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Expression of the heparin-binding growth factors Midkine and Pleiotrophin during ocular development

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Abstract

Midkine (MDK) and Pleiotrophin (PTN) belong to a group of heparin-binding growth factors that has been shown to have pleiotropic functions in various biological processes during development and disease. Development of the vertebrate eye is a multistep process that involves coordinated interactions between neuronal and non-neuronal cells, but very little is known about the potential function of MDK and PTN in these processes. In this study, we demonstrate by section *in situ* hybridization, the spatiotemporal expression of *MDK* and *PTN* during ocular development in chick and mouse. We show that *MDK* and *PTN* are expressed in dynamic patterns that overlap in a few non-neuronal tissues in the anterior eye and in neuronal cell layers of the posterior eye. We show that the expression patterns of *MDK* and *PTN* are only conserved in a few tissues in chick and mouse but they overlap with the expression of some of their receptors *LRP1, RPTPZ, ALK, NOTCH2,ITGβ1, SDC1*, and *SDC3*. The dynamic expression patterns of *MDK, PTN* and their receptors suggest that they function together during the multistep process of ocular development and they may play important roles in cell proliferation, adhesion, and migration of neuronal and non-neuronal cells.

Keywords

Midkine; Pleiotrophin; ocular development; cornea; lens; retina

Introduction

Midkine (MDK) and pleiotrophin (PTN) are closely related genes that form a two-member family of heparin binding growth factors. MDK was identified as a retinoic acid-induced gene in carcinoma cells (Kadomatsu et al., 1990). PTN was discovered as a neurite outgrowth-promoting factor in neonatal rat brains (Rauvala and Pihlaskari, 1989), and also as a mitogen for bovine uterus fibroblasts (Li et al., 1990). Since their discovery, several studies have shown that MDK and PTN have pleiotropic functions in various biological processes including cell proliferation, migration, differentiation and survival, and in neural

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development, angiogenesis, cancer, and inflammation (Choudhuri et al., 1997; Garver et al., 1993; Li et al., 1990; Ohta et al., 1999; Qi et al., 2001; Weckbach et al., 2011; Weckbach et al., 2012), but their function in ocular development remain unclear.

MDK and PTN function by binding various receptors that include receptor protein tyrosine phosphatase beta/zeta (RPTPZ), anaplastic lymphoma kinase (ALK), integrin beta 1 (ITGα1), NOTCH2, lipoprotein receptor-related protein 1 (LRP1), and co-receptors syndecan-1 (SDC1) and syndecan-3 (SDC3) (Huang et al., 2008; Kaspiris et al., 2013; Muramatsu et al., 2000; Muramatsu et al., 2004; Raulo et al., 1994; Reiff et al., 2011; Sakaguchi et al, 2003; Stoica et al., 2002). Through interactions with these receptors, MDK and PTN promote neuronal cell migration and differentiation of neural stem cells (Jung et al., 2004; Maeda et al., 1999), enhance endothelial cell proliferation and angiogenesis (Choudhuri et al., 1997; Laaroubi et al., 1994; Mikelis et al., 2009), act as mitogens for fibroblasts, hepatocytes, and osteoblasts (Asahina et al., 2002; Li et al., 1990; Yang et al., 2003), and play various roles in cancer (see review by Jono and Ando, 2010).

Studies in mice indicate that *Mdk* is ubiquitously expressed during early embryogenesis and it becomes progressively restricted to the central nervous system, ocular tissues, jaws, somites, and kidneys where it is transiently expressed during organogenesis (Kadomatsu et al., 1990). Also in mice, *Ptn* is expressed in the central and peripheral nervous system during development (Fan et al., 2000; Nakamoto et al., 1992). Both Mdk and Ptn transcripts and proteins are expressed during branching morphogenesis in salivary glands, lungs, and kidneys (Mitsiadis et al., 1995). Expression of MDK and PTN is conserved between mouse, zebrafish, xenopus, and chick embryos during early development (Cockshutt et al., 1994; Sekiguchi et al., 1995; Winkler and Moon, 2001; Winkler et al., 2014), suggesting that they have similar functions in these model organisms. Studies involving single knockout of either Mdk or Ptn in mice reveal only subtle physical defects with no noticeable malformation in the major organs (Muramatsu et al., 2006). The differences between wild type and Mdk or Ptn single mutants relate to response to neurotoxicity and pharmacological agents in the adults (Herradon and Perez-Garcia, 2014). However, Mdk/Ptn double mutants have low embryonic viability, with only 1/3 of the expected double knockout embryos surviving to birth. The surviving double knockout mutants are smaller than their wild type littermates and they have defects in female reproduction, lower intestinal tract, locomotion, and deficit in auditory response due to loss of β-tectorin (Muramatsu et al., 2006; Zou et al, 2006).

The above studies indicate that MDK and PTN function during early development and organogenesis in tissues that involve epithelial-mesenchymal interactions. Although ocular development involves initial interaction between the neural and cranial ectoderm to form the lens and optic cup, which later interact with the neural crest mesenchyme to form the cornea, iris and other ocular tissues (Beebe and Coats, 2000; Evans and Gage, 2005; Hyer et al, 2003; Lwigale and Bronner-Fraser, 2009), very little is known about the expression and function of MDK and PTN during ocular development. In this study, we examined the spatiotemporal expression of *MDK* and *PTN* during ocular development and compare their expression patterns between chick and mice. Our results show that *MDK* and *PTN* are expressed in dynamic patterns that partially overlap during ocular development, and that their expression is only conserved in a few ocular tissues between chick and mouse. This

work sets the foundation for further investigation of the function of MDK and PTN during ocular development in chick and mouse.

Results

To determine the expression of *MDK* and *PTN*, we performed section *in situ* hybridization on chick and mouse eyes at different stages of development, beginning at the separation of the ectoderm from the lens vesicle through the formation of the cornea, eyelids, and retina. These processes occur between embryonic day (E)3 and E18 in chick (Creuzet et al., 2005; Doh et al., 2010; Lwigale et al., 2005) and between E10.5 and P10 in mice (Pei and Rhodin, 1970; Turner et al., 1990; Young, 1985).

