mediate cell–cell adhesion through homophilic interactions. *N*-cadherin is critically involved in neurulation [Radice et al., 1997]. *N*-cadherin is hemophilic binding protein and is a potent substrate for the rapid induction of neurite outgrowth [Bixby and Zhang, 1990]. *N*-cadherin is predominantly expressed in neuronal cells [Radice et al., 1997; Karkkainen et al., 2000], where it promotes axonal outgrowth [Huntley, 2002]. In the neural retina, undifferentiated neuroblasts contact each other through cell–cell adhesion molecules, such as *N*-cadherin [Liu et al., 1997]. In some retinal layers, *N*-cadherin is co-expressed with the ADAM10 protein [Yan et al., 2011]. The "a disintegrin and metalloproteinase" (ADAM) family of transmembrane and secreted multi-domain proteins are unique among the cell-surface proteins in possessing both a potential adhesion domain and a potential protease domain [Wolfsberg et al., 1995; Edwards et al., 2008]. Their metalloproteinase domains can induce ectodomain shedding and cleave extracellular matrix (ECM) proteins, while their disintegrin and cysteine-rich domains have adhesive activities [White, 2003]. To date, more than 30 ADAMs have been characterized; they are involved in diverse biological functions, including cell signaling, cell adhesion, fertilization, migration, proliferation, differentiation, and the ectodomain shedding of growth factors [McCulloch et al., 2004; Alfandari et al., 2009; Duffy et al., 2009]. ADAM10 was the first metalloproteinase shown to function in vertebrate axon guidance; it is responsible for the shedding of proteins that are important for brain development, including *N*-cadherin [Chen et al., 2007; Jorissen et al., 2010; Esteve et al., 2011], which can function as a substrate-attached neurite outgrowth-promoting molecule and participates in the regulation of axonal growth [Bixby and Zhang, 1990]. The metalloproteinase-mediated cleavage of *N*-cadherin forms adherens junction between cells by interacting with other cadherin molecules via its extracellular domain, leading to the release of soluble extracellular domains and providing a mechanism for extracellular signaling [Kohutek et al., 2009; Reiss and Saftig, 2009]. However, although ADAM10 is known to play an important role in neurogenesis and axon extension [Lin et al., 2008], and several ADAMs are known to be important for neural development [Yang et al., 2006], the precise function of ADAM10 is still not well understood within the field of neuroscience.

The transcription factor Pax6 plays a pivotal role in eye development, and controls timing of retinal neuron differentiation along with regulation of cell proliferation and is required for specification of early retinal cell type [Philips et al., 2005]. Pax6 expression is maintained throughout retinal development, including early expression in all retinal progenitor cells (RPCs) in the developing chick retina [Canto-Soler et al., 2008], continued expression in the proliferative margin of the retina, and expression predominantly confined to the retinal ganglion cell (RGC) [Walther and Gruss, 1991; Puschel et al., 1992; Riesenberg et al., 2009]. Importantly, Pax6 function may change at different stages of retinal development as development proceeds [Philips et al., 2005].

In the present study, we examined the potential roles of ADAM10 in neurite outgrowth and neuronal cell differentiation. We cultured retinal neuroblasts and treated them with the ADAM10-specific inhibitor, GI254023X- and ADAM10-specific siRNA. Here, we demonstrate that the inhibition of ADAM10 expression or activity modulates RGC differentiation by inhibiting *N*-cadherin ectodomain shedding, but does not affect the differentiation of cone photoreceptor cells.

MATERIALS AND METHODS

REAGENTS

Fertilized eggs of the white leghorn chicken were obtained from a local hatchery and incubated at 37°C in a humidified egg incubator. The monoclonal anti-Tuj-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-ADAM10 antibody was obtained from Chemicon (Temecula, CA). The polyclonal anti-Tuj-1 antibody was obtained from Sigma (St. Louis, MO). The monoclonal anti-visinin and anti-Pax6 antibodies, and the monoclonal antibodies against the N- and C-termini of *N*-cadherin, were all purchased from DSHB (University of Iowa, Iowa, IA). The monoclonal anti-BrdU antibody was obtained from Cell Signaling Technology (Danvers, MA). The monoclonal anti-β-actin was purchased from Santa Cruz Biotechnology. The specific ADAM10 inhibitor (GI254023X) was a kind gift from GlaxoSmithKline (Harlow, UK). DAPI and Prolong Gold were purchased from Invitrogen (Carlsbad, CA).

RETINAL CELL PREPARATION AND CULTURE

Embryonic day (ED) 6 chick retinas were carefully dissected to be free of retinal pigment epithelia, and dissociated retinal cell cultures were prepared as previously described [Saga et al., 1996]. Briefly, neural retinas were incubated for 15 min at 37°C in 0.1% trypsin in Ca^{2+}- and Mg^{2+}-free HBSS (CMF-HBSS) and gently triturated for 5 min to yield a single-cell suspension. Digestion was stopped by the addition of heat-inactivated fetal calf serum. Prior to cell culture, the culture dishes (Corning, Rochester, NY) were coated overnight with 1 mg/ml of poly-DL-ornithine (Sigma) and washed with distilled water. Cells were seeded at 3.5×10^6 per 60-mm culture dish and cultured for 6 h in F-12 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% chick serum (Gibco, Grand Island, NY) and 1× ITS (Sigma). Thereafter, the cells were treated with or without 5 μM of GI254023X in F-12 media containing 10% FBS for 6 and 24 h.

TRANSFECTION OF CHICKEN ADAM10 siRNA

The siRNA-ADAM10 (GenBank accession number, NM_204261) sequences were obtained from siDESIGN center (Dharmacon RNAi Technologies). Two siRNA-GFP duplexes targeting two regions of the ADAM10 (siRNA1: 5′-GGGACAAACUUAAUAACAA-3′ and siRNA2: 5′-CAGUAUUGCUUAUGGGAAU-3′) were obtained from Bioneer (Daejeon, Korea). For siRNA knock-down of ADAM10, retinal cells cultured for 6 h were transfected with siRNA at a final concentration of 33 nM using Lipofectamin 2000 (Invitrogen)

according to the manufacturer's instructions. After 12 h, medium was replaced by F-12 medium supplemented with 10% FBS without antibiotics, and cells were cultured for 12 h. The efficiency of knockdown of ADAM10 expression with siRNA2 was higher than that of siRNA1. Therefore, we used siRNA2 for further experiments.

RNA EXTRACTION AND RT-PCR ANALYSIS

Total RNA from cultured cells, 24 h after transfection, was extracted using RNA extraction kit according to the manufacturer's protocol (Qiagen, Germany). Equal amounts (1 μg) of total RNA was reverse transcribed into cDNA with random primer by using reverse transcriptase (SuperScript II; Invitrogen). Transcribed complementary DNAs were used for polymerase chain reaction (PCR) amplification. The conditions for which were as follows: heat denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 1 min. The following primer sequences for ADAM10 gene sequences were utilized as follows, 5′-GCCAAGGCCCTTGCTGTAC-3′ (forward) and 5′-TCAATGTCTCATATGTCCCA-3′ (reverse), and 5′-GATGGGTGTCAACCATGAGAAA-3′ (forward) and 5′-ATCAAAGGTGGAAGAATGGCTG-3′ (reverse) for GAPDH as an internal loading control. This method resulted in amplification of a single major cDNA fragment (731 bp), as visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

PREPARATION OF CONDITIONED MEDIA

After serum culture for 6 h as described above, cells were washed twice with serum-free F-12 media. Thereafter, cells were treated with or without GI254023X (5 μM) in serum-free F-12 media for 6 and 24 h, and serum-free conditioned media were collected. Similarly cells were transfected with or without ADAM10-siRNA in serum-free media and supernatants were collected after 24 h of transfection. To examine the levels of cleaved *N*-cadherin ectodomain, proteins were precipitated from equal-volume aliquots of supernatant using 10% ice-cold trichloroacetic acid (TCA). The precipitates were washed twice with 100% acetone, air-dried, dissolved in RIPA buffer, and stored at −20°C until use.

WESTERN BLOT ANALYSIS

Western blot analysis was performed using standard techniques. Equal amounts of conditioned media or protein lysates (30 μg) in RIPA buffer were separated by 8% SDS–PAGE under reducing conditions, and the proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 h, and then incubated overnight at 4°C with polyclonal anti-ADAM10 or anti-Tuj-1 antibodies, or monoclonal antibodies against Tuj-1, visinin, *N*-cadherin (C-terminal and N-terminal), Pax6, or β-actin, all diluted in TBS-T containing 5% dried milk. The primary antibodies were detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for a further 1 h. Specific antibody binding was visualized using an enhanced chemiluminescence detection kit (ELPIS Biotech, Korea) and X-ray film exposure. Experiments were performed in triplicate.

PARAFFIN EMBEDDING AND IMMUNOHISTOCHEMISTRY

Developing eyeballs were harvested at ED5, ED7, and ED9, placed in cold PBS, fixed in 4% paraformaldehyde (PFA) in PBS for 3 h at 4°C, and then washed twice in PBS. The samples were then dehydrated through an ethanol series, cleared by soaking in xylene, embedded in paraffin, and sectioned (6 μm) using a microtome RM 2125RT (Leica, Wetzlar, Germany). Slides containing paraffin sections were deparaffinized in xylene and rehydrated through an ethanol series, and endogenous peroxidase activity was inactivated by incubation in 0.3% H_2O_2 in methanol for 10 min. The sections were then rinsed in 0.1 M TBS (pH 7.4) and boiled in citrate buffer (pH 6.0) containing 0.03% Tween-20 for 4 min. Finally, the sections were incubated with blocking solution (5% normal goat serum and bovine serum albumin in TBS) at room temperature (RT) for 1 h, and subjected to indirect immunohistochemistry using an antibody against ADAM10 for 1 h. For the negative control, the primary antibody was omitted and the slides were incubated with blocking solution. For the visualization of results, the sections were incubated with HRP-conjugated anti-rabbit IgG for 1 h at RT, stained with VECTOR® NovaRED (Vector, Burlingame, CA), and counterstained with hematoxylin. The sections were dehydrated, cleared, and mounted with Permount (Fisher, Fair Lawn, NJ). Images were captured using a Zeiss microscope (Carl Zeiss, Germany).

