Nephronectin-Integrin $\alpha 8$ signaling is required for proper migration of periocular neural crest cells during chick corneal development

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ABSTRACT

During development, cells aggregate at tissue boundaries to form normal tissue architecture of organs. However, how cells are segregated into tissue precursors remains largely unknown. Cornea development is a perfect example of this process whereby neural crest cells aggregate in the periocular region prior to their migration and differentiation into corneal cells. Our recent RNA-Seq analysis identified upregulation of Nephronectin (Npnt) transcripts during early stages of corneal development where its function has not been investigated. We found that Npnt mRNA and protein are expressed by various ocular tissues including the migratory periocular neural crest (pNC), which also express the integrin alpha 8 (Itga8) receptor. Knockdown of either *Npnt* or *Itga8* attenuated cornea development, whereas overexpression of *Npnt* resulted in cornea thickening. Moreover, overexpression of Npnt variants lacking RGD binding sites did not affect corneal thickness. Neither the knockdown or augmentation of Npnt caused significant changes in cell proliferation, suggesting that Npnt directs pNC migration into the cornea. *In vitro* analyses showed that Npnt promotes pNC migration from explanted periocular mesenchyme, which requires Itga8, focal adhesion kinase (FAK) and Rho kinase (ROCK). Combined, these data suggest that Npnt augments cell migration into the presumptive cornea ECM by functioning as a substrate for Itga8-positive pNC cells.

INTRODUCTION

In vertebrates, the cornea is comprised of three cellular layers: the epithelium, stroma and endothelium. The corneal epithelium is derived from the ectoderm, whereas the stromal keratocytes and corneal endothelium are derived from cranial neural crest cells (Lwigale et al., 2005). In birds and humans, neural crest migration from the periocular region into the presumptive cornea occurs in two waves (Feneck et al., 2020; E. D. Hay, 1980). The first wave forms a monolayer of the nascent corneal endothelium, which is accompanied by a second wave of mesenchymal migration into the acellular matrix between endothelium and ectoderm to form the cornea stroma (Hay and Revel, 1969; Noden, 1978; Lwigale et al., 2005; Creuzet et al., 2005; Feneck et al., 2020). Several mechanisms pertaining to the role of growth factors and guidance cues have been identified (Beebe and Coats, 2000; Saika et al., 2001; Lwigale and Bronner-Fraser, 2009; Choi et al., 2014), but the functional role of ECM/integrin signaling in establishing the cornea remains unclear.

Among the initial effects of the signaling that takes place between the nascent ocular tissues is the synthesis of the corneal extracellular matrix (ECM) by the corneal epithelium in response to inductive signals from the lens vesicle to form the primary stroma (Hay and Revel, 1969; Hendrix et al., 1982; Fitch et al., 1988). In birds and humans, the primary stroma serves an important role as a scaffold for pNC migration throughout the process of corneal development (Hay and Revel, 1969; Bard and Hay, 1975; Quantock and Young, 2008). It consists of multiple ECM proteins including hyaluronan (Toole and Trelstad, 1971), collagen type I and II (Hayashi et al., 1988; Fitch et al., 1988), laminin (Doane et al., 1996), and fibronectin (Fn) (Fitch et al., 1991). Following the second wave of pNC migration into the developing cornea, the primary stroma is concomitantly replaced by the secondary stroma that is synthesized by differentiating

stromal keratocytes. The secondary stroma comprises the bulk of the adult cornea and consists of collagens and proteoglycans (Hayashi et al., 1988; Quantock and Young, 2008) that are arranged in patterns that result in transparency (Chen et al., 2015). Over the past decades, the role of collagens and proteoglycans have been the focus of several investigations due to their indispensable functions in corneal transparency. However, most of these studies were conducted in rodents, in which early corneal development does not involve the primary stroma (Pei and Rhodin, 1970; Haustein, 1983; Feneck et al., 2019). Although the ECM plays a critical role during organogenesis by providing cell adhesion substrate, sequestering signaling molecules, providing structural support and mechanical cues (Hynes, 2014), the function of the primary stroma during early corneal development remains to be elucidated.

Our recent RNASeq analysis of pNC differentiation into corneal cells identified novel expression and upregulation of Nephronectin (Npnt) transcripts during corneal development. Npnt was discovered as an ECM ligand for integrin $\alpha 8 \beta 1$ ($\alpha 8 \beta 1$) during mouse kidney development, consisting of 70-90 kDa proteins (Brandenberger et al., 2001) generated by alternate splicing (561 to 609 amino acids). At the same time, it was also identified as preosteoblast epidermal growth factor-like repeat protein with, meprin A5 protein and receptor protein-tyrosine phosphatase μ domain (POEM) (Morimura et al., 2001). Npnt consists of five epidermal growth factor (EGF)-like domains in the N-terminal, a central region containing an Arg-Gly-Asp (RGD) sequence, and a meprin-A5 protein-receptor protein tyrosine phosphatase μ (MAM) domain in the C-terminal. The EGF-like and RGD domains have been functionally characterized and shown to play critical roles in development and tissue homeostasis. The RGD domain signals through $\alpha 8 \beta 1$ receptor during epithelial-mesenchymal interactions involved in kidney development and maintenance (Miner, 2001, Linton et al., 2007; Müller et al., 1997, Sato 2009,