Expression of MDK and PTN during development of the anterior eye in chick

Cornea development begins with the migration of periocular neural crest cells into the presumptive corneal region located between the lens vesicle and the overlying ectoderm. In chick, this process occurs in two successive waves, whereby the initial migration of periocular neural crest cells at E4.5 forms the corneal endothelium, which is followed by a second wave at E6 into the primary stroma that forms the keratocytes (Creuzet et al., 2005; Hay, 1980; Johnston et al., 1979; Lwigale et al., 2005). Our analyses show that in chick, *MDK* is robustly expressed at E3 in the periocular mesenchyme, optic cup, lens vesicle, and overlying ectoderm (Fig. 1A). At E5, *MDK* is vividly expressed in the corneal epithelium, but it persists at low levels in the periocular mesenchyme during the formation of the corneal endothelium, in the optic cup region that will later become the ciliary margin, and in the lens epithelium (Fig. 1B). By E7, *MDK* is localized to the corneal epithelium and endothelium, with the stroma showing weak signals. *MDK* is also vividly expressed in the iris stroma, ciliary margin, lens epithelium, and in the mesenchyme surrounding the ocular blood vessels (Fig. 1C).

In contrast, expression of *PTN* is undetectable in the ocular tissues at E3 (Fig. 1D). Its expression is first detected in the periocular mesenchyme, corneal endothelium, and lens epithelium at E5 (Fig. 1E). By E7, *PTN* is strongly expressed in the corneal stroma and in the condensed mesenchyme that marks the boundary between the presumptive drainage angle and trabecular meshwork (Fig. 1F, arrowheads). *PTN* is also expressed at low levels in the corneal endothelium, iris stroma, ciliary margin, and lens epithelium at E7.

Expression of Mdk and Ptn during development of the anterior eye in mouse

Morphogenesis of the lens and optic cup follow a similar pattern in chick and mice, but corneal development follows a different process. In the mouse, the periocular mesenchyme migrates in a single wave at about E11.5, which becomes the corneal mesenchyme that later differentiates to form the corneal stroma and endothelium (Gage et al., 2005; Pei and Rhodin, 1970). Similar to E3 chick, *Mdk* is robustly expressed in the periocular mesenchyme, optic cup, lens vesicle, and overlying ectoderm at E10.5 (Fig. 2A). However, by E14.5, vivid expression is only observed in the optic cup and adjacent periocular mesenchyme, whereas the presumptive corneal mesenchyme and lens epithelium show weak

staining (Fig. 2B). Similar pattern of *Mdk* expression is observed at a diminished level at E16.5, with strong staining in the presumptive iris stroma (Fig. 2C).

Expression of *Ptn* is very low and mostly localized to the optic cup at E10.5 (Fig. 2D), where it persists in the anterior region at E14.5 and E16.5 (Fig. 2E and 2F). Also at E14.5, *Ptn* is expressed in few cells adjacent to the retinal pigment epithelium that comprise the presumptive iris stroma (Fig. 2E, arrowheads), where it becomes strongly expressed at E16.5 (Fig. 2F, arrowheads). Sporadic staining is also observed in the lens epithelium at E16.5 (Fig. 2F).

Expression of MDK and PTN during development of the chick retina

Development of the retina follows a sequence that is conserved in vertebrates. This process begins with multipotent progenitor cells in the optic cup that undergo temporal differentiation into retinal ganglion cells, horizontal cells, cone photoreceptors, amacrine cells, bipolar cells, rod photoreceptors, and then Müller glia cells (see review by Agathocleous and Harris, 2009). In the chick, development of the neural retina begins with the differentiation of retinal ganglion cells at about E6, and well-defined boundaries of all layers are observed by E18 (Doh et al., 2010). To determine whether MDK and PTN have a potential role during development of the chick neural retina, we examined their expression patterns between E5-E18. Analysis of the posterior eye at E5 shows that the expression of *MDK* remains evenly robust in the neural retina and the mesenchyme surrounding the retinal pigment epithelium, but it is diminished in the optic nerve (Fig. 3A). Retina sections at subsequent time points show that *MDK* remains robust at E7 (Fig. 3B), but it becomes restricted to the inner nuclear layer (INL) at E10, where it is maintained at E15 and E18 (Fig. 3C, D, E).

At E5, *PTN* is strongly expressed in the optic nerve and by a few cells that line the boundary of the inner retina, prior to the formation of the retinal ganglion cells (Fig. 3F). The strong expression of *PTN* persists in the optic nerve during subsequent stages of development, and it is still detectable at E18 (data not shown). By E7, expression of *PTN* is observed throughout the neural retina, with strong staining persisting in the ganglion cell layer (GCL; Fig. 3G). By E10, *PTN* is localized to the GCL and INL (Fig. 3H), and this pattern remains at E15 with diminished staining of the GCL (Fig. 3I). By E18, *PTN* is not detectable in the GCL, although the staining remains in the INL and new staining is observed in the outer nuclear layer (ONL; Fig. 3J).

Expression of Mdk and Ptn during development of the mouse retina

In the mouse, retina development occurs between E11 and E18 (Turner et al., 1990). We examined expression patterns of *Mdk* and *Ptn* between E13.5-P12 and found that by E13.5, *Mdk* is expressed in the posterior ocular mesenchyme and the outer neuroblastic layer of the neural retina, with negligible expression in the optic nerve (Fig. 4A). By E16.5, expression of *Mdk* is maintained in the outer neuroblastic layer and extends at low levels into the inner neuroblastic layer (Fig. 4C). At P0, *Mdk* is expressed in the INL (Fig. 4D), and it is not detected in the retina by P12 (Fig. 4E).

Similar to chick, strong expression of *Ptn* is observed in the mouse optic nerve by E13.5 (Fig. 4F). At this time, *Ptn* is also expressed in the posterior ocular mesenchyme and neural retina. Between E14.5 and E16.5, *Ptn* is expressed throughout the neural retina in salt-and-pepper pattern (Fig. 4G and 4H). At P0, expression of *Ptn* appears to localize in the INL and GCL (Fig. 4I), and it is not detected in the retina by P12 (Fig. 4J).

Expression of MDK and PTN receptors during ocular development

To determine the potential receptors for MDK and PTN during ocular development, we chose the chick eye at E7 because both *MDK* and *PTN* are expressed in the anterior and posterior ocular structures at this time. We screened for receptor expression by RTPCR, which indicated that only *NOTCH2, ITGβ1, SDC1*, and *SDC3* were expressed in the cornea, whereas all receptors including *LRP1, RPTPZ*, and *ALK*were expressed in the retina (Fig. 5L). Analysis by *in situ* hybridization confirmed that *NOTCH2* and *ITGβ1* are robustly expressed in the cornea, and observed that they are also expressed in the lens epithelium and presumptive iris (Fig. 5A and 5B). In addition, *NOTCH2* is expressed in the condensed mesenchyme (Fig. 5A, arrowheads) and in the mesenchyme surrounding the ocular blood vessels. *ITGβ1* is also expressed in the lens fiber cells (Fig 5B). *SDC1* is strongly expressed in the condensed mesenchyme (Fig. 5C, arrowheads), and at lower levels in the corneal stroma, iris stroma, and lens epithelium (Fig. 5C). *SDC3* is sparsely expressed in the cornea and iris stroma (Fig. 5D).