IMMUNOFLUORESCENCE STAINING

Equal numbers of cells were cultured with or without GI254023X for 6 and 24 h, and then fixed with 4% PFA in PBS (pH 7.4) at RT for 15 min. The fixed cells were permeabilized with 0.3% Triton-X for 10 min, and blocked overnight with 5% normal goat serum and BSA in TBS at 4°C. The cells were then incubated overnight with monoclonal antibodies against Tuj-1, visinin, or Pax6 (diluted 1:100) in 5% BSA, and washed three times in TBS. Double-labeling immunofluorescence was performed using primary antibodies of different species. The cells were further incubated with the appropriate Alexa555- or/and Alexa488-conjugated secondary antibodies at RT for 1 h, and nuclei were counterstained with DAPI (1 μg/ml). The cells were washed three times with PBS and mounted, and images were captured using a Zeiss fluorescence microscope.

CELL PROLIFERATION ASSAY

Cells were cultured with or without GI254023X for 6 and 24 h, treated with BrdU (25 μg/ml) for 2 h, and harvested. The cells were then fixed with 4% PFA in PBS for 15 min, permeabilized with 0.1% Triton in PBS, and exposed to 2 M HCl at RT for 30 min. For double-labeling immunofluorescence, the cells were exposed to a polyclonal anti-Tuj-1 antibody. All other experimental steps were as described above for immunofluorescence staining.

MEASUREMENT OF NEURITE OUTGROWTH

After 6 and 24 h of drug treatment, cells were fixed and immunocytochemistry was performed as described above. Images were taken randomly and selected three areas from three independent experiments per condition. Length of neurites was measured using Image J software. Neurite processes larger than the cell soma diameter in length were considered as the neurites.

STATISTICAL ANALYSIS

Data are expressed as the mean ± SEM from three independent experiments. The statistical analyses were performed using one-way ANOVA, followed by the Student's *t*-test in Microsoft Excel. $P < 0.05$ was taken as indicating statistical significance.

RESULTS

EXPRESSION OF ADAM10 PROTEINS IN THE DEVELOPING CHICKEN RETINA AND PRIMARY CULTURES OF RETINAL CELLS

Immunohistochemistry (Fig. 1A) showed that the ADAM10 protein could be detected in the entire neuroblastic layer (NBL) at ED5 (a). It was notable in the inner zone (arrow) of the NBL at ED5 (a), and high levels of ADAM10 signal (arrows) were also detected in the ganglion cells of the GCL at ED7 (b) and ED9 (c). There was no ADAM10 signal in the negative control prepared using ED7 retina and no primary antibody (d). This pattern of ADAM10 expression was similar to that found in a previous study [Yan et al., 2011]. ADAM10 is expressed as an inactive pro-form (97 kDa) that is processed to a shorter, catalytically active form (68 kDa) [Nagano et al., 2004; Sahin et al., 2004], and cell and tissue type specific expression of different size of ADAM10 has been observed in many studies. To examine ADAM10 at the protein level, Western blot analysis was performed on cell lysates taken from cultured retinal cells from ED6 for 6 and 24 h, and five ADAM10 bands with ≈110, ≈90, ≈80, ≈67, and ≈56 kDa were detected (Fig. 1B). Previously, an inactive form (110 kDa) and an active form (82 kDa) of ADAM10 have been found in several tissues of chicken embryo from ED2-ED6 [Hall and Erickson, 2003]. On the other hand, an inactive (114 kDa) and an active form (67 kDa) of ADAM10 are present in the developing chick brain from ED10 to ED20, suggesting that the form of the ADAM10 is dynamically regulated during different stages of development [Markus et al., 2011]. Our previous study shows that pro (≈82 kDa) and active ADAM10 (≈68 kDa) bands are detected in the early developing chick corneas [Huh et al., 2007]. In addition, active ADAM10 (56–58 kDa) purified from bovine kidney could cleave a basement membrane type IV collagen and also a myelin basic protein (MBP) [Millichip et al., 1998]. Other study suggests that 56- and 58-kDa immunoreactive ADAM10 proteins may represent either post-translational processing of the mature ADAM10 protein, or glycosylation variants of the active protein [Dallas et al., 1999]. Therefore, we suspect that ADAM10 band (≈56 kDa) appears to be the processed mature ADAM10 protein (≈67 kDa) in this study. Compared to control culture, GI254023X treatment induced a time dependent decrease of the presumably mature form of ADAM10 (≈56 and ≈67 kDa) while increasing the ≈80 kDa of ADAM10 band, which could clearly be seen 6 h after GI254023X addition and was evident after 24 h (Fig. 1B). Glycosylation is one of the most important post-translational modifications in newly synthesized proteins, and four potential *N*-glycosylation sites of ADAM10 are found [Escrevente et al., 2008]. Likewise, high molecular weight of ADAM10 bands (≈90 and ≈110 kDa), presumably representing the premature full-length protein with different glycosylation rate [Escrevente et al., 2008], were not altered by GI254023X treatment (Fig. 1B). Taken together, our findings suggest that activation of ADAM10 is inhibited by GI254023X treatment in short-term culture condition, resulting in the down-regulation of the mature form of ADAM10, which may affect ganglion cell differentiation.

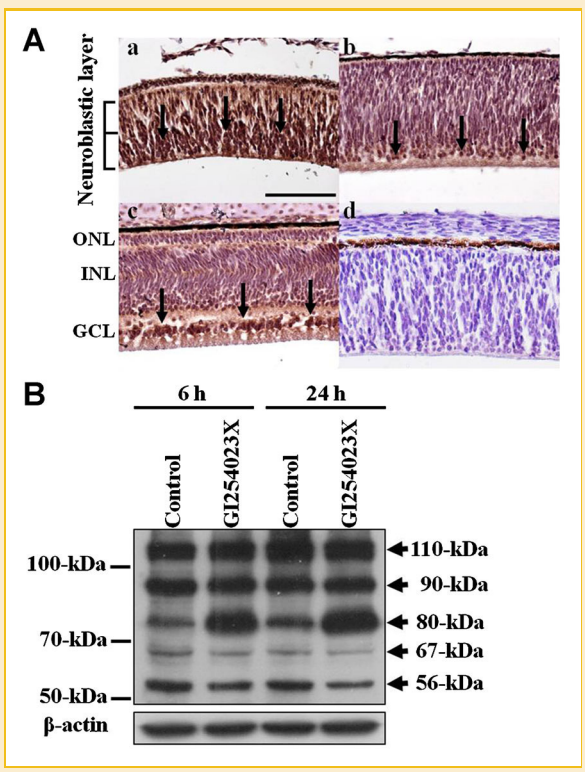

Fig. 1. Expression patterns of ADAM10 in the developing retina and primary cultured retinal cells. A: The temporal and spatial expression patterns of ADAM10 in the developing retina were examined by immunohistochemistry using an anti-ADAM10 antibody. ADAM10 was detected throughout the entire neuroblastic layer on embryonic day (ED) 5 (a), but it was concentrated in the ganglion cell bodies on ED7 and ED9 (b and c). The negative control, which was an ED7 sample incubated with anti-rabbit IgG, showed only hematoxylin and eosin (H&E) staining (d). B: Cells were cultured for 6 and 24 h with or without the specific ADAM10 inhibitor, GI254023X (5 μM). Equal amounts of cell lysates were subjected to Western blot analysis using an anti-ADAM10 antibody. Note that five ADAM10 bands with ≈110, ≈90, ≈80, ≈67, and ≈56 kDa were detected. GI254023X treatment decreased the presumably mature form of ADAM10 (≈56 and ≈67 kDa) while increasing the ≈80 kDa of pro form of ADAM10 band. β-actin was used as the loading control. The presented data are representative of at least three independent experiments.

INHIBITION OF ADAM10 ACTIVITY SUPPRESSES NEURITE OUTGROWTH

The morphologies of cultured chick retinal cells [200× (A) and 400× (B) magnifications] were observed at 6 and 24 h after culture by phase-contrast microscopy (Fig. 2). At 6 h of culture (upper panel), both control cultured cells and GI254023X (5 μM)-treated cells were relatively similar in morphology and did not show much differentiation. At 24 h (lower panel), however, we observed longer neurite extensions (black arrows) in untreated cells compared to GI254023X-treated cells, suggesting that ADAM10 activity might be associated with neurite outgrowth.

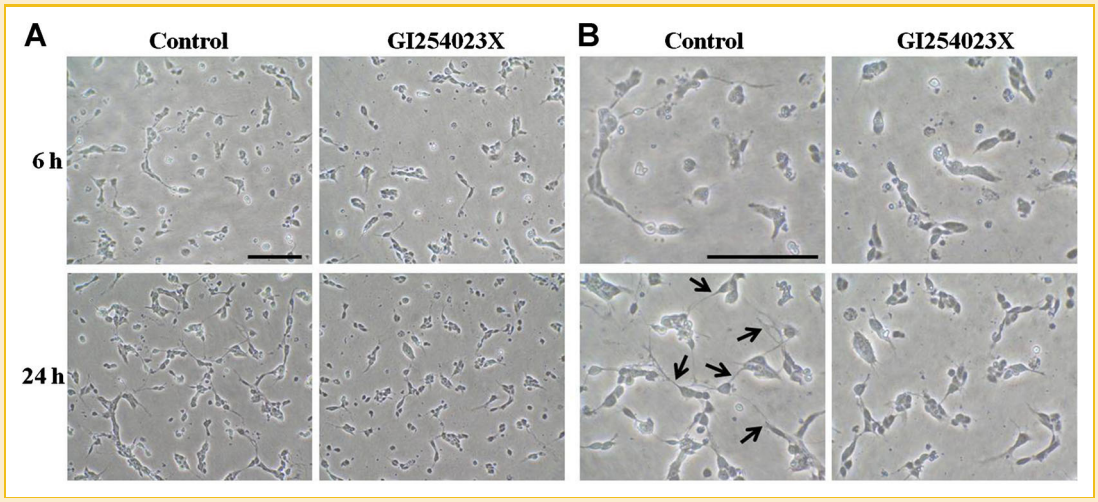

Fig. 2. Inhibition of ADAM10 decreases neurite outgrowth. Cells were cultured with or without GI254023X (5 μM) for 6 and 24 h, and morphological features were observed by phase-contrast microscopy. The development of neurite outgrowth (black arrows) was evident in control cultured cells for 24 h (B), but was inhibited by GI254023X. A: 200× Magnification. B: 400× Magnification. Scale bar: 100 μm.