Cheng et al., 2008, Inagi et al., 2017 , Müller-Deile et al., 2017, Zimmerman et al., 2018). These observations in mice were recently confirmed by the identification of recessive mutation in the *Npnt* gene that caused bilateral kidney agenesis in human fetuses (Dai et al., 2021). The EGF-like domains of *Npnt* are associated with inducing differentiation and proliferation in osteoblasts and dental stem cells through the activation of mitogen-activated protein kinase (MAPK) pathways (Fang et al., 2010; Kahai et al., 2010; Arai et al., 2017), and to induce vascular endothelial cell migration via phosphorylation of extracellular signal-regulated kinases (ERK) and p38MAPK (Kuek et al., 2016). Recent studies have also implicated *Npnt* to play a role in heart development (Patra et al., 2011), attachment of the arrector pili muscle to hair follicles (Fujiwara et al., 2011), and during forelimb formation in amphibians (Abu-Daya et al., 2011). Other studies have also demonstrated that *Npnt* plays a potential role in diseases such as chronic obstructive pulmonary disease (COPD) (Saferali et al., 2020), diabetic glomerulosclerosis (Nakatani et al., 2012), Fraser syndrome (Kiyozumi et al., 2012), and in various cancers (Magnussen et al., 2021). Despite the pleiotropic functions of *Npnt* in development and disease, its expression and function have not yet been described in the cornea.

Inspired by our observation that *Npnt* transcripts were upregulated during corneal development, we sought to establish its role during pNC migration into the developing avian cornea. We first characterized the spatiotemporal expression of *Npnt* mRNA and protein at stages that correspond with pNC migration. Next, we used RCAS-mediated gene knockdown to investigate the role of *Npnt*. We identified *Itga8* as a potential receptor for *Npnt* during cornea development and confirmed its role in pNC using an *in vitro* migration assay coupled with inhibitors for $\alpha 8\beta 1$, focal adhesion kinase, Rho signaling pathway, and *in vivo* using gene knockdown. Lastly, we performed misexpression studies using either the full-length, RGD mutant, and truncated

versions of *Npnt* to further confirm its role during pNC migration and that it functions through the RGD domain. Together, our findings show that *Npnt* secreted into the ECM of the nascent cornea provides a substrate that promotes migration of Itg α 8-positive pNC during development.

RESULTS

***Npnt* mRNA and Protein are expressed during early ocular development**

Our previous RNA-seq analysis of gene expression during pNC differentiation into corneal cells identified upregulation of *Npnt* transcripts during chick corneal development (Bi and Lwigale, 2019). To define the expression of *Npnt* mRNA and protein distribution during this process, we performed section *in situ* hybridization and immunohistology, respectively. Consistent with our RNA-seq data (Bi & Lwigale, 2019), we found that *Npnt* is undetectable in the pNC by embryonic day (E) 4 and in the presumptive corneal endothelium at E5, but it is primarily expressed in the retinal pigment epithelium (RPE) and presumptive lens fiber cells at these time points (Figure 1A, B). However, by E6, we found that in addition to the RPE and lens, *Npnt* is vividly expressed by the second wave of migratory pNC that eventually differentiate into the stromal keratocytes (Figure 1C, arrowhead). Expression of *Npnt* persisted in the corneal stroma through E12 (Figure 1-figure supplement 1A-C), but it is undetectable at E15 (data not shown).

Because *Npnt* is a secreted protein that plays a role in cell migration (Kuek et al., 2016; Magnussen et al., 2021; Yamada & Kamijo, 2016), we examined its localization at similar time points during cornea development. At E4, *Npnt* is localized in the optic cup, lens epithelium, surrounding the pNC cells adjacent to the nascent cornea (Figure 1D-F, arrow), and in the primary stroma of the cornea (Figure 1D-F, asterisk). At E5, strong *Npnt* staining is observed in the primary stroma (Figure 1G-I, asterisk). By E6, vivid *Npnt* staining was localized in the

anterior region of the cornea and diffusely throughout the primary stroma (Figure 1J-L). At this time, the migratory pNC also stained positive for *Npnt* (Figure 1L, arrowheads). Strong expression of *Npnt* persists in the basement membrane of the corneal epithelium through (Figure 1-figure supplement 1C,D), and it is also faintly detectable in the corneal epithelial cells by E12 (Figure 1-figure supplement 1E). Interestingly, the protein expression did not correlate with the strong mRNA expression in the stroma during later stages of cornea development. This mismatch could be due to post-transcriptional regulation that prevents protein expression. It is also possible that post-translational modification by enzymes such as matrix metalloproteinases (MMPs), which are temporally and spatially regulated in the corneal ECM during development (Huh et al., 2007), could lead to protein degradation. A previous study showed that *Npnt* can be modified by MMP cleavage (Toraskar et al., 2019). Given that the changes in protein localization occur after the second wave of migration, we can conclude from our results that expression of *Npnt* coincides with pNC ingress into the cornea, implicating a potential role during development.

Knockdown of *Npnt* disrupts corneal thickness during early development

To investigate the function of *Npnt* during early corneal development, we generated several RCAS-GFP-*Npnt*-shRNA (*Npnt^{kd}*) constructs in chick DF-1 cells (Figure 2-figure supplement 1). The construct with the highest knockdown of *Npnt* was used for the experiments. Constructs containing only GFP (RCAS-GFP) or scrambled shRNA (RCAS-GFP-Scr-shRNA) were used as control. The viral constructs were injected to cover the entire cranial region of HH7-8 (Hamburger & Hamilton, 1951) chick embryos (Figure 2A). Using this approach, the viral constructs affect all the ocular regions where *Npnt* is expressed including the neural progenitors

of the optic cup and neural crest cells, and the ectodermal progenitors of the lens (Figure 1A-C). Embryos were re-incubated and collected at E7, then the anterior eyes were screened for GFP as an indicator for the extent of viral infection. Only the eyes that showed robust GFP expression were evaluated for knockdown of *Npnt* expression (Figure 2A). Histological analysis of E7 corneas based on Hematoxylin and Eosin staining revealed that knockdown of *Npnt* (N=4) resulted in reduction of corneal thickness (Figure 2C) compared to control (N=5; Figure 2B). Measurements taken in the mid-corneal regions (Figure 2D, 2E; arrowheads) showed significant reduction in corneal thickness in *Npnt*^{kd} corneas (Figure 2F). These data suggest that *Npnt* plays a critical role during early development of the cornea.