In the neural retina, *NOTCH2* is detected in the progenitor cells located in the mid and peripheral regions (Fig. 5E). *SDC3* is strongly expressed in the GCL and in the progenitor cells located in the mid and peripheral regions of the retina (Fig. 5H). *RPTPZ* is expressed in the progenitor cells located in the mid region of the retina and sparsely in the GCL (Fig. 5J). The remaining receptors *ITGβ1 SDC1, LRP1*, and *ALK* are diffusely expressed at low levels throughout the neural retina (Fig. 5F, 5G, 5I, 5K).

Expression of MDK and PTN during late development of the chick anterior eye

Most of the ocular tissues are formed by E12 in chick. At this time, *MDK* and *PTN* appear to be exclusively expressed in the epithelial and mesenchymal tissues, respectively (Fig. 6A and 6B). *MDK* is localized to epithelial layers of the eyelids, nictitating membrane, and cornea (Fig. 6A, arrowheads). It is also detected in the mesenchyme surrounding the blood vessels in the iris and adjacent to the trabecular meshwork, and in the ciliary body and ciliary muscle (Fig. 6A). *PTN* is expressed in the mesenchyme of the eyelid and nictitating membrane, and at low levels in the mesenchyme adjacent to the iridocorneal angle, in the iris stroma, and in the ciliary body (Fig. 6B).

Given that both MDK and PTN are involved in promoting neurite outgrowth (Michikawa et al., 1993; Nakanishi et al., 1997; Raulo et al., 1994) and that ocular tissues are highly innervated by trigeminal sensory nerves at this time (Lwigale, 2001; Lwigale and Bronner-Fraser, 2007), we analyzed the expression of MDK and PTN receptors in the trigeminal ganglion. Initial analysis by RTPCR indicated that only *ITGβ1* and *RPTPZ* are expressed in the trigeminal ganglion (Fig. 6C). This was confirmed by *in situ* hybridization, which revealed robust expression of both *ITGβ1* and *RPTPZ* by the small support cells, but not the

cell bodies of the larger neural crest-derived sensory neurons (Lwigale et al., 2004) (Fig. 6D, 6D' and 6E, 6E'). To determine whether the receptors were directly involved in cellular interactions within the trigeminal ganglion, we examined the expression of *MDK* and *PTN*. Our results indicate that *MDK* is expressed at low levels in the trigeminal ganglion compared to *PTN* (Fig. 6F and 6G). Nonetheless, both *MDK* and *PTN* are expressed by sensory neurons located in the proximal region of the trigeminal ganglion (Fig. 6E' and 6F'). Combined, these expression patterns indicate potential MDK/PTN signaling between the neurons and support cells via ITGβ1 and RPTPZ receptors.

Discussion

The cellular and molecular mechanisms regulating the morphogenesis of ocular tissues are not well known. In the present study, we analyzed the expression of *MDK* and *PTN* during ocular development in chick and mouse. Our results reveal that *MDK* and *PTN* are differentially expressed during ocular development and only overlap in a few areas. Comparison between chick and mouse also show differences in the expression patterns of *MDK* and *PTN*. Despite the differences in expression patterns, our results indicate potential roles for *MDK* and *PTN* in epithelial-mesenchymal interactions, cell migration and differentiation, and during neurogenesis.

Our results show that *MDK* is ubiquitously expressed shortly after the formation of the rudimentary eye in both chick and mouse. Given that *MDK* is induced by retinoic acid (RA) signaling (Kadomatsu et al., 1988), it is likely that its expression mirrors the relatively high levels of RA signaling during early ocular development (Duester, 2009; Matt et al., 2005; Mic et al., 2004; Molotkov et al., 2006). In the anterior eye, expression of *MDK* becomes localized to the corneal epithelium and endothelium and to the mesenchyme of the presumptive iris in chick, whereas in the mouse, *Mdk* is downregulated in most of the anterior eye except for the presumptive iris. Conserved expression of *MDK* in the presumptive iris coupled with the colocalization with *NOTCH2, ITGβ1,* and *SDC1* suggests its potential function in iris development that may involve cell adhesion between the stroma and optic cup. *MDK* and *NOTCH2* are expressed in the mesenchyme surrounding the ocular blood vessels in chick, suggesting a potential role during ocular vasculogenesis. Strong expression of *MDK* in the corneal epithelium and endothelium may be a result of high RA levels during ocular development. MDK may also play a role in cell adhesion of the chick corneal epithelium and endothelium via *NOTCH2* and *ITGβ1*.

Contrary to *MDK, PTN* is not detectable in the rudimentary eye of the chick, and it is expressed at low levels in the mouse optic cup. Previously it was shown that Ptn is involved in recruitment of precursor cells during osteogenesis (Imai et al., 1998). Thus, the upregulation of *PTN* during chick corneal development suggests its potential role in recruiting periocular neural crest cells into the cornea. In the mouse *Ptn* is not expressed during corneal development, but it is upregulated in the mesenchyme of the presumptive iris similar to *Mdk* expression. Overlap between *Mdk* and *Ptn* during iris development in the mouse suggest that they play a similar role in promoting cell adhesion by signaling through NOTCH2, ITGα1, or SDC1 receptors. We also show that in chick *PTN* is expressed in the condensed mesenchyme at the boundary between the presumptive drainage angle and

trabecular meshwork, where *NOTCH2* and *SDC1* are also expressed, suggesting that potential signaling between PTN and these two receptors may be involved in promoting condensation of the periocular mesenchyme in this region.

Our results also show differential expression of *MDK* and *PTN* in the posterior eye and neural retina. The most striking difference is observed in the optic nerve, where *PTN* is strongly expressed whereas *MDK* is expressed at low levels in both chick and mouse, suggesting that PTN plays an important role during optic nerve development, and that it may also be involved in guiding axons from the RGC to the central nervous system. In the chick both *MDK* and *PTN* are ubiquitously expressed in the neural retina at E7, and they are both restricted to the INL at subsequent stages of development. *PTN* is also transiently expressed in the GCL and it localizes to the ONL at E18. The spatiotemporal expression of *PTN* suggests its potential function during neurogenesis in the various layers of the chick neural retina. In the mouse, *Mdk* is localized in the outer neuroblastic layer and *Ptn* is maintained at low levels throughout the neural retina. These expression patterns indicate that both MDK and PTN play important roles during chick retinal development, whereas Mdk is the dominant player in the mouse.