INHIBITION OF ADAM10 ACTIVITY BLOCKS GANGLION CELL DIFFERENTIATION

We next examined whether ADAM10 inhibition influenced the differentiation of ganglion cells, using Western blotting and immunofluorescence analysis of the ganglion cell marker, Tuj-1. Tuj-1-positive cells with short neurites were detected at 6 h of culture in both control and GI254023X-treated cells. The number of strongly Tuj-1-positive cells with longer neuritic extensions (indicating ganglion cell differentiation) increased in control cultures by 24 h (Fig. 3A), whereas GI254023X (5 μM)-treated cultures showed remarkably fewer Tuj-1-positive cells with shorter neurites at 24 h. Quantitative analysis revealed that the length of neurites and the number of Tuj-1-positive cells were significantly decreased by GI254023X treatment (Fig. 3B). Consistent with these findings, Western blot analysis showed that GI254023X treatment decreased Tuj-1 expression at 24 h of culture (Fig. 3C). We also examined the proliferating cells by BrdU labeling at 6 and 24 h of culture. As expected, the post-mitotic Tuj-1-positive cells did not co-localize with the BrdU-labeled proliferating cells (Fig. 3D). The control and GI254023X-treated cultures appeared to have comparable numbers of BrdU-positive (purple) cells during the culture period (Fig. 3D). Therefore, it seems likely that ADAM10 activity may be associated with RGC differentiation, but not proliferation.

INHIBITION OF ADAM10 ACTIVITY DOES NOT AFFECT PHOTORECEPTOR CELL DIFFERENTIATION

We next used immunofluorescence staining to analyze the expression of visinin, a cone photoreceptor marker. The number of visinin-positive cells was higher at 24 h compared to 6 h, but comparable numbers of visinin-positive cells were seen in control and GI254023X-treated cells at both time points (Fig. 4A). Quantification of the visinin-positive cells (Fig. 4B) and Western blot analysis of visinin protein levels (Fig. 4C) confirmed that photoreceptor cells were not affected by ADAM10 inhibition. Thus, inhibition of ADAM10 does not appear to affect photoreceptor cell differentiation.

ADAM10 KNOCKDOWN SUPPRESSES THE GANGLION CELL DIFFERENTIATION BUT NOT THE PHOTORECEPTOR CELL DIFFERENTIATION

To test whether inhibition of ADAM10 mRNA expression interferes with RGC differentiation normally occurring in the control cultures, we carried out transfection of cells with ADAM10-siRNA for 24 h (Fig. 5). RT-PCR analysis showed that ADAM10 siRNA significantly diminished the endogenous ADAM10 mRNA levels in cultured cells compare to control cultures (Fig. 5A). In addition, the ADAM10 siRNA reduced the protein level of mature ADAM10, as determined by Western blot analysis (Fig. 5B). As expected, ADAM10 siRNA decreased the Tuj-1 expression but not the visinin expression when compared with that observed in the control culture for 24 h by Western blotting (Fig. 5C). We next examined whether ADAM10 expression influenced the differentiation of ganglion cells and photoreceptor cells by using immunofluorescence analysis (Fig. 5D). After the 24 h of transfection, GFP-labeled siRNA was seen around the nucleus (DAPI). In no cases was modification in the viability of the cultures observed. Tuj-1-positive cells with long neurite were observed in control cultures by 24 h, whereas ADAM10 siRNA-GFP transfected cells without neurite (green arrow; a and a′) showed weak Tuj-1 signal (upper panel). In addition, there were many siRNA-GFP labeled cells did not co-localize with Tuj-1. Importantly, siRNA-GFP-positive signal was not detected in strong Tuj-1-positive cell with neurites (red arrow; b and b′). The images of a′ and b′ were the enlarged images of a and b, respectively. Interestingly, the number of visinin-positive cells in control culture was similar with ADAM10-siRNA-GFP transfected cultures (lower panel). In addition, strong GFP-positive signals were clearly observed in visinin-positive photoreceptor cells (green arrows; c and d). The enlarged images of c and d, indicated by green arrows are shown as

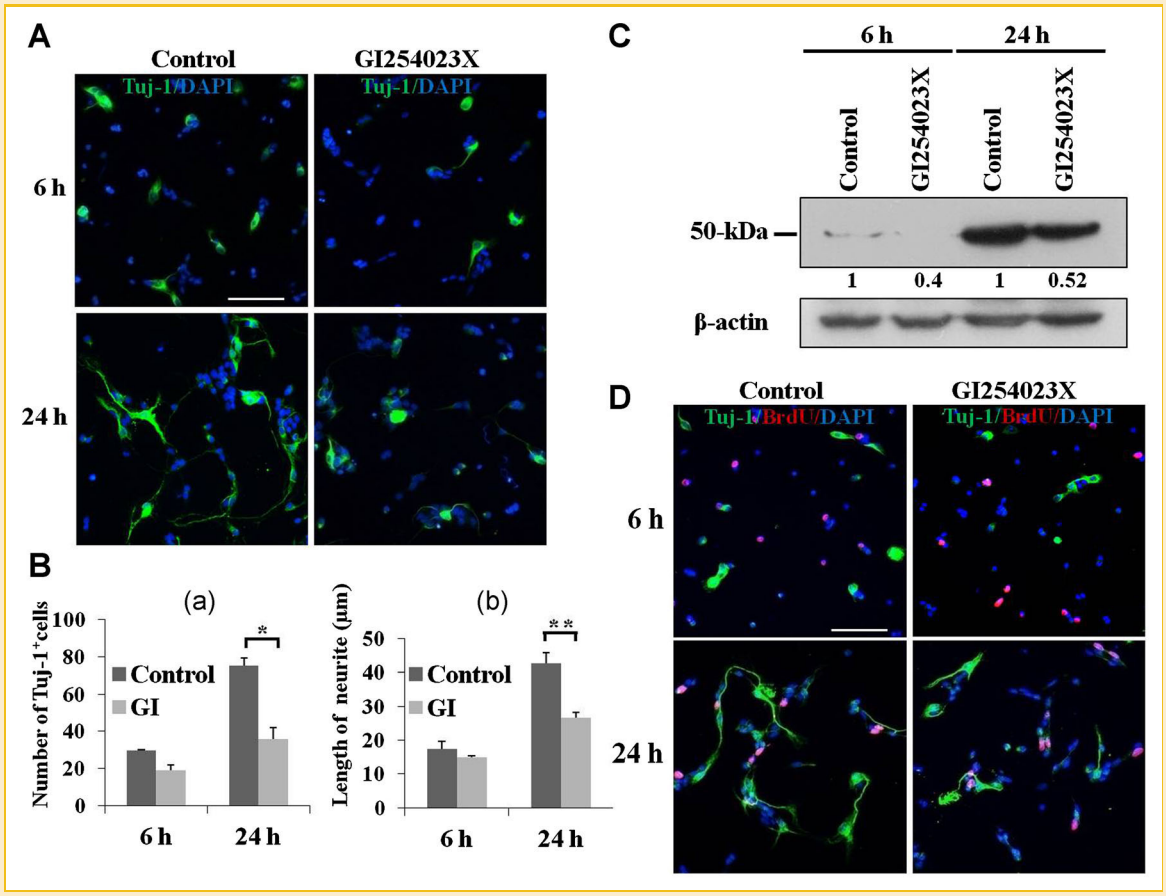

Fig. 3. Inhibition of ADAM10 decreases ganglion cell differentiation. A: Cells were cultured with or without GI254023X (5 μM) for 6 and 24 h and subjected to immunofluorescence staining using a monoclonal anti-Tuj-1 antibody. GI254023X treatment reduced the number of Tuj-1-positive cells and inhibited neurite extension. Scale bar: 50 μm. B: Inhibition of ADAM10 decreases ganglion cell differentiation (a) Quantification of Tuj-1-positive cells. (b) Measurement of neurite outgrowth. The number of Tuj-1-positive cells and the mean length of neurite outgrowth were reduced in GI254023X-treated cultures. The graphs show the mean values calculated in three different areas of three different experiments; $^{*}P < 0.001$, $^{**}P < 0.005$. C: Cells were cultured for 6 and 24 h in serum containing media with or without 5 μM of GI254023X. Equal amounts of cell lysates were subjected to Western blot analysis using an anti-Tuj-1 antibody. The protein expression of Tuj-1 was down-regulated by GI254023X treatment. β-actin was used as the loading control. D: Cells were exposed to BrdU for 2 h and fixed, and the proliferating cells were observed using a monoclonal antibody against BrdU, followed by fluorescence microscopy. Notably, the BrdU-positive cells (purple) did not co-localize with the Tuj-1-positive cells (green). Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm.

c′ and d′. These data confirmed that ADAM10 knockdown does appear to affect RGC differentiation but not photoreceptor cell differentiation.