***Itga8* is the receptor for *Npnt* during cornea development and plays a role in pNC migration**

Next, we reasoned that because knockdown of *Npnt* causes a significant reduction in corneal thickness, one or more of its receptors should be expressed by the migratory pNC during cornea development. In this study, we focused on $\alpha 8\beta 1$ because of its strong affinity for *Npnt* (Brandenberger et al., 2001). Although $\alpha 8\beta 1$ was observed to promote spreading of trunk neural crest *in vitro* (Testaz et al., 1999), little is known about its expression and function in the cranial neural crest. Our analysis by *in situ* hybridization revealed that *Itga8* was expressed in the leading edge of the periocular mesenchyme prior to pNC migration into the primary stroma of the nascent cornea (Figure 3A, arrow). Subsequently, *Itga8* was maintained in the periocular mesenchyme but also expressed in the corneal endothelium at E5 (Figure 3B, arrows) and stroma at E6 (Figure 3C).

Given the striking expression of *Itga8* by the migratory pNC, we first assessed the potential for *Itga8*-Npnt signaling *in vitro* using explanted periocular mesenchyme from the leading edge adjacent to the presumptive cornea (Figure 3A and 3D). Mesenchyme explants were cultured on slides coated with Npnt in the presence or absence of a peptide inhibitor previously shown to specifically inhibit binding of $\alpha8\beta1$ with Npnt (Figure 3D) (Sato et al., 2009). In the absence of the inhibitor, explants attached to the Npnt-coated slides and numerous migratory cells formed a halo around the explant within 12-17 hours of incubation (Figure 3E; Figure 3-Video 1). In contrast, we observed that treatment with the $\alpha8\beta1$ inhibitor significantly decreased the density of migratory cells from the explant (Figure 3F, 3G; Figure 3-Video 2). These results indicate that $\alpha8\beta1$ functions as a receptor for Npnt signaling in the presumptive corneal pNC, and that it is required for their migration.

Knockdown of *Itga8* disrupts corneal thickness during development

Given that *Itga8*-expressing pNC appear to be in direct contact with Npnt secreted into the primary stroma, we wanted to characterize the role of *Itga8* during pNC migration. For this analysis, we generated and tested several RCAS-GFP-*Itga8*-shRNA constructs and validated one that showed highest knockdown of *Itga8* in DF-1 cells (Figure 2-figure supplement 1C) and *in vivo* (Figure 4-figure supplement 1). As a first step, we analyzed the effect of *Itga8* knockdown on the first wave of pNC migration that forms the corneal endothelium at E5. We found that at this time point relatively fewer GFP-positive cells expressing the *Itga8*^{kd} construct appeared to migrate into the cornea (Figure 4B, arrows) compared to RCAS-GFP control, which showed robust occupation of GFP-positive cells in the corneal endothelium (Figure 4A). Despite the

attenuated migration of pNC expressing the *Itga8^{kd}* construct during the first wave, we did not observe defects in the corneal endothelium. One possibility is that pNC which do not endogenously express *Itga8* (Figure 3A), may also contribute to the corneal endothelium albeit at a lower level, but they are able to compensate for the *Itga8* knockdown resulting in the formation of a normal endothelial layer. Nonetheless, histological analysis at E7 revealed reduction in corneal thickness following *Itga8* knockdown (Figure 4D) compared to control corneas (Figure 4C). Measurements and quantification of cells in the mid-corneal regions showed an overall significant reduction in corneal thickness and cell number, but no difference in cell density between *Itga8^{kd}* and control corneas (Figure 4E). Furthermore, we performed a BrdU assay and observed no significant differences in cell proliferation between *Itga8^{kd}* (Figure 4G) and control corneas (Figure 4F). As indicated in our analysis at E5, it is likely that non-*Itga8* expressing pNC may compensate during the second wave of pNC migration, but not to the extent that abrogates the corneal thinning defect, possibly due to the relatively large number of cells required for the formation of the stroma. These results indicate that *Itga8* is required for pNC migration into the cornea. Given that the corneal thinning defect following knockdown of *Itga8* correlates with *Npnt* knockdown, our results suggest that *Npnt-Itga8* signaling plays an important role during pNC migration into the cornea.

Overexpression of *Npnt* causes corneal thickening

Given that knockdown of both *Npnt* and *Itga8* resulted in corneal thinning, combined with our observation that *Npnt* is expressed during the second wave of migration at E6, we investigated whether overexpression of *Npnt* affects cornea development. We generated and tested viral constructs containing the full-length *Npnt* gene (*Npnt^{oe}*) in DF1 cells (Figure 2-figure

supplement 1C). Control and *Npnt^{oe}* constructs were injected in HH7-8 embryos as described (Figure 2), and the corneas were collected for analysis at E7, E9, and E15. First, we confirmed the overexpression of *Npnt* mRNA and protein in the corneas. At E9, expression of *Npnt* was localized in the corneal stroma during normal development (Figure 5A). However, *Npnt^{oe}* corneas showed robust expression of *Npnt* in the stroma, as well as ectopic expression in the corneal epithelium and endothelium, and the lens (Figure 5D). At this stage, strong staining for *Npnt* protein was only observed in the basement membrane of the corneal epithelium and low diffuse staining in the stroma of control corneas (Figure 5B). In contrast, the *Npnt^{oe}* corneas showed strong ectopic staining for *Npnt* in the corneal epithelium and endothelium, and a substantial increase in protein expression in the stroma compared to the control (Figure 5E).