Our data also show that the receptors *SDC3* and *RPTPZ* are expressed in the chick GCL and INL. In the mouse both *SDC3* and *RPTPZ* and is expressed in the GCL and outer neuroblastic layer (Inatani et al., 2002; Klausmeyer et al., 2007). *SDC3* is expressed in the developing central nervous system and it is involved in MDK and PTN signaling that induce neurite outgrowth (Raulo et al., 1994; Nakanishi et al., 1997). *RPTPZ* is expressed in the central nervous system (Canoll et al., 1993; Shintani et al., 1998) and it is also involved in neuron and osteoblast cell migration (Maeda and Noda, 1998; Qi et al., 2001). Based on the expression patterns, it is likely that both MDK and PTN play important roles in neurogenesis, neural migration, and neurite outgrowth during chick retinal development. Mdk is dominant in the mouse, where it may play a role in cell proliferation or maintenance of the progenitor cells in the neuroblastic layer. Although the role of MDK and PTN during avian and mammalian ocular development is yet to be determined, functional studies in the zebrafish indicate that a paralogue of MDK, *mdka*, is expressed in retinal progenitor cells (Calinescu et al., 2009). Knockout of *mdka* attenuates cell cycle kinetics, which results in few progenitors whereas its overexpression accelerates the cell cycle and increases their number (Luo et al., 2012).

Our analysis of *MDK* and *PTN* expression in the chick anterior eye at E12 show that they are expressed in non-overlapping patterns in non-neural tissues, where they could be involved in epithelial-mesenchymal interactions or cell adhesion. Anterior ocular tissues are highly innervated by trigeminal sensory nerves (Lwigale, 2001; Lwigale and Bronner-Fraser, 2007). Although MDK and PTN have been shown to have neurotrophic properties in cultured sensory neurons and to stimulate neurite outgrowth (Michikawa et al., 1993; Raulo et al., 1994; Nakanishi et al., 1997), it is unlikely that they play these roles during sensory innervation of the anterior ocular tissues. We show that *ITGβ1* and *RPTPZ*, the only receptors present in the trigeminal ganglion, are not expressed by the neurons, but label the support cells. Our results also show that *MDK* and *PTN* are expressed by the neural crest-derived neurons located in the proximal region of the trigeminal ganglion (Johnston, 1966;

Lwigale, 2001), suggesting that MDK and PTN signal to the support cells through ITGα1 and RPTPZ, and may play a role in their maintenance and adherence to the neurons for proper myelination. Previous studies have shown that *RPTPZ* is expressed by astrocyte progenitors in the central nervous system and by oligodendrocytes and Schwann cells (Ivanova et al, 2004; Canoll et al., 1996; Shintani et al., 1998). Knockout mice without Rptpz exhibit delayed response to nociception, but it was not clear whether this defect was caused by inadequate myelination of the sensory neurons (Lafont et al., 2009).

In conclusion, our study reveals the spatiotemporal expression patterns of *MDK, PTN*, and their receptors during ocular development. Despite the differences in expression patterns observed between chick and mouse, our results suggest potential roles for MDK and PTN in both neuronal and non-neuronal processes of ocular development.

Experimental procedures

Embryos

Fertilized white leghorn chicken eggs are obtained from the Department of Poultry Science at Texas A&M University (College Station, TX). Eggs are incubated at 37 °C until the appropriate stages. Timed pregnant C57BL/6J mice were obtained from Jackson Laboratory. All animals were handled in accordance with guidelines from Institutional Animal Care and Use Committee (IACUC) at Rice University.

In situ hybridization

Chick and mouse heads or eyes were collected at time points that correspond with when the corneal layers are forming and neural retinal progenitor cells are differentiating (chick, E3-E18; mouse, E11.5-P12). Freshly isolated tissues were rinsed in Ringer's solution then fixed overnight at 4°C in modified Carnoy's fixative (60% ethanol, 30% formaldehyde, 10% glacial acetic acid). Fixed tissues were dehydrated through an ethanol series, cleared with Histosol, and embedded in paraffin. Tissues were sectioned at 10-12 μm.

Section *in situ* hybridization was performed as previously described (Etchevers et al., 2001). RNA probes were synthesized using cDNA sequences obtained from GenBank (accession numbers in Table 1) and amplified using primers listed in Table 1. The PCR products were cloned into pCRII-TOPO® or pCRIV-TOPO® cloning vectors (ThermoFisher Scientific), and linearized with appropriate restriction enzymes (HindIII, BamHI, SpeI, KpnI, EcoRV, NotI, XbaI, PmeI, or PstI). Digoxigenin-labeled sense and antisense probes were transcribed using Sp6, T3, or T7 polymerases. Sense probes were used in parallel with each gene as controls and they showed no specific signals.

Reverse Transcription PCR

RNA was extracted from chick E7 corneas, E7 retinas, and E12 trigeminal ganglia. Corneas were trimmed and included only the epithelium, stroma and endothelium. Retinal tissue included both neural retina and pigmented epithelium. The trigeminal ganglion tissue included the maxillomandibular and ophthalmic branches. Any attached mesenchyme was removed from the retina and trigeminal tissues before extraction. Samples containing three

tissues each were collected induplicate. Tissues were collected in TRIzol Reagent (Invitrogen) and RNA was isolated according to manufacturer's protocol. cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). PCRs were run with GoTaq Polymerase (Promega) and normalized to GAPDH. Primers used for *in situ* probe synthesis were also used for RT-PCR.

Imaging

Stained sections were imaged using a Zeiss AxioImager Z1 microscope with AxioCam MRc5 camera and AxioVision program (Carl Zeiss AG, Oberkochen, Germany).

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Key findings:

- *MDK* and *PTN* show unique expression patterns in neuronal and non-neuronal tissues during ocular development.
- Expression patterns of *MDK and PTN* are dynamic and only conserved between chick and mouse in a few ocular tissues.
- The receptors *RPTPZ, ALK, NOTCH2, ITGβ1, LRP1, SDC1*, and *SDC3* are expressed various patterns that overlap with *MDK* and *PTN*.
- The expression profiles of *MDK, PTN*, and their receptors are consistent with potential functions in cell proliferation, adhesion, migration, and neurite outgrowth during ocular development.