INHIBITION OF ADAM10 DECREASES Pax6 EXPRESSION DURING RGC DIFFERENTIATION

During the preneurogenic-to-neurogenic transition, RPCs undergo a dramatic down-regulation of Pax6, while neurogenic progenitors maintain low but detectable levels. Furthermore, Pax6 expression is seen in post-mitotic differentiating RGCs but not in cone photoreceptor cells [Hsieh and Yang, 2009]. Our immunofluorescence staining showed numerous strong nuclear Pax6 signals (purple), presumably representing preneurogenic RPCs, in control cultures at 6 h. In contrast, fewer Pax6 signals were observed in GI254023X-treated cultures (Fig. 6A). Furthermore, Pax6 immunoreactivity was strongly detected in the nucleus (magenta; arrows) of ganglion cells in control cultures after 24 h (Fig. 6A), but far fewer Tuj-1- and Pax6-expressing cells were seen in GI254023X-treated cultures. The differentiated ganglion cells strongly showed Pax6 (magenta) expression in nucleus in magnified images (yellow arrows). The images of a′, b′, and c′ were the enlarged images of a, b, and c, respectively (Fig. 6A). Similarly, we found that Pax6 expression was weakly detected in ADAM10-siRNA-GFP transfected cells, but large numbers of strong Pax6-positive signals were detected in control cultured cells (Fig. 6B). Importantly, strong Pax6 signal was detected in the siRNA-GFP-negative cells indicated by red arrow (a and a′), whereas cell bearing with GFP signal showed very weak Pax6 signal (green arrow; b and b′) or no Pax6 expression. The images of a′ and b′ were the enlarged images of a and b, respectively. Consistent with these findings, Western blot analysis illustrated that GI254023X treatment greatly reduced the protein expression of Pax6 resulting in the decreased RGC differentiation (Fig. 6C). Furthermore, the protein level of Pax6 is also down-regulated by ADAM10-siRNA transfected cultures compared with

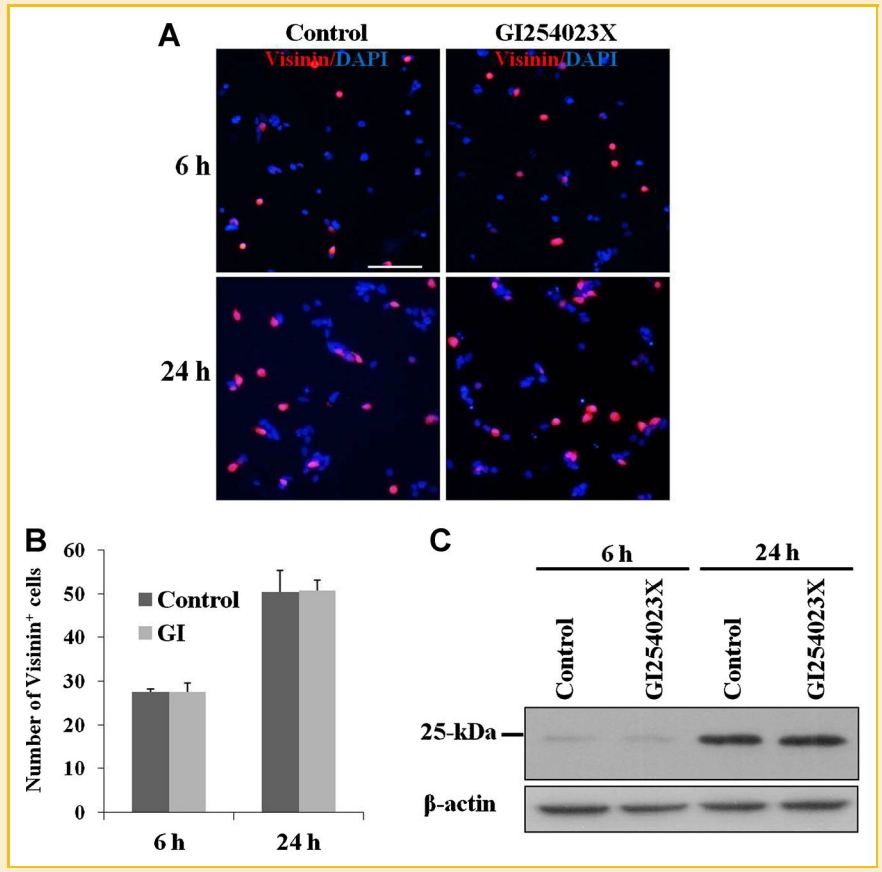

Fig. 4. ADAM10 inhibition does not affect photoreceptor cell differentiation. A: Cells were cultured with or without GI254023X for 6 and 24 h, and stained for visinin. Control and GI254023X-treated cultures showed similar numbers of visinin-positive cells. B: Quantification of visinin-positive cells using the Image-j program. C: Western blot analysis confirmed that visinin expression was not changed by GI254023X treatment. β-actin was used as the loading control. Scale bar: 50 μm.

control culture for 24 h (Fig. 6D). Therefore, it seems likely that the role of ADAM10 in ganglion cell differentiation may be associated with Pax6 expression.

SECRETION OF *N*-CADHERIN ECTODOMAIN IS INHIBITED BY ADAM10 INHIBITION

A previous study showed that full-length *N*-cadherin (135 kDa) is cleaved by ADAM10 activity, generating a 90-kDa extracellular N-terminal fragment (NTF) and a 40-kDa intracellular C-terminal fragment (CTF1) [Reiss et al., 2005]. To examine whether *N*-cadherin cleavage was mediated by ADAM10 in our system, we used Western blotting to examine *N*-cadherin cleavage products in cultured cells treated with or without GI254023X for 6 and 24 h (Fig. 7A). The full-length, un-cleaved form of *N*-cadherin (135 kDa) was highly detected under all tested culture conditions. CTF1 (40 kDa) was detectable at 6 h and increased at 24 h in control cultures, but it was undetectable in GI254023X-treated cultures. Importantly, the secreted form of the NTF (90 kDa) was highly detected in conditioned media from control cultures at 24 h when neuronal cell differentiation was predominantly observed, but it was completely absent from the conditioned media of GI254023X-treated cells. Similarly, ADAM10 knockdown not only resulted in an inhibition of the CTF1 cleavage product, it also led to an inhibition of secreted NTF (Fig. 7B). Together, these results suggest that *N*-cadherin cleavage is regulated by an ADAM10-dependent manner in retinal cells and may be involved in mediating ganglion cell differentiation.

NTF STAINING IS DETECTED IN VISININ-POSITIVE PHOTORECEPTOR CELLS BUT NOT IN TUJ-1-POSITIVE GANGLION CELLS

Since ADAM10 inhibition blocked the secretion of the NTF, we examined whether ADAM10-mediated *N*-cadherin shedding is important for the differentiation of retinal ganglion or photoreceptor cells by immunofluorescence analysis (Fig. 8). Consistent with previous studies showing that *N*-cadherin is not expressed in RGCs [Matsunaga et al., 1988; Inuzuka et al., 1991a; Wohrn et al., 1998; Yan et al., 2011], we also observed that *N*-cadherin staining did not co-localize with strong Tuj-1-positive cells (white arrows) at 24 h in both control and-GI254023X-treated retinal cells (Fig. 8A). The images of a′, b′, and c′ were the enlarged images of a, b, and c, respectively (yellow arrows). In contrast, consistent with the observation that *N*-cadherin is always abundant only in the outer limiting membrane [Matsunaga et al., 1988; Inuzuka et al., 1991a], the visinin-positive signals (white arrows) co-localized with *N*-cadherin staining in merged images at 24 of both control and GI254023X-treated cells (Fig. 8B). In detail, *N*-cadherin was present largely at the cell surface of a single visinin-positive photoreceptor