Next, we performed histological analysis on E7, E9, and E15 corneas to determine whether there were any morphological differences between the control and *Npnt^{oe}* corneas. Our analysis did not reveal any significant differences in corneal thickness and cell number at E7 (Figure 5G, H). By contrast, cross-sections of E9 *Npnt^{oe}* corneas revealed significant thickening and increase in the cell number (Figure 5F, G, H) compared with control (Figure 5C, G, H). Similar increases in corneal thickness and cell number were observed at E15 (Figure 5G, H). Given that the keratocyte density is higher in the anterior stroma than the posterior stroma (Patel et al., 2001; Berlau et al., 2002), we quantified the cell densities in these regions. Overall, we observed relatively higher cell densities in the anterior stroma of E7, E9, and E15 corneas and there were no significant differences between *Npnt^{oe}* and control corneas (Figure 5I). Posterior cell density was not affected in E7 *Npnt^{oe}* corneas, but there was significant reduction at E9 and E15.

To determine whether elevated cell proliferation was a contributing factor to the increased corneal thickness in *Npnt^{oe}*, we examined corneas at E7, E9, and E15 by performing BrdU

labeling and immunofluorescent detection. Overall, we observed cell proliferation in all corneal layers, and there was a decreasing trend in the percentage of labeled cells in the stroma as development progressed from E7 to E15 (Figure 5J, K). However, our results revealed no significant increase in cell proliferation at E7 and E15. Surprisingly, there was a significant reduction in cell proliferation in E9 *Npnt*^{oe} corneas despite their increase in thickness (Figure 5K). Combined, our data indicate that overexpression of *Npnt* increases transcript and protein expression in the cornea, which results in increased cell number in the stroma and corneal thickness. Since these increases were not correlated with increased cell proliferation, our data suggest that the changes in corneal thickness are due to augmented pNC migration into the cornea caused by excessive expression of *Npnt*.

The RGD domain mediates *Npnt* signaling during pNC migration into the cornea

Npnt mediates signal transduction in development and cancer by binding to various receptors through the EGF-like and RGD domains (Arai et al., 2017; Kahai et al., 2010; Kuek et al., 2016; Linton et al., 2007). The marked reductions in corneal thickness observed following knockdown of both *Npnt* and *Itga8* (Figure 2 and Figure 3) suggest that *Npnt* functions via the RGD domain during pNC migration. Given that overexpression of the full-length *Npnt* protein caused corneal thickening, we generated an *Npnt*-RAE version of the protein in which the RGD domain was mutated by substitution with RAE sequence, and an *Npnt*-EGF version in which both the RGD and MAM domains were truncated (Figure 6A). Constructs were injected in HH7-8 embryos and E9 corneas were collected, sectioned, and analyzed following DAPI staining. Contrary to our observations following overexpression of the full-length protein (Figure 5), neither the *Npnt*-RAE or *Npnt*-EGF versions caused a significant change in corneal thickness compared with the

control (Figure 6B,C). Interestingly, there was a significant reduction in cell count and density in Npnt-RAE corneas although no difference in these values was observed for Npnt-EGF (Figure 6D,E). The absence of corneal thickening following overexpression of either Npnt-RAE or Npnt-EGF constructs further suggest that the RGD domain plays an essential role in this process. Our results also indicate that the EGF-like domain does not play an essential role during for pNC migration into the cornea.

Npnt-Itg α 8 signaling mediates pNC migration via a Rho-mediated mechanism

Previous studies have suggested that the Rho associated kinase (ROCK) pathway is involved with integrin α 8 β 1 modulation of actin stress fibers in vascular smooth muscles and intestinal crypt cells (Benoit et al., 2009; Zargham et al., 2007). To explore the mechanism by which Npnt-Itg α 8 signaling modulates pNC migration, we performed explant culture experiments for 24 hours on Npnt substrate as control (Figure 7A) and compared them to explants cultured in the presence of Npnt combined with various inhibitors. Given that focal adhesion kinases (FAK) play a role in integrin-mediated migration (Ilić et al., 1995; Parsons, 2003), we first wanted to verify if Npnt-Itg α 8-induced migration is mediated through the modulation of focal adhesions. We compared explants cultured on Npnt with the inhibitor for α 8 β 1 or FAK and observed that similar to the α 8 β 1 inhibitor (Figure 7-figure supplement 1C,D), inhibition of FAK reduced pNC migration from the explant but also severely attenuated cell attachment (Figure 7-figure supplement 1E,F).

Next, to examine if Npnt-Itg α 8 signaling induces assembly of the cytoskeletal machinery required for pNC migration via the Rho pathway, we treated the explants with the ROCK inhibitor Y27632. Under these conditions, the cells dissociated from the explant, attached to the

Npnt substrate, and transformed into a rounded phenotype with significantly increased surface area compared to Npnt alone (Figure 7A-C). In addition, our analysis indicated that the ROCK inhibitor significantly decreased the density of migratory cells (Figure 7D). Given that the cells covered a wider area, it is possible that the decrease in cell density could be due to the increased surface area or decreased cell migration. We therefore tested for linear patterns of cell dispersion from the explant. Our results revealed that in the presence of the ROCK inhibitor, cells exhibited a significantly reduced preferred orientation ($90^{\circ} \pm 30^{\circ}$) compared to Npnt alone (Figure 7E). Thus, in the presence of the ROCK inhibitor, the pNC spread out on the Npnt substrate with limited directional migration. To test if loss of directionality was caused by changes to the cytoskeletal structure, we examined the cytoskeletal machinery of the cells using phalloidin staining and focal adhesions by immunostaining for pY118 paxillin, which provides a docking site for assembly of the actin fibers that is required for cell adhesion, spreading, and migration (Nakamura et al., 2000; Turner, 2000). As previously reported (Katoh et al., 2001; Masamune et al., 2003; Takamura et al., 2001), the ROCK inhibitor attenuated the formation of actin stress fibers in pNC as indicated by the substantial reduction in the intensity of phalloidin staining (Figure 7F and 7G). Cells in both conditions stained positive for pY118 paxillin, but the number of puncta per cell significantly increased in the presence of the ROCK inhibitor. This result indicates that disruption of Rho kinase prevents pNC from forming the actin stress fibers and that the cells adhere to the Npnt substrate using multiple foci. Collectively, these data show that Npnt/Itg α 8 signaling activates FAK/paxillin for adhesion and that the Rho signaling pathway plays an important role during pNC migration on Npnt.