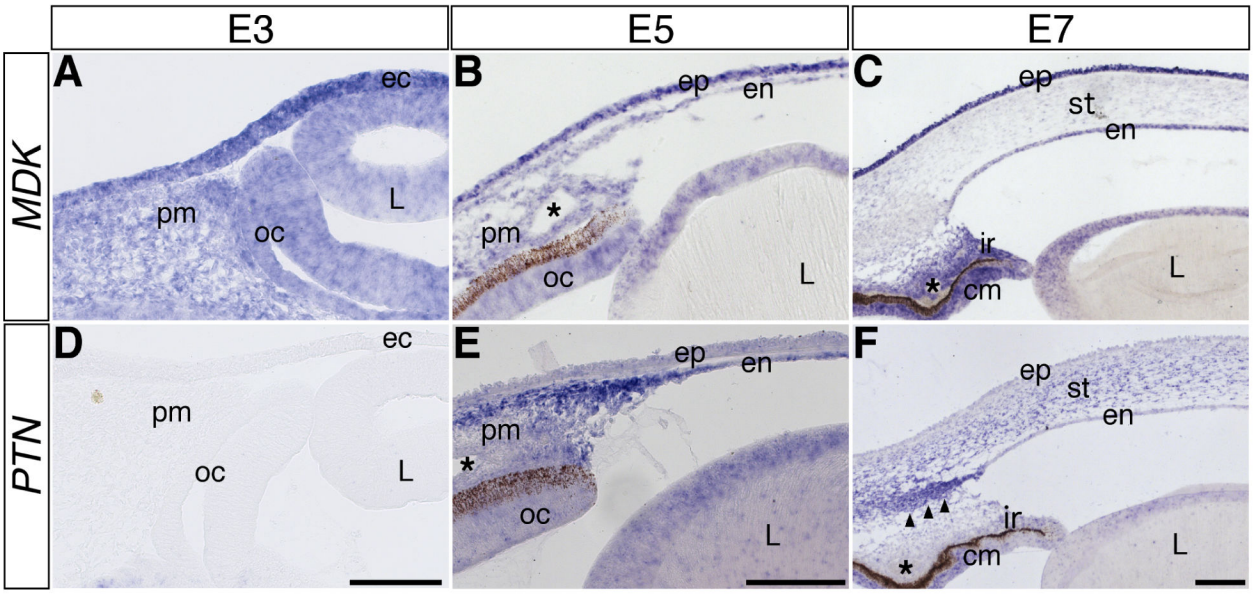

Figure 1. Expression pattern of *MDK* and *PTN* during development of the chick anterior eye.

Transverse sections through E3, E5, and E7 eyes showing the spatiotemporal expression of *MDK* (A-C) and *PTN* (D-F). Asterisks in B, C, E, and F indicate ocular blood vessels. Arrowheads in F indicate condensed mesenchyme at the boundary between the presumptive drainage angle and trabecular meshwork. Abbreviations: ec, ectoderm; pm, periocular mesenchyme; oc, optic cup; L, lens; ep, corneal epithelium; en, corneal endothelium; st, corneal stroma; ir, iris; cm, ciliary margin. Scale bars represent 100 μm in A, D; B, E; and C, F.

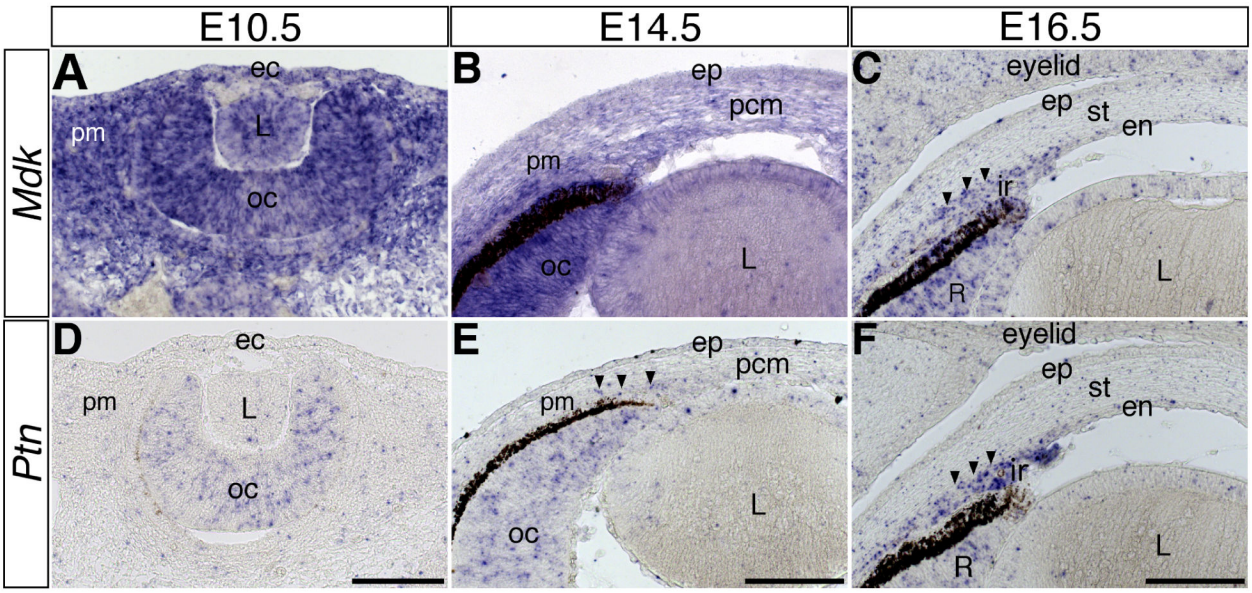

Figure 2. Expression pattern of *Mdk* and *Ptn* during development of the mouse anterior eye.

Transverse sections through E10.5, E14.5, and E16.5 eyes showing the spatiotemporal expression of *Mdk* (A-C) and *Ptn* (D-F). Arrowheads in E and F indicate expression of Ptn in mesenchyme of the presumptive iris. Abbreviations: ec, ectoderm; pm, periocular mesenchyme; oc, optic cup; L, lens; ep, corneal epithelium; pcm, presumptive corneal mesenchyme; en, corneal endothelium; st, corneal stroma; ir, iris; pm, periocular mesenchyme; R, neural retina. Scale bars represent 100 μm in A, D; B, E; and C, F.