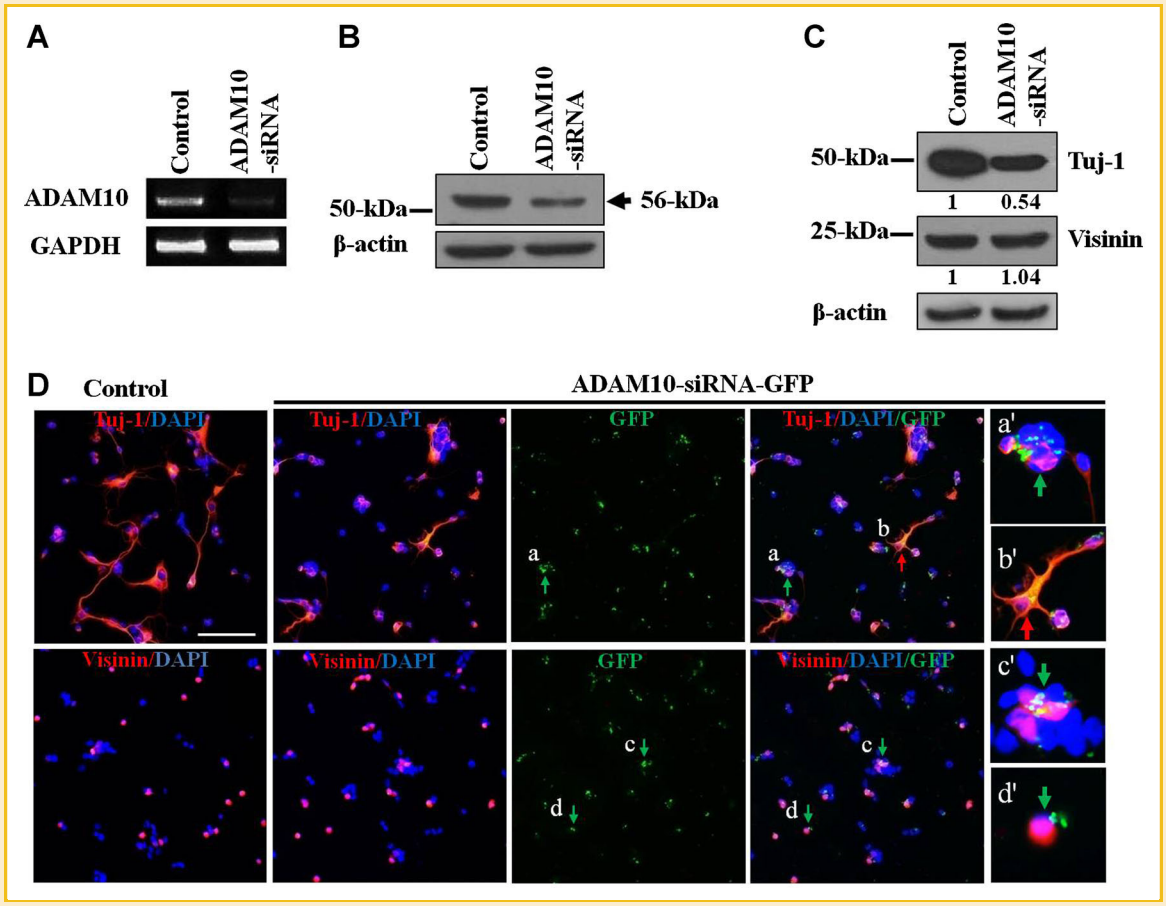

Fig. 5. Knockdown of ADAM10 suppresses ganglion cell differentiation. A: RT-PCR analysis of mRNA of ADAM10. Cells treated with transfection reagent alone was used as control. Specific GAPDH RT-PCR was used as loading control. B: Western blot analysis of total lysates from control cells and ADAM10-siRNA transfected cells. Equal amount of lysates were investigated with antibody against ADAM10. Western blot analysis using an anti ADAM10 antibody confirmed the expression level of mature form of ADAM10 was decreased by ADAM10-siRNA. β-actin was used as loading control. C: Equal amount of cell lysates from control cells and ADAM-10 siRNA transfected cells were subjected to Western blot analysis using monoclonal anti Tuj-1 and anti visinin antibodies. The protein expression of Tuj-1 was down regulated by ADAM10-siRNA but the protein level of visinin was not affected. β-actin was used as the loading control. D: Immunofluorescence analysis of cultured retinal cell with or without transfection with ADAM10-siRNA using anti Tuj-1 antibody (upper panel) and anti visinin antibody (lower panel). ADAM-10 siRNA inhibited neuritic extensions of ganglion cells. The Images of a′, b′, c′, and d′ were the enlarged images of a, b, c, and d, respectively. Figures were the representative of three independent experiments. Scale bar: 50 μm

cell (a, c, e, a′, c′, and e′). When dissociated chick ciliary ganglion neurons are grown in culture, some of the dissociated cells migrate sufficiently to join in small clumps composed usually two to four neurons [Conroy et al., 2000]. Interestingly, *N*-cadherin staining was also highly concentrated at the interface (asterisks) between visinin-positive photoreceptor cell and other cells (b, d, f, b′, d′, and f′). The images of a′, b′, c′, d′, e′, and f′ were the enlarged images of a, b, c, d, e, and f, respectively. The principal finding in this study is that *N*-cadherin is highly detected in the photoreceptor cells membrane, but is not in the ganglion cells at all. Therefore, it seems likely that ADAM10-mediated *N*-cadherin shedding may be associated with the differentiation of ganglion cells but not photoreceptor cells.

DISCUSSION

In this study, we identified ADAM10 as an important regulatory factor for neurite outgrowth during the differentiation of retinal neuroblasts in vitro. To the best of our knowledge, this is the first report showing that ADAM10-mediated *N*-cadherin ectodomain shedding is involved in the differentiation of RGCs but not photoreceptor cells.

INVOLVEMENT OF ADAM10 IN RGC DIFFERENTIATION

During the period of active neurogenesis in the retina, neuroblasts enter the post-mitotic state and then differentiate into retinal neurons [Tanaka et al., 2002]. ADAM10 is expressed and spatiotemporally regulated in the developing retinal layers [Yan et al., 2011], and its function is required for the targeting of RGC axons in the tectum [Chen et al., 2007]. Similar to previous findings [Yan et al., 2011], we observed strong and abundant ADAM10 protein expression among neuroblasts in the inner zone of the NBL at ED5 (Fig. 1). After their final mitosis, RGCs are located in the innermost layer of the retina [Prada et al., 1981]. Consistent with this, post-mitotic Tuj-1-positive ganglion cells failed to label with

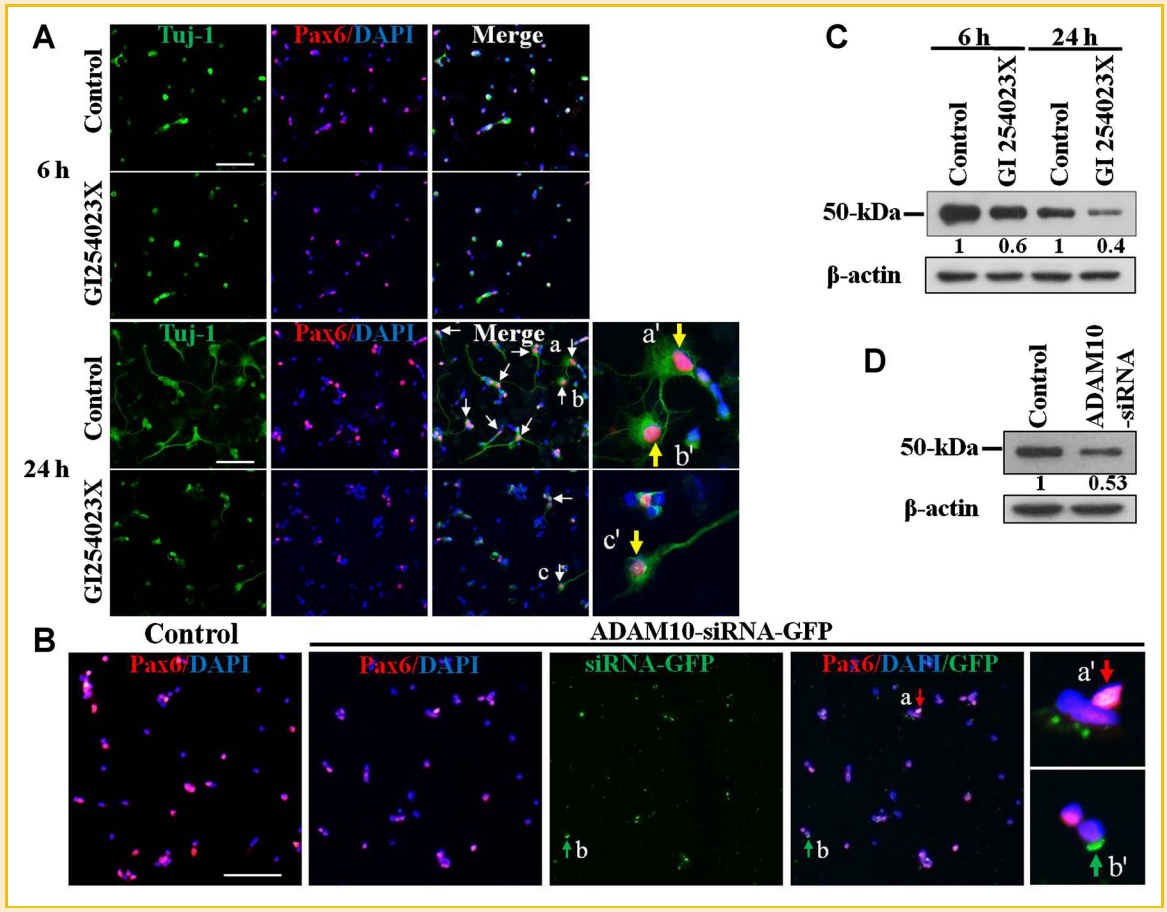

Fig. 6. ADAM10 inhibition blocks Pax6 expression and ganglion cell differentiation. A: Cells were cultured with or without GI254023X for 6 and 24 h and then stained for Tuj-1 (green), Pax6 (RED) and DAPI (blue). Strong and mainly nuclear Pax6 signals (magenta), presumably representing preneurogenic retinal progenitor cells, were observed in control cultures at 6 h, but the signal and the number of Pax6-positive cells were reduced in GI254023X-treated cultures. Pax6 was highly observed in the nucleus (magenta; arrows) of ganglion cells at 24 h in control cultures, but both-Tuj-1 and Pax6-expressing cells were greatly reduced in GI254023X-treated cultures. The images a′, b′, and c′ are the enlarged images of a, b, and c, respectively. B: Immunofluorescence analysis of cultured retinal cells with or without transfection with ADAM10-siRNA using anti-Pax6 antibody. Cells treated with transfection reagent alone were used as control. Pax6-positive cells were more strongly detected in control cultured cells than that of ADAM10 siRNA transfected cells. Note that ADAM-10 siRNA down-regulated Pax6 expression. The images of a′ and b′ were the enlarged images of a and b, respectively. C: Western blot analysis using an anti-Pax6 antibody confirmed that Pax6 protein expression is down-regulated in GI254023X-treated cultures at 6 and 24 h. β-actin was used as the loading control. D: Western blot analysis using an anti-Pax6 antibody confirmed that Pax6 protein expression is down-regulated by ADAM10-siRNA. β-actin was used as the loading control. Scale bar: 50 μm.

BrdU in our in vitro experiments (Fig. 3D). Strong ADAM10 signals were restricted to the ganglion cells at ED7, and were highly detected in the ganglion cell layer by ED9. Furthermore, we detected the active form of ADAM10 in our cultured cell system (Fig. 1), and found that inhibition of ADAM10 activity or expression decreased neurite outgrowth and ganglion cell differentiation (Figs. 2, 3, and 5). Importantly, the differentiation of visinin-positive cone photoreceptor cells was not affected by the inhibition of ADAM10 expression or activity (Figs. 4 and 5). Taken together, our present data suggest that ADAM10 may play a role in ganglion cell differentiation during chicken retinal development. In fact, a recent study has demonstrated ADAM9 and ADAM17 are also widely expressed in the differential layers of the developing chick retina [Yan et al., 2011]. In addition, ADAM22 and ADAM23 mRNAs are expressed strongly in the NBL at E5 and the GCL at E7. Therefore, it seems likely that several ADAMs may function together to contribute RGC differentiation during chick retinal development.