DISCUSSION

Very little is known about the function of the corneal ECM during early development. Here, we focus on *Npnt*, which we recently found in our RNA-seq study (Bi & Lwigale, 2019) to be upregulated during early development of the chick cornea. Specifically, we have identified novel expression of *Npnt* and its *Itga8* receptor at critical time points during pNC migration into the nascent cornea. Our data reveal that disruption of *Npnt/Itga8* signaling either *in ovo* or *in vitro* perturbs pNC migration, which subsequently results in corneal thickness defects, but formation of the cellular layers is not affected. Our model (Figure 8) provides a context in which *Npnt* functions in the presence of Fn during corneal development. Fn is a well-known substrate for neural crest cell migration (Alfandari et al., 2003; Bronner-Fraser, 1986; Newgreen & Thiery, 1980) that is robustly expressed in the periocular mesenchyme and cornea (Doane et al., 1996; Kurkinen et al., 1979). In comparison, *Npnt* is expressed in an increasing gradient from the edge of the periocular region towards the cornea (Figure 8A). We posit that all pNC cells express $\alpha5\beta1$ and previously showed that they robustly migrate on Fn substrate *in vitro* (Lwigale & Bronner-Fraser, 2009). However, *in vivo*, it is most likely that the pNC which express both $\alpha5\beta1$ and $\alpha8\beta1$, and thus can respond to *Npnt* and Fn, migrate into the cornea. Based on our current study, we conclude that *Npnt/Itga8* signaling plays an essential role in pNC migration during corneal development.

***Npnt* and *Itga8* in the cornea**

While *Npnt* is associated with kidney, teeth, and bone development (Arai et al., 2017; Kuek et al., 2016; Linton et al., 2007), only one reference was made to its expression in the mouse lens during ocular development (Brandenberger et al., 2001). First, we confirmed that *Npnt* is expressed in the migratory pNC and showed that it is also localized in the optic cup and lens

343 vesicle. Our immunohistochemistry analysis revealed that *Npnt* protein staining consistently
344 corresponded with the mRNA expression during pNC migration into the developing cornea. In
345 addition, we observed that *Npnt* was localized in the acellular primary stroma suggesting that
346 prior to its expression by the migratory pNC in the cornea, the optic cup and lens vesicle are the
347 primary sources of *Npnt* in the nascent corneal ECM. The link between the primary stroma and
348 the presumptive corneal epithelium and endothelium was established in classical studies, which
349 showed that the lens and corneal endothelium induce the corneal epithelium to synthesize
350 collagen and glycosaminoglycans into the underlying space (Fitch et al., 1994; E. Hay et al.,
351 1979; E. D. Hay & Revel, 1969; Toole & Trelstad, 1971). Our expression analyses suggest that
352 the primary stroma sequesters *Npnt* initially secreted by the optic cup and the lens, and exposes it
353 to pNC during early corneal development.

354 *Itga8* is expressed in the spinal cord, optic nerve, retina, urogenital and digestive systems, and in
355 the head epidermis (Bossy et al., 1991; Ogawa et al., 2018). $\alpha 8\beta 1$ is known to modulate
356 epithelial-mesenchymal interactions during kidney and pharyngeal development (Müller et al.,
357 1997; Talbot et al., 2016) and to promote migration of mesangial and smooth muscle cells
358 (Bieritz et al., 2003; Zargham & Thibault, 2006). We report for the first time that *Itga8* is
359 expressed by pNC during ocular development. Since *Itga8* heterodimerizes with *Itgb1* subunit
360 (Bossy et al., 1991), which is robustly expressed in the periocular mesenchyme and migratory
361 pNC (Doane & Birk, 1994), we infer that $\alpha 8\beta 1$ signaling functions during corneal development
362 in response to *Npnt* secreted in the primary stroma. Following delamination from the neural tube,
363 cranial neural crest migrate in response to Fn and laminin (Duband & Thiery, 1982; Newgreen &
364 Thiery, 1980; Sternberg & Kimber, 1986) via integrin receptors such as $\alpha 1\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$,
365 $\alpha V\beta 1$ (Alfandari et al., 2003; Delannet et al., 1994; Desban & Duband, 1997; Kil et al., 1996;

Lallier et al., 1994). Although neural crest cells are initially highly migratory, they remain relatively stationary upon their localization in the periocular region. Given that *Itga8* expression coincides with the onset of pNC migration, our results indicate its potential role in their ingression into the developing cornea.