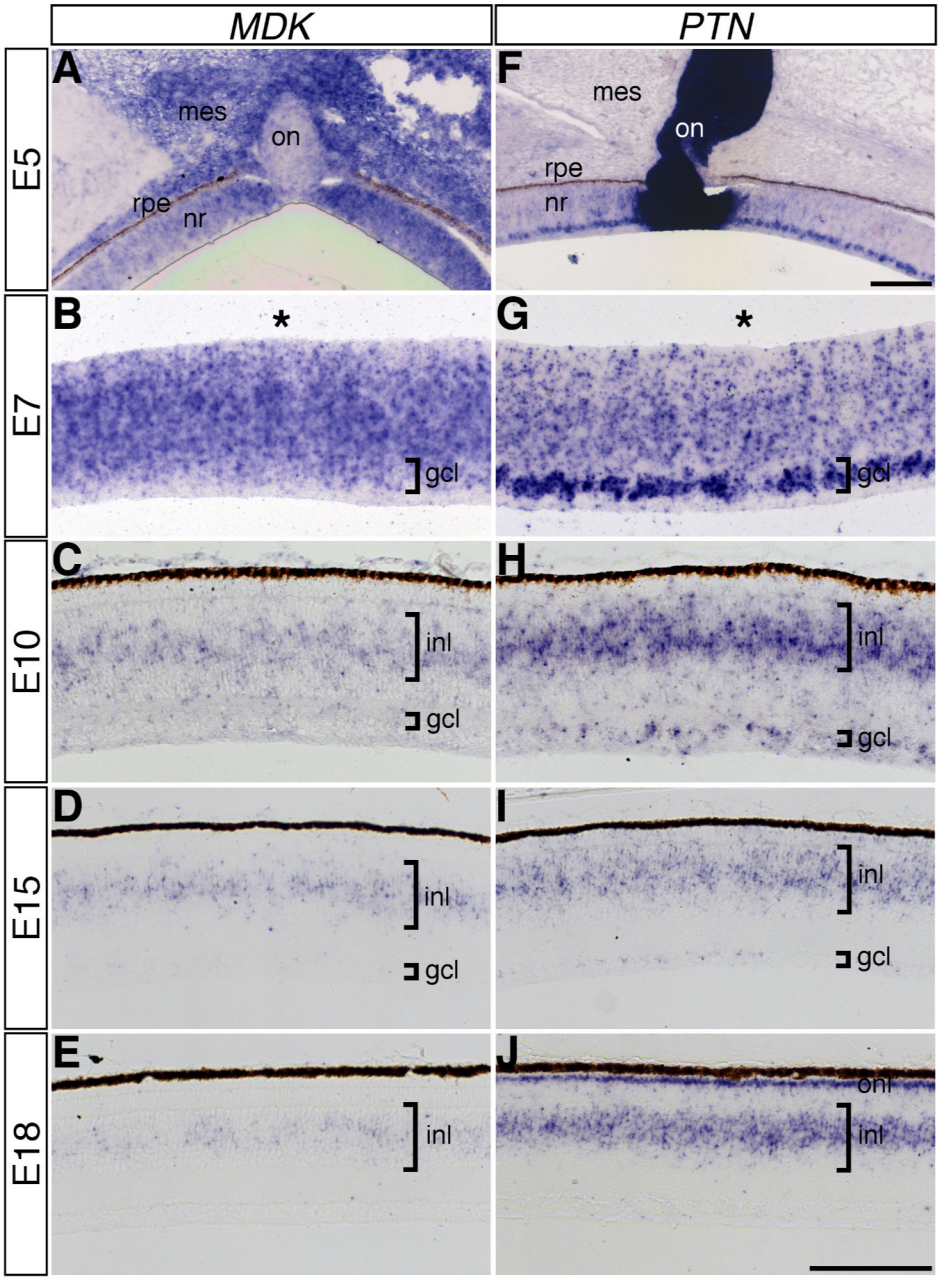

Figure 3. Expression pattern of *MDK* and *PTN* during retinal development in chick.

Transverse sections through E5, E7, E10, E15 and E18 eyes showing the spatiotemporal expression of *MDK* (A-E) and *PTN* (F-J). All images oriented with the retinal pigmented epithelium (rpe) at the top. Images for A and F represent posterior retina. Rest of the images were acquired from the dorsal retina in the region between the optic nerve and ciliary body. Asterisks in B and G indicate detachment of the rpe (not shown) from the neural retina during tissue processing. Abbreviations: nr, neural retina; mes, mesenchyme; on, optic nerve; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer. Scale bars represent 100 μm in A,F and B,C,D,E,G,H,I,J.