INHIBITION OF ADAM10 BLOCKS BOTH Pax6 EXPRESSION AND RGC DIFFERENTIATION

Pax6 controls timing of retinal neuron differentiation and determination of specific neuronal cell type [Philips et al., 2005]. Pax6 functions at multiple levels to integrate extracellular information and execute cell-intrinsic differentiation programs [Shaham et al., 2012] in a tissue- and stage-specific manner [Chow et al., 1999]. Pax6 function may change at different stages of retinal development as development proceeds [Philips et al., 2005]. RPCs express high levels of Pax6 in the neural retina preceding differentiation in the developing chick retina by E6 [Canto-Soler et al., 2008; Riesenberg et al., 2009], which is probably required to maintain the retinal progenitors and to inhibit premature onset of

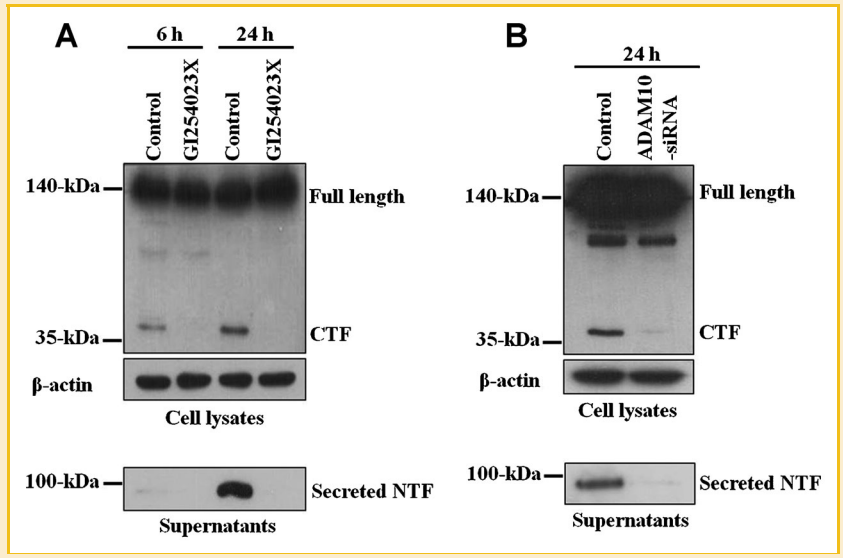

Fig. 7. GI254023X treatment inhibits *N*-cadherin ectodomain shedding. A: Western blot analysis was used to examine *N*-cadherin expression in cell lysates and conditioned media. Comparable high levels of un-cleaved full-length *N*-cadherin (135 kDa) were detected in control and GI254023X-treated cells at 6 and 24 h. The secreted ectodomain-cleaved form (90 kDa) was highly detected only in control cultures at 24 h; it was not detected at all in GI254023X-treated cultures. The C-terminal intracellular fragment (40 kDa) was detected at 6 h and increased at 24 h in control cultures; it was not detected at all in GI256023X-treated cultures. β-actin was used as the loading control. B: Western blot analysis to examine *N*-cadherin expression in cell lysates after 24 h of transfection. Cells treated with transfection reagent alone were used as control. Comparable high level of cleaved CTF *N*-cadherin (40 kDa) was detected in control and it was inhibited by ADAM10-siRNA. The secreted ectodomain-cleaved form was not detected in siRNA transfected cells. β-actin was used as loading control.

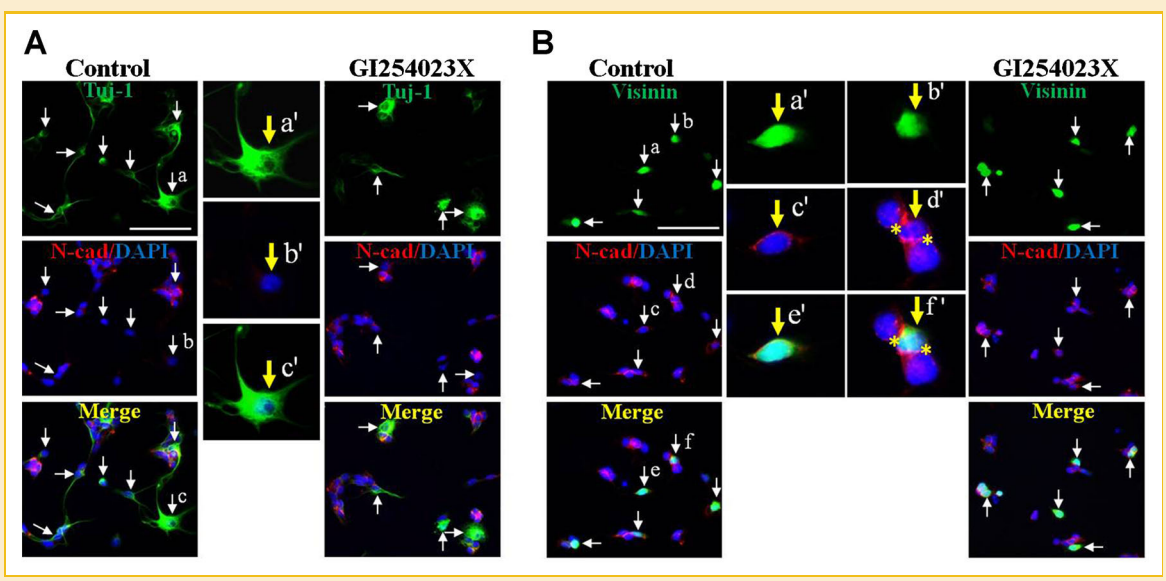

Fig. 8. *N*-cadherin expression in photoreceptor cells membrane, but not in ganglion cells. Dissociated ED6 retinal cells were cultured with or without GI254023X for 24 h and subjected to immunofluorescence staining. A: Cells were co-stained with using monoclonal anti-Tuj-1(green) and polyclonal anti-*N*-cadherin (red) antibodies. Nuclei were counterstained with DAPI (blue). Note that the *N*-cadherin signal did not co-localize with Tuj-1-positive differentiated ganglion cells (arrows) in both control and GI254023X-treated cultures. The Images of a′, b′, and c′ were the enlarged images of a, b, and c, respectively. B: Cells were co-stained with monoclonal anti-visinin (green) and polyclonal anti-*N*-cadherin (red) antibodies. Nuclei were counterstained with DAPI (blue). Note that *N*-cadherin signals co-localized with the visinin-positive photoreceptor cells (arrows) in both control and GI254023X-treated cultures. *N*-cadherin was present at the visinin-positive single photoreceptor cell membrane (a, c, e, a′, c′, and e′). In addition, *N*-cadherin staining was also highly concentrated at the interface (asterisks) between visinin-positive photoreceptor cell and other cells (b, d, f, b′, d′, and f′). The images of a′, b′, c′, d′, e′, f′ were the enlarged images of a, b, c, d, e, and f, respectively. Scale bar: 50 μm.

abrogated neurogenic program [Gumbiner, 1996]. During retinogenesis, RPCs appear to be retained in a progenitor state by the action of Notch-Delta signaling [Henrique et al., 1997]. As a result of Notch activation, RGC differentiation is inhibited in neighboring cells and they remain neural progenitor cells in retinal development in vivo [Austin et al., 1995]. Pax6 action is counteracted by the Notch-Delta signaling pathway, which keeps RPCs in the cell cycle by activating the bHLH transcriptional repressors Hes1 and Hes5 [Agathocleous and Harris, 2009]. Pax6 is thereafter substantially down-regulated when the cells enter the neurogenic phase [Ferreiro-Galve et al., 2010, 2011]. Its expression is abolished in post-mitotic precursors of cone photoreceptor cells [Oron-Karni et al., 2008; Hsieh and Yang, 2009]. However, Pax6 expression is up-regulated in differentiated RGCs [Oron-Karni et al., 2008; Hsieh and Yang, 2009]. For RGC commitment to occur, a population of Pax6-expressing RPCs must down-regulate the Notch signaling pathway, exit the cell cycle, and express the proneural bHLH gene Math5 [Mu and Klein, 2004; Agathocleous and Harris, 2009]. Previous study demonstrated that Pax6 expression in differentiated neurons stimulates process elongation [Sebastian-Serrano et al., 2012]. Consistent with these previous findings, we detected high levels of Pax6 in differentiated ganglion cells with long neurites of control cultures at 24 h. In contrast, Pax6 expression was decreased along with the number of ganglion cells with short neurites in cultures treated with the ADAM10 inhibitor or ADAM10 siRNA (Fig. 6), suggesting that Pax6-associated RGC differentiation may be mediated, in part, by ADAM10.

RGCs are generated in stages E4, E4.5, and E6 in chick as Delta-Notch signaling decreases from center to the periphery presumably due to a gradient decrease in Notch and Delta expression in vivo [Ahmad et al., 1997]. During retinal development in vivo, ADAM10 may directly participate in the proteolytic activation of Notch to regulate signaling [Rooke et al., 1996; Bozkulak and Weinmaster, 2009; Glomski et al., 2011]. Of note, there are mixture of stem and early committed precursor cells, and differentiating cells in culture of chick retinal cells at E6 in this study. Therefore, it is not sufficient to induce Notch activation by Delta expressing cells because cell–cell interaction through Notch-Delta signal is broken in dissociated culture [Austin et al., 1995]. In addition, some RPCs may be insensitive to Notch signaling and a Notch-independent ganglion cell inducing signal may explain the ganglion cell persistence [Austin et al., 1995]. Taken together, although the association of ADAM10 and pax6 during RGC differentiation is not clear at this moment, ADAM10-mediated regulation of Pax6 may play an important role in ganglion cell differentiation. Future functional studies will be necessary to elucidate how activities of ADAM10 integrate with Pax6's role in RGC differentiation.