Npnt promotes pNC migration via RGD domain, *Itga8*, and the Rho/Rock pathway

The three major factors that contribute to corneal thickness during development are pNC migration, cell proliferation, and synthesis of the secondary stroma by the keratocytes. We showed that disruption of *Npnt* expression in the anterior ocular tissues via RCAS-mediated knockdown resulted in decreased corneal thickness. In addition to functioning in epithelial-mesenchymal interactions, *Npnt* has been shown to promote migration in various tissues including vascular endothelial cells during osteogenesis (Kuek et al., 2016), infiltration of immune cells into the liver (Hong et al., 2020; Inagaki et al., 2013), and cancer metastasis (Magnussen et al., 2020; Mei et al., 2020; Wang et al., 2018). Given that *Npnt* is localized in the primary stroma during corneal development, we hypothesized that it may play a potential role in pNC migration. In agreement with our hypothesis, our *in vitro* migration assays confirmed that robust pNC migration from mesenchyme explant occurred on *Npnt* substrate but was abrogated in the presence of the *Itga8* inhibitor. We also observed that knockdown of *Itga8* reduced pNC migration into the cornea and phenocopied the reduction of corneal thickness observed following *Npnt* knockdown. In addition, we found that reduction in corneal thickness was accompanied by decrease in cell count, but cell density and proliferation were not affected, suggesting that *Npnt/Itga8* signaling mediates pNC migration during corneal development. Given that the primary function of keratocytes is to synthesize the corneal ECM comprised of collagens and

389 proteoglycans, and represents approximately 90% of the corneal thickness (Fini, 1999;
390 Funderburgh et al., 2003; Kao, 2010), our observation that there was no change in stromal cell
391 density raises a possibility that matrix synthesis by the pNC that differentiated into keratocytes is
392 not a major contributing factor to the reduction in corneal thickness. However, additional studies
393 may be required to determine how the disruption in corneal thickness observed in the current
394 study may affect the corneal ECM and transparency at later stages of development.

395 Our overexpression studies showed the opposite effect whereby the full-length construct caused
396 increased corneal thickness at E9. We also found that increased corneal thickness persisted at
397 E15 when *Npnt* appears to be downregulated in the cornea. Surprisingly, the E7 corneas were not
398 affected in the overexpression studies. This could be caused by limited pNC migration potential
399 due to their expression of Neuropilin1, which prevents them from entering the corneal
400 environment that contains a repulsive Semaphorin3A signal (Lwigale & Bronner-Fraser, 2009).

401 As observed in the knockdown studies, there were no differences in cell proliferation between
402 control and *Npnt^{oe}* corneas, suggesting that there was an increase of pNC migration. The mutant
403 *Npnt*-RAE construct overexpresses approximately the same size protein as the endogenous *Npnt*,
404 while maintaining its MAM domain to support ECM-ECM interactions, but it did not impact
405 cornea thickness. This indicates that expression of high levels of protein does not affect cornea
406 thickness, further implicating that *Npnt* functions in directing cell migration specifically through
407 its RGD sequence during early cornea development. The reduction in cell count and density
408 could be attributed to the mutant RAE domain outcompeting the expression of the endogenous
409 RGD by the pNC. Similarly, the truncated version of *Npnt* containing only the EGF-like repeats
410 did not affect cornea thickness, further confirming that *Npnt* signals through the RGD domain
411 and *Itga8* to modulate pNC migration into the cornea.

412 Furthermore, our *in vitro* culture experiments revealed that directed migration of pNC from the
413 explant requires activation of FAK by $\alpha 8 \beta 1$ to promote focal adhesions. Previous studies have
414 shown that activation of the Rho/Rock pathway via integrin signaling drives actin remodeling
415 that is necessary for cell adhesion and migration (Clark et al., 1998; Cox et al., 2001; Price et al.,
416 1998). Our data also revealed that in the presence of the ROCK inhibitor, pNC transformed into
417 a rounded morphology in which assembly of the actin cytoskeletal machinery was disrupted and
418 the number of focal adhesions increased. These results indicate a drastic change in migratory
419 behavior that was less polarized from the explant, implying a potential involvement of the
420 Rho/Rock pathway in the directed migration pNC into the cornea. Given that pNC migrate on
421 both Fn and Npnt *in vitro*, and that both $\alpha 5 \beta 1$ and $\alpha 8 \beta 1$ activate the Rho/Rock pathway (Benoit
422 et al., 2009; Danen et al., 2005; White et al., 2007; Zargham et al., 2007), it is possible that Rho
423 activity increases as the cells migrate from the periocular region into the matrix of the
424 developing cornea due to the presence of additional cues from Npnt.

425 Precise coordination of multiple signals from surrounding ocular tissues orchestrate the
426 spatiotemporal migration and differentiation of multipotent pNC during the formation of the
427 avian corneal endothelium and stromal keratocytes. Disruptions in the sequence of these early
428 events can lead to significant defects in corneal development. Our study provides the first
429 characterization of Npnt/Itg $\alpha 8$ function in pNC that contribute to the cornea. Despite being
430 surrounded by an ECM rich in Fn, pNC remain relatively immobile in the periocular region until
431 some become competent to respond to Npnt cues via the expression of $\alpha 8 \beta 1$. This
432 transformation may be crucial for the timely induction of directional migration of pNC towards
433 the gradient of additional cues generated by Npnt in the primary stroma (Figure 8A,B), and
434 therefore plays a vital role in segregating the cornea progenitors from the rest of the periocular

mesenchyme. In this study, we focused on the role of Npnt/Itg α 8 signaling during pNC migration. Our observation that the Npnt protein subsequently localizes to the basement membrane of the epithelial layer combined with its pleiotropic functions during development and in disease (Yamada & Kamijo, 2016), imply that Npnt may have other functions at later stages of cornea development. One possibility is that Npnt mediates epithelial-mesenchymal interactions between the cornea epithelium and stroma by signaling through the EGF-like domain to EGFR receptors in the epithelial cells. Future studies will expand on these findings to determine whether the corneal thinning and thickening phenotypes observed in the current study affect transparency, which develops at later stages. Elucidation of the link between Npnt signaling and corneal cell differentiation may provide useful insights for future therapeutic applications for wound healing and regeneration studies.