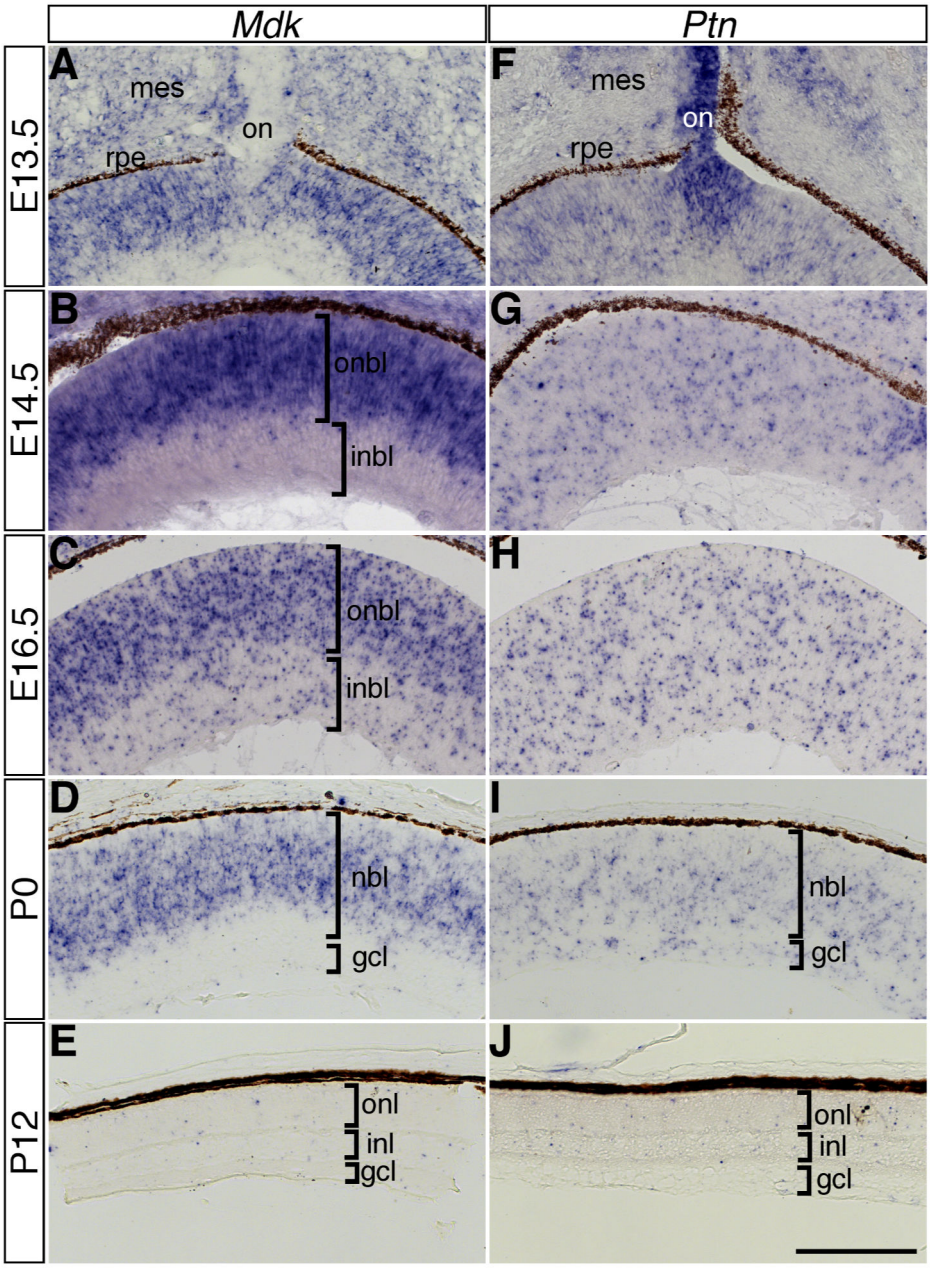

Figure 4. Expression pattern of *Mdk* and *Ptn* during retinal development in mouse.

Transverse sections through E13.5, E14.5, E16.5, P0 and P12 eyes showing the spatiotemporal expression of *Mdk* (A-E) and *Ptn* (F-J). All images oriented with the retinal pigmented epithelium (rpe) at the top. Images for A and F represent the posterior retina. Rest of the images were acquired from the dorsal retina in the region between the optic nerve and ciliary body. Abbreviations: mes, mesenchyme; on, optic nerve; onbl, outer neuroblastic layer; inbl, inner neuroblastic layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bar represents 100 μm.

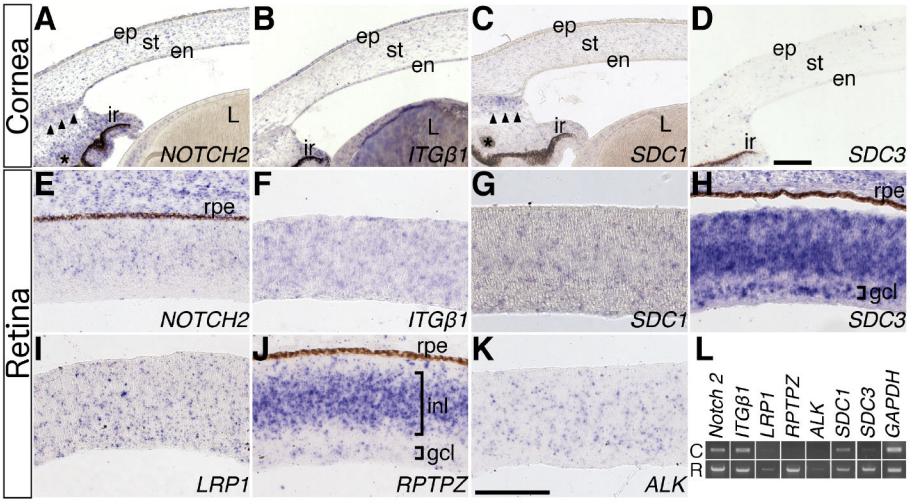

Figure 5. Expression of MDK and PTN receptors during chick ocular development.

(A-D) Transverse sections through E7 anterior eye showing the expression of *NOTCH2, ITGβ1, SDC1*, and *SDC3*. (E-K) Transverse sections through E7 retina showing the expression of *NOTCH2, ITGβ1, SDC1, SDC3, LRP1, RPTPZ*, and *ALK*. (L) RTPCR results showing amplification of *NOTCH2, ITGβ1, LRP1, RPTPZ, ALK, SDC1, SDC3*, and *GAPDH* (control) using cDNA obtained from E7 chick corneas and retinas. All retina images were acquired from the dorsal retina in the region between the optic nerve and ciliary body. Abbreviations: ep, corneal epithelium; st, corneal stroma; en, corneal endothelium; ir, iris; L, lens; rpe, retinal pigmented epithelium; gcl, ganglion cell layer; inl, inner nuclear layer; C, cornea; R, retina. Arrowheads indicate condensed mesenchyme that marks the boundary between the presumptive drainage angle and trabecular meshwork. Asterisks in A and C indicate ocular blood vessels. Scale bar represents 100 μm in A,B,C,D; and 100μm in E,F,G,H,I,J,K.