ADAM10-MEDIATED REGULATION OF SOLUBLE FORM OF THE CLEAVED *N*-CADHERIN ECTODOMAIN MAY BE ASSOCIATED WITH NEURITE OUTGROWTH AND GANGLION CELL DIFFERENTIATION

A variety studies have indicated that neurogenesis involves cell–ECM and cell-to-cell interactions and communication. There are multiple findings supporting a role for Pax6 in the regulation of cell-adhesion molecules (NCAM and Cadherins) and ECM molecules, which are required for axon outgrowth [Holst et al., 1997; Stoykova et al., 1997; Gotz et al., 1998; Andrews and Mastick, 2003]. As a consequence of Pax6 knockdown, the homologue of R-cadherin, *N*-cadherin, as well as other cell-adhesion molecules, was shown to be down-regulated [Rungger-Brandle et al., 2010]. In addition, loss of Pax6 disturbs cytoskeletal organization, thus affecting cell migration and neurite outgrowth in the cerebellum [Engelkamp et al., 1999; Yamasaki et al., 2001]. Therefore, it seems likely that Pax6-mediated *N*-cadherin regulation may play a role for RGC differentiation in cultured retinal cells.

Undifferentiated retinal neuroblasts contact each other through with *N*-cadherin from the beginning of the formation of the retina. However, *N*-cadherin gradually disappeared from the ganglion process layer [Matsunaga et al., 1988] and finally disappears from the most parts of the retina during development [Inuzuka et al., 1991ab]. However, *N*-cadherin is eventually localized only in the outer limiting membrane of retina [Matsunaga et al., 1988]. Notably, the ADAM10 protein is co-expressed with *N*-cadherin in distinct retinal layers [Yan et al., 2011]. *N*-cadherin homophilic binding may generate a specific signal within the neuronal cell body, axon, or growth cone [Rosdahl et al., 2002]. *N*-cadherin contains a large *N*-terminal extracellular region which consists of five tandem repeated domains (EC1–EC5), and the cytoplasmic domain of *N*-cadherin interacts with β-catenin, which in turn is linked to the cytoskeleton [Reiss et al., 2005]. ADAM10 can cleave the ectodomain of *N*-cadherin, which modulates cell–cell adhesion and β-catenin nuclear signaling [Reiss et al., 2005]. The released ectodomain of the *N*-cadherin is functionally important for the regulation of cell adhesion, cell migration and neurite outgrowth [Paradies and Grunwald, 1993; Bixby et al., 1994; Nakagawa and Takeichi, 1998], and a previous study showed that the soluble form of *N*-cadherin (NCAD90) induced calcium increases in ciliary ganglion cell bodies and neuronal growth cones [Bixby et al., 1994]. NCAD90, which is generated by proteolysis at the cell surface during chick retinal development, promotes neurite outgrowth [Paradies and Grunwald, 1993]. Shed *N*-cadherin stimulates neurite outgrowth through activation of Ca^{2+}/calmodulin kinase II (CaMKII) [Hansen et al., 2008]. In addition, NCAD can also associate with and activate FGF receptors, resulting in the activation of PI3K and AKT signaling, which also promote neurite outgrowth [McCusker and Alfandari, 2009]. Previous studies showed that *N*-cadherin is expressed by the ADAM10-positive photoreceptor cells in the developing chick retinas at ED16 [Yan et al., 2011]. *N*-cadherin mutations disrupt maintenance of adherens junctions in the neural retina, and photoreceptors are the neuronal subtype least affected by *N*-cadherin mutation [Masai et al., 2003]. The entire structure of the retina is disordered by culturing with the *N*-cadherin antibody (Fab), especially the photoreceptor cell layer [Matsunaga et al., 1988]. The position-specific differences in the expression of *N*-cadherin may contribute to establishing and maintaining the segregated distributions of different retinal cells [Inuzuka et al., 1991a]. Such heterogeneity in *N*-cadherin expression in visinin-positive photoreceptor cells was also observed in vitro cultures of retinal cells (Fig. 8). By contrast, ADAM10 protein is expressed in RGCs, where Cadherin-6B and Cadherin-7, but not *N*-cadherin, are co-expressed [Yan et al., 2011]. In the present study, *N*-cadherin cleavage was abrogated by pharmacological inhibitor and siRNA specific for

ADAM10 (Fig. 7). In addition, *N*-cadherin staining was not detected in ganglion cells, but was detected in photoreceptor cells (Fig. 8), suggesting that ganglion cell differentiation is closely related to ADAM10-mediated *N*-cadherin ectodomain shedding. Although it is not known whether *N*-cadherin cleavage is solely responsible for ADAM10's role in RGC differentiation, our study suggests that the ADAM10-mediated shedding of the soluble form of cleaved *N*-cadherin ectodomain may trigger neurite outgrowth among RGCs in vitro.

Taken together, our findings demonstrate that inhibition of ADAM10 activity restrained the differentiation of ganglion cells but had no effect on photoreceptor cell differentiation. Our findings may provide new insight into the role of ADAM10 in RGC differentiation in vitro.

REFERENCES

Adler R. 2000. A model of retinal cell differentiation in the chick embryo. Prog Retin Eye Res 19:529–557.

Agathocleous M, Harris WA. 2009. From progenitors to differentiated cells in the vertebrate retina. Annu Rev Cell Dev Biol 25:45–69.

Ahmad I, Dooley CM, Polk DL. 1997. Delta-1 is a regulator of neurogenesis in the vertebrate retina. Dev Biol 185:92–103.

Alfandari D, McCusker C, Cousin H. 2009. ADAM function in embryogenesis. Semin Cell Dev Biol 20:153–163.

Andrews GL, Mastick GS. 2003. R-cadherin is a Pax6-regulated, growth-promoting cue for pioneer axons. J Neurosci 23:9873–9880.

Austin CP, Feldman DE, Ida JA, Jr., Cepko CL. 1995. Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development 121:3637–3650.

Bixby JL, Zhang R. 1990. Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J Cell Biol 110:1253–1260.

Bixby JL, Grunwald GB, Bookman RJ. 1994. Ca^{2+} influx and neurite growth in response to purified N-cadherin and laminin. J Cell Biol 127:1461–1475.

Bozkulak EC, Weinmaster G. 2009. Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling. Mol Cell Biol 29:5679–5695.

Canto-Soler MV, Huang H, Romero MS, Adler R. 2008. Transcription factors CTCF and Pax6 are segregated to different cell types during retinal cell differentiation. Dev Dyn 237:758–767.

Chen YY, Hehr CL, Atkinson-Leadbeater K, Hocking JC, McFarlane S. 2007. Targeting of retinal axons requires the metalloproteinase ADAM10. J Neurosci 27:8448–8456.

Chow RL, Altmann CR, Lang RA, Hemmati-Brivanlou A. 1999. Pax6 induces ectopic eyes in a vertebrate. Development 126:4213–4222.

Conroy WG, Ogden LF, Berg DK. 2000. Cluster formation of alpha7-containing nicotinic receptors at interneuronal interfaces in cell culture. Neuropharmacology 39:2699–2705.

Dallas DJ, Genever PG, Patton AJ, Millichip MI, McKie N, Skerry TM. 1999. Localization of ADAM10 and Notch receptors in bone. Bone 25:9–15.

Duffy MJ, McKiernan E, O'Donovan N, McGowan PM. 2009. Role of ADAMs in cancer formation and progression. Clin Cancer Res 15:1140–1144.

Edwards DR, Handsley MM, Pennington CJ. 2008. The ADAM metalloproteinases. Mol Aspects Med 29:258–289.

Engelkamp D, Rashbass P, Seawright A, van Heyningen V. 1999. Role of Pax6 in development of the cerebellar system. Development 126:3585–3596.

Escrevente C, Morais VA, Keller S, Soares CM, Altevogt P, Costa J. 2008. Functional role of N-glycosylation from ADAM10 in processing, localization and activity of the enzyme. Biochim Biophys Acta 1780:905–913.

Esteve P, Sandonis A, Cardozo M, Malapeira J, Ibanez C, Crespo I, Marcos S, Gonzalez-Garcia S, Toribio ML, Arribas J, Shimono A, Guerrero I, Bovolenta P. 2011. SFRPs act as negative modulators of ADAM10 to regulate retinal neurogenesis. Nat Neurosci 14:562–569.

Fanarraga ML, Avila J, Zabala JC. 1999. Expression of unphosphorylated class III beta-tubulin isotype in neuroepithelial cells demonstrates neuroblast commitment and differentiation. Eur J Neurosci 11:517–527.

Ferreiro-Galve S, Rodriguez-Moldes I, Anadon R, Candal E. 2010. Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas. J Chem Neuroanat 39:1–14.

Ferreiro-Galve S, Rodriguez-Moldes I, Candal E. 2011. Pax6 expression during retinogenesis in sharks: Comparison with markers of cell proliferation and neuronal differentiation. J Exp Zool B Mol Dev Evol 318:91–108.

Glomski K, Monette S, Manova K, De Strooper B, Saftig P, Blobel CP. 2011. Deletion of Adam10 in endothelial cells leads to defects in organ-specific vascular structures. Blood 118:1163–1174.

Gotz M, Stoykova A, Gruss P. 1998. Pax6 controls radial glia differentiation in the cerebral cortex. Neuron 21:1031–1044.

Gumbiner BM. 1996. Cell adhesion: The molecular basis of tissue architecture and morphogenesis. Cell 84:345–357.

Hall RJ, Erickson CA. 2003. ADAM 10: An active metalloprotease expressed during avian epithelial morphogenesis. Dev Biol 256:146–159.