MATERIALS AND METHODS

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
transfected construct (<i>Gallus gallus</i>)	shRNA: Npnt	This paper		See Material and methods, section 5
transfected construct (<i>Gallus gallus</i>)	shRNA: Itga8	This paper		See Material and methods, section 5

biological sample (<i>Gallus gallus</i>)	Primary periocular neural crest	This paper		See Material and methods, section 7
biological sample (<i>Gallus gallus</i>)	DF-1 cells	ATCC	Cat# CRL12203	Lot# 58217603, no mycoplasma contamination detected
antibody	anti-Npnt (Rabbit polyclonal)	Biorbyt	Cat# orb221700; RRID: AB_2905548	IF “(1:100)”
antibody	anti-GFP (Mouse monoclonal)	Invitrogen	Cat# A-6455; RRID: AB_221570	IF “(1:500)”
antibody	anti-fibronectin (Mouse monoclonal)	DHSB	Cat#: B3/D6; RRID: AB_2105970	IF “(1:30)”
antibody	anti-phosphorylated paxillin (Rabbit polyclonal)	Invitrogen	Cat#: 44-722G; RRID: AB_2533733	IF “(1:200)”
antibody	anti-BrdU (Mouse monoclonal)	DHSB	Cat#: G3G4; AB_2618097	IF “(1:30)”
chemical compound, drug	Bromodeoxyuridine	Sigma	Cat#: B5002	

chemical compound, drug	Poly-D-Lysine	Sigma	Cat#: P6407	
recombinant DNA reagent	pSLAX13	Addgene	Cat#: CT#232	
recombinant DNA reagent	RCAS plasmid	Hughes et al., 1987 doi.org/10.1128/jvi.61.10.3004-3012.1987		
recombinant RNA reagent	Integrin $\alpha 8$ RNA probe	This paper	PCR primers	Supplementary File 1
recombinant RNA reagent	Nephronectin RNA probe	This paper	PCR primers	Supplementary File 1
peptide, recombinant protein	$\alpha 8\beta 1$ inhibitor	Sato et al., 2009 doi.org/10.1074/jbc.M900200200	Genscript	
Peptide, recombinant protein	Nephronectin (human)	R&D systems	9560-NP-050	1.5 $\mu\text{g}/\text{cm}^2$
Peptide, recombinant protein	Nephronectin (mouse)	R&D systems	AF4298-NP-50	1.5 $\mu\text{g}/\text{cm}^2$
commercial assay or kit	CloneEZ PCR cloning kit	Genscript	Cat#: L00339	
commercial assay or kit	DIG RNA Labelling Kit (SP6/T7)	Roche	Cat#: 11175025910	

chemical compound, drug	PF-573228	Sigma Aldrich	Cat#: PZ0117-5MG	10 μ M
chemical compound, drug	Y27632	Sigma Aldrich	Cat#: Y0503	10 μ M
other	DAPI stain	Roche	D8417	(1 μ g/mL)
other	Phalloidin	Invitrogen	A-12380	“(1:200)”

448

449 **Chick embryos**

450 All experiments were performed using fertilized White Leghorn chicken eggs (*Gallus gallus*
451 *domesticus*) obtained from Texas A&M Poultry Center (College Station, TX). Eggs were
452 incubated at 38 °C in humidified conditions until the desired stages. Animal studies were
453 approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University.

454 For Npnt and Itga8 knockdown and overexpression studies, eggs were incubated for
455 approximately 24-26hrs to obtain 3-somite stage or HH8 (Hamburger & Hamilton, 1951), then
456 windowed as previously described (Spurlin & Lwigale, 2013). A few drops of Ringer’s solution
457 containing 100 U/mL penicillin and 100 μ g/mL streptomycin (PenStrep, ThermoFisher
458 Scientific) were added to embryos to maintain hydration. Embryos were injected with viral
459 constructs (see below) using a Picospritzer III pneumatic microinjection system (Parker
460 Hannifin) in the space between the vitelline membrane and cranial region, ensuring that the
461 neural tube and adjacent ectoderm were completely covered (Figure 2A). Injected embryos were
462 re-incubated and collected for GFP screening at the desired stages of development ranging

between embryonic day (E)5-E15. Only the corneas showing robust GFP expression were used for subsequent analyses.

Histology, Hematoxylin and Eosin staining, and Immunohistochemistry

Embryos were collected at desired stages and eyes were dissected in Ringer's saline solution. Samples collected for Hematoxylin and Eosin (H&E) staining were fixed overnight at 4 °C in modified Carnoy's fixative (60% ethanol, 30% formaldehyde, and 10% glacial acetic acid). Afterwards, the tissues were dehydrated in ethanol series, cleared in HistoSol (National Diagnostics), embedded in paraffin blocks, and sectioned at 10 µm thickness. Sections were stained with Hematoxylin for 15 seconds, counterstained with Eosin for 40 seconds, dehydrated in ethanol series, and mounted with Cytoseal (ThermoFisher Scientific) for imaging. For Npnt immunohistochemistry, eyes were fixed in methanol-acetic acid (MAA) fixative (98% methanol, 2% glacial acetic acid) that was chilled on dry ice. Samples were maintained in MAA fixative at -80 °C for at least two days, then were gradually warmed to room temperature before further dehydration in ethanol series and embedding in paraffin blocks. Samples were sectioned at 10 µm, rehydrated, then immunostained with Npnt antibody (1:100; orb221700, Biorbyt) following standard procedures. For all the other immunohistochemistry and phalloidin staining, samples were fixed in 4% PFA overnight at 4 °C, embedded in paraffin, and sectioned as described above. Immunostaining with anti-GFP (1:500; A-6455, Invitrogen), and anti-fibronectin (1:30; B3/D6, Developmental Studies Hybridoma Bank) was used to detect protein expression. Explant cultures were immunostained with anti-phosphorylated paxillin (1:200; 44-722G, Invitrogen). Phalloidin-568 (1:200; A-12380, Invitrogen) and DAPI (Roche) were used to show total cell distribution and actin organization in whole-mount, sectioned tissue, and explant cultures. The

following secondary antibodies (Invitrogen) were used at 1:200: Alexa-488 goat anti-rabbit IgG, Alexa-594 goat anti-rabbit IgG, Alexa-594 goat anti-mouse IgG2a, and Alexa-488 goat anti-mouse IgG1.

Section *In situ* Hybridization

Eyes were fixed in modified Carnoy's fixative, embedded in paraffin, and sectioned as described above. Riboprobes were generated from gene fragments using cDNA pooled from E7 anterior eyes and cloned into TOPO-PCR II (Invitrogen). Digoxigenin (DIG)-labeled riboprobes were synthesized following the manufacturer's protocol (DIG Labeling Kit, Roche). Primers used to generate the riboprobes are listed in Supplementary File 1. Sections were hybridized with riboprobe at 52 °C (*Npnt*) and 60 °C (*Itga8*) overnight. Hybridization was detected using anti-DIG antibody conjugated with alkaline phosphatase (Roche) and color was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma). Following color development, sections were fixed with 4% PFA, mounted in Cytoseal and imaged using an AxioCam mounted on an AxioImager2 microscope (Carl Zeiss).

Production of RCAS virus

Replication-Competent ASLV long terminal repeat with a Splice acceptor (RCAS) (Hughes et al., 1987) virus was used for stable and prolonged expression of short hairpin (sh)RNA constructs used for knockdown and for the overexpression studies. To produce RCAS viral stocks, chick fibroblasts (DF-1 cells; Lot 58217603 with no mycoplasma contamination detected, ATCC) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin

(referred to here as complete DMEM, ThermoFisher Scientific). Cells were transfected at about 70% confluency with plasmid DNA containing the RCAS constructs using Lipofectamine 3000 (Invitrogen). Transfected cells were grown for four days to enable viral replication. Media containing viral particles were collected on subsequent days, pooled, and centrifuged at 21,000 rpm (Beckman) for 1.5 hour at 4 °C to concentrate the virus. Virus pellets were resuspended in DMEM and stocks at approximately $1-7 \times 10^6$ Ifu/ml were stored in -80 °C until use.

Generation of shRNA and viral constructs

shRNA target sequences used for *Npnt*^{kd} and *Itga8*^{kd} (Supplementary File 2), were designed using the BLOCK-iT RNAi Designer tool (ThermoFisher Scientific) and linked to their reverse complementary sequence by a loop sequence, TTCAAGAGA. A short termination sequence and restriction enzyme sites were added at each end to create fragments that were ligated into a pSLAX shuttle vector (Hughes et al., 1987) and to add a chick U6 (Cu6) promoter and a GFP reporter. The shRNA sequences were cloned into the RCAS vector (Figure 2-figure supplement 1A,B) by homologous recombination using a CloneEZ kit (GenScript) as previously described (Kwiatkowski et al., 2017; Ojeda et al., 2017). The *Npnt*^{oe} overexpression constructs were either generated by substitution of the shRNA with a full-length coding sequence of *Npnt* in the vector above or using a modified vector where the full-length *Npnt* protein was driven by a promoter within the viral long terminal repeats (LTRs) with eGFP linked by an IRES motif (Figure 2-figure supplement 1B). Constructs containing either mutated *Npnt* with the RGD sequence changed to RAE (PRGDVFIPRQPGVSNNLFEILEIER to PRAEVFIPRQPGVSNNLFEILAIAR) or the truncated version of *Npnt* containing only the N-terminal EGF-like repeats domain (*Npnt*-EGF) were commercially synthesized (Genscript) and

cloned into the modified RCAS vector. All constructs were validated using primers for Npnt and Itga8 (Supplementary File 3).

BrdU staining

Bromodeoxyuridine (BrdU) was used to assay for cell proliferation. The BrdU solution was prepared at a final concentration of 10 μ M in complete media. Eyes were collected at desired stages and injected into the anterior chamber between the cornea and lens with BrdU solution (approximately 50-100 μ l depending on development stage), then cultured in BrdU solution for 2 hours at 37 °C. Eyes were rinsed in phosphate buffered solution (PBS), fixed in 4% PFA, embedded in paraffin, and sectioned as described above. BrdU-positive cells were identified by immunohistochemistry using anti-BrdU antibody (1:30; G3G4, Developmental Studies Hybridoma Bank). Sections were counterstained with DAPI.

***In vitro* explant culture**

Embryos were collected at E4 in Ringer's solution and the anterior eyes were dissected and digested in dispase (1.5mg/mL, Worthington) for 10 minutes at 37 °C. The periocular mesenchyme was isolated by physically removing the presumptive cornea epithelium, lens, and the optic cup. The mesenchyme ring was further trimmed using tungsten needles to obtain cells that are proximal to the presumptive cornea, then dissected into approximately 120x120 μ m explants. Nunc Lab-tek II 8-well chamber slides (Sigma) were coated with 1.5 μ g/cm² with poly-D lysine (MP Biomedicals) for 1 hour at room temperature, followed by recombinant mouse or human Npnt (R&D Systems) at 1.5 μ g/cm² for 2 hours at 37 °C. Mesenchyme explants were transferred to the coated slides containing complete media, with and without inhibitors and

incubated at 37 °C in a humidified tissue culture incubator with 5% CO₂ for 12-24 hours. The $\alpha 8\beta 1$ peptide inhibitor following the 23-mer sequence **PRGDVFIPRQPTNDLFEIFEIER** (Sato et al., 2009) was commercially generated (Genscript) and used at a 10 μ M working concentration. The small molecule FAK inhibitor PF-573228 (Sigma; Slack-Davis et al., 2007) and ROCK inhibitor Y27632 (Sigma; Uehata et al., 1997) were used at 10 μ M working concentration. Explants with migratory cells were imaged with a Rebel T