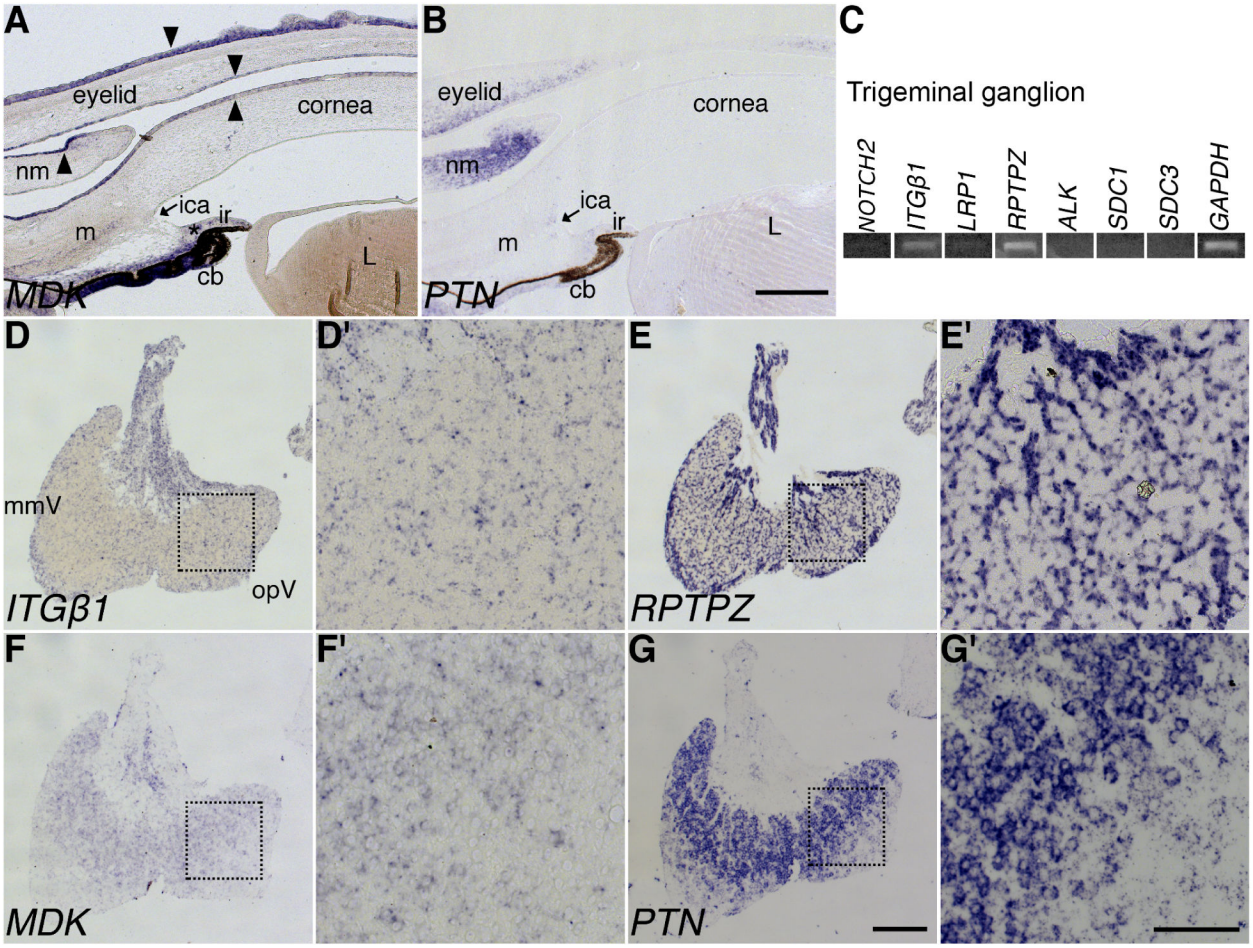

Figure 6. Expression pattern of *MDK* and *PTN* in the anterior eye of E12 chick and receptor expression in the trigeminal ganglion.

Transverse sections through E12 anterior eye showing the expression of (A) *MDK* in mostly epithelial tissues (arrowheads) and (B) *PTN* in mesenchymal tissues. (C) RTPCR results showing amplification of *NOTCH2, ITGβ1, LRP1, RPTPZ, ALK, SDC1, SDC3*, and *GAPDH* (control) using cDNA obtained from E12 chick trigeminal ganglia. Cross-sections through E12 trigeminal ganglion showing the expression of *ITGβ1* (D, D') and *RPTPZ* (E, E'), and *MDK* (F, F') and *PTN* (G, G'). Asterisk in A indicates ocular blood vessel..Abbreviations: nm, nictitating membrane; ir, iris; cb, ciliary body; ica, iridocorneal angle; L, lens; m, ciliary muscle; opV, ophthalmic branch of the trigeminal ganglion; mmV, maxillomandibular branch of the trigeminal ganglion. Scale bars represent 250μm in A,B; 250μm in D,E,F,G; and 100μm in D',E',F',G'.

Table 1.

Primers used for in situ hybridization and RT-PCR.

Species	Gene	NCBI ID	Forward Primer	Reverse Primer
chick	MDK	NM_001113289.1	5'- CTGCCAAAGCCAAGAAAGGT −3'	5'- ACCACCTCCTCACATTCAGC −3'
	PTN	NM_001276362.1	5'- ATGCCACAGCAACAACAG −3'	5'- TTAATCCAGCATCTTCTC −3'
	ALK	XM_025148917.1	5'- ACTGGCTGTTCACAACATGTGGTG −3'	5'- GATCTTCCTCCAGTAGCACCTTCCAG - 3'
	NOTCH2	NM_001252033.1	5'- GTGTCGAGAAGGCTATCTTG −3'	5'- GTATCACACAGAGCTCCCTTC −3'
	LRP1	NM_205242.2	5'- GTCAAGGCACTGGTAAAACA −3'	5'- GGAGATTCTGAAGAGAGGGA −3'
	ITGβ1	NM_001039254.2	5'- GTTGCTTGATATGGAGTGGA −3'	5'- GGTCACCTGTACAAGGATTTC −3'
	RPTPZ	NM_001199312.1	5'- AGCTCGTCTGTATCCTCCGA −3'	5'- TTCCCACGTAACCAGAAGGC −3'
	SDC1	XM_419972.5	5'- GTCCCGCAAACTACAAATCT −3'	5'- GTTCATCCAGTGAATAGCTTCC −3'
	SDC3	NM_205383.1	5'- GATAACAGAAGCACCAGTGATCC −3'	5'- GACTTCCAGGTCACTGTCGA −3'
	GAPDH*	NM_204305.1	5'- GATTCTACACACGGACACTTCA −3'	5'- CTGAGGGAGCTGAGATGATAAC −3'
mouse	Mdk	NM_010784.5	5'- AAGATGCAGCACCGAGGCTT −3'	5'- TTGTACCGCGCCTTCTTCAG −3'
	Ptn	NM_008973.3	5'- ATGTCGTCCCAGCAATATC −3'	5'- ATCCAGCATCTTCTCCTGTTTC −3'
	Alk	NM_007439.2	5'- GCTTTGACTTCCCCTGTGAG −3'	5'- GAAGCGAGGATGTCAGTGGT −3'
	Notch2	D32210.1	5'- CCTTATGTGAGGGGTCTGCC −3'	5'- AATGTACTGCCCGTTCAGGG −3'
	Lrp 1	NM_008512.2	5'- TATGAAGGTGGAGAGCCCGA −3'	5'- TTCCAGGGGTATGCTCGGTA −3'
	Itgβ1	NM_010578.2	5'- CTCCGCAAGCCGAGGTC −3'	5'- TGGAAAACACCAGCAGTCGT −3'
	Rptpz	NM_011219.2	5'- GACGTTAGCCAGGCCTATCC −3'	5'- GTGAAGGTCTGCTGGTGGAC −3'
	Sdc1	NM_011519.2	5'- CCTAACGCAGAGGAAGGGACC −3'	5'- GAGGCTGATGGTCAGGTTGA −3'
	Sdc3	NM_011520.3	5'- TGGACACCGCCACCCAT −3'	5'- ACCTCCTTCCGCTCCAGTAT −3'

* Golden and Dansen, 2012

KEY RESOURCES TABLE

Reagent or resource	Source	Identifier
Experimental Models: Organisms/Strains		
Chicks: White leghorn (*Gallus gallus domesticus*)	Texas A&M University, Department of Poultry Science, College Station, Texas, USA	
Mouse: *Mus musculus* C57BL/6J	Jackson Labs	JAX: 006494
Oligonucleotides		
Primers for *in situ* and RT-PCR, see Table 1	This paper	N/A
Recombinant DNA		
pCRII-TOPO cloning kit	Invitrogen	K466001
pCRIV-TOPO cloning kit	Invitrogen	450030