Hansen SM, Berezin V, Bock E. 2008. Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-cadherin. Cell Mol Life Sci 65:3809–3821.

Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquie O, Ish-Horowicz D, Lewis J. 1997. Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol 7:661–670.

Holst BD, Wang Y, Jones FS, Edelman GM. 1997. A binding site for Pax proteins regulates expression of the gene for the neural cell adhesion molecule in the embryonic spinal cord. Proc Natl Acad Sci USA 94:1465–1470.

Hsieh YW, Yang XJ. 2009. Dynamic Pax6 expression during the neurogenic cell cycle influences proliferation and cell fate choices of retinal progenitors. Neural Dev 4:32.

Huh MI, Lee YM, Seo SK, Kang BS, Chang Y, Lee YS, Fini ME, Kang SS, Jung JC. 2007. Roles of MMP/TIMP in regulating matrix swelling and cell migration during chick corneal development. J Cell Biochem 101:1222–1237.

Huntley GW. 2002. Dynamic aspects of cadherin-mediated adhesion in synapse development and plasticity. Biol Cell 94:335–344.

Inuzuka H, Miyatani S, Takeichi M. 1991a. R-cadherin: A novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina. Neuron 7: 69–79.

Inuzuka H, Redies C, Takeichi M. 1991b. Differential expression of R- and N-cadherin in neural and mesodermal tissues during early chicken development. Development 113:959–967.

Jorissen E, Prox J, Bernreuther C, Weber S, Schwanbeck R, Serneels L, Snellinx A, Craessaerts K, Thathiah A, Tesseur I, Bartsch U, Weskamp G, Blobel CP, Glatzel M, De Strooper B, Saftig P. 2010. The disintegrin/metalloproteinase ADAM10 is essential for the establishment of the brain cortex. J Neurosci 30:4833–4844.

Karkkainen I, Rybnikova E, Pelto-Huikko M, Huovila AP. 2000. Metalloprotease-disintegrin (ADAM) genes are widely and differentially expressed in the adult CNS. Mol Cell Neurosci 15:547–560.

Kim YH, Chang Y, Jung JC. 2012. Staurosporine induces ganglion cell differentiation in part by stimulating urokinase-type plasminogen activator expression and activation in the developing chick retina. Biochem Biophys Res Commun 423:67–72.

Kohutek ZA, diPierro CG, Redpath GT, Hussaini IM. 2009. ADAM-10-mediated N-cadherin cleavage is protein kinase C-alpha dependent and promotes glioblastoma cell migration. J Neurosci 29:4605–4615.

Lin J, Luo J, Redies C. 2008. Differential expression of five members of the ADAM family in the developing chicken brain. Neuroscience 157: 360–375.

Liu X, Mizoguchi A, Takeichi M, Honda Y, Ide C. 1997. Developmental changes in the subcellular localization of R-cadherin in chick retinal pigment epithelium. Histochem Cell Biol 108:35–43.

Markus A, Yan X, Rolfs A, Luo J. 2011. Quantitative and dynamic expression profile of premature and active forms of the regional ADAM proteins during chicken brain development. Cell Mol Biol Lett 16:431–451.

Masai I, Lele Z, Yamaguchi M, Komori A, Nakata A, Nishiwaki Y, Wada H, Tanaka H, Nojima Y, Hammerschmidt M, Wilson SW, Okamoto H. 2003. N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. Development 130:2479–2494.

Matsunaga M, Hatta K, Takeichi M. 1988. Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina. Neuron 1:289–295.

McCulloch DR, Akl P, Samaratunga H, Herington AC, Odorico DM. 2004. Expression of the disintegrin metalloprotease, ADAM-10, in prostate cancer and its regulation by dihydrotestosterone, insulin-like growth factor I, and epidermal growth factor in the prostate cancer cell model LNCaP. Clin Cancer Res 10:314–323.

McCusker CD, Alfandari D. 2009. Life after proteolysis: Exploring the signaling capabilities of classical cadherin cleavage fragments. Commun Integr Biol 2:155–157.

Millichip MI, Dallas DJ, Wu E, Dale S, McKie N. 1998. The metallo-disintegrin ADAM10 (MADM) from bovine kidney has type IV collagenase activity in vitro. Biochem Biophys Res Commun 245:594–598.

Mu X, Klein WH. 2004. A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. Semin Cell Dev Biol 15:115–123.

Nagano O, Murakami D, Hartmann D, De Strooper B, Saftig P, Iwatsubo T, Nakajima M, Shinohara M, Saya H. 2004. Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. J Cell Biol 165: 893–902.

Nakagawa S, Takeichi M. 1998. Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125:2963–2971.

Oron-Karni V, Farhy C, Elgart M, Marquardt T, Remizova L, Yaron O, Xie Q, Cvekl A, Ashery-Padan R. 2008. Dual requirement for Pax6 in retinal progenitor cells. Development 135:4037–4047.

Paradies NE, Grunwald GB. 1993. Purification and characterization of NCAD90, a soluble endogenous form of N-cadherin, which is generated by proteolysis during retinal development and retains adhesive and neurite-promoting function. J Neurosci Res 36:33–45.

Philips GT, Stair CN, Young Lee H, Wroblewski E, Berberoglu MA, Brown NL, Mastick GS. 2005. Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type. Dev Biol 279:308–321.

Pimentel B, Sanz C, Varela-Nieto I, Rapp UR, De Pablo F, de La Rosa EJ. 2000. c-Raf regulates cell survival and retinal ganglion cell morphogenesis during neurogenesis. J Neurosci 20:3254–3262.

Prada C, Puelles L, Genis-Galvez JM. 1981. A golgi study on the early sequence of differentiation of ganglion cells in the chick embryo retina. Anat Embryol (Berl) 161:305–317.

Puschel AW, Gruss P, Westerfield M. 1992. Sequence and expression pattern of pax-6 are highly conserved between zebrafish and mice. Development 114:643–651.

Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO. 1997. Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181:64–78.

Reiss K, Saftig P. 2009. The ''a disintegrin and metalloprotease'' (ADAM) family of sheddases: Physiological and cellular functions. Semin Cell Dev Biol 20:126–137.

Reiss K, Maretzky T, Ludwig A, Tousseyn T, de Strooper B, Hartmann D, Saftig P. 2005. ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. EMBO J 24:742–752.

Riesenberg AN, Liu Z, Kopan R, Brown NL. 2009. Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J Neurosci 29:12865–12877.

Rooke J, Pan D, Xu T, Rubin GM. 1996. KUZ, a conserved metalloprotease-disintegrin protein with two roles in Drosophila neurogenesis. Science 273:1227–1231.

Rosdahl JA, Mourton TL, Brady-Kalnay SM. 2002. Protein kinase C delta (PKCdelta) is required for protein tyrosine phosphatase mu (PTPmu)-dependent neurite outgrowth. Mol Cell Neurosci 19:292–306.

Rungger-Brandle E, Ripperger JA, Steiner K, Conti A, Stieger A, Soltanieh S, Rungger D. 2010. Retinal patterning by Pax6-dependent cell adhesion molecules. Dev Neurobiol 70:764–780.

Saga T, Scheurer D, Adler R. 1996. Development and maintenance of outer segments by isolated chick embryo photoreceptor cells in culture. Invest Ophthalmol Vis Sci 37:561–573.

Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP. 2004. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol 164:769–779.

Schulte D, Bumsted-O'Brien KM. 2008. Molecular mechanisms of vertebrate retina development: Implications for ganglion cell and photoreceptor patterning. Brain Res 1192:151–164.

Sebastian-Serrano A, Sandonis A, Cardozo M, Rodriguez-Tornos FM, Bovolenta P, Nieto M. 2012. Pax6 expression in postmitotic neurons mediates the growth of axons in response to SFRP1. PLoS ONE 7:e31590.

Shaham O, Menuchin Y, Farhy C, Ashery-Padan R. 2012. Pax6: A multi-level regulator of ocular development. Prog Retin Eye Res 31:351–376.

Snow RL, Robson JA. 1994. Ganglion cell neurogenesis, migration and early differentiation in the chick retina. Neuroscience 58:399–409.

Stoykova A, Gotz M, Gruss P, Price J. 1997. Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. Development 124:3765–3777.

Tanaka H, Yamashita T, Asada M, Mizutani S, Yoshikawa H, Tohyama M. 2002. Cytoplasmic p21(Cip1/WAF1) regulates neurite remodeling by inhibiting Rho-kinase activity. J Cell Biol 158:321–329.

Walther C, Gruss P. 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113:1435–1449.

Watanabe M, Rutishauser U, Silver J. 1991. Formation of the retinal ganglion cell and optic fiber layers. J Neurobiol 22:85–96.

White JM. 2003. ADAMs: Modulators of cell-cell and cell-matrix interactions. Curr Opin Cell Biol 15:598–606.

Wohrn JC, Puelles L, Nakagawa S, Takeichi M, Redies C. 1998. Cadherin expression in the retina and retinofugal pathways of the chicken embryo. J Comp Neurol 396:20–38.

Wolfsberg TG, Primakoff P, Myles DG, White JM. 1995. ADAM, a novel family of membrane proteins containing A Disintegrin And Metalloprotease domain: Multipotential functions in cell-cell and cell-matrix interactions. J Cell Biol 131:275–278.

Yamasaki T, Kawaji K, Ono K, Bito H, Hirano T, Osumi N, Kengaku M. 2001. Pax6 regulates granule cell polarization during parallel fiber formation in the developing cerebellum. Development 128:3133–3144.

Yan X, Lin J, Rolfs A, Luo J. 2011. Differential expression of the ADAMs in developing chicken retina. Dev Growth Differ 53:726–739.

Yang P, Baker KA, Hagg T. 2006. The ADAMs family: Coordinators of nervous system development, plasticity and repair. Prog Neurobiol 79:73–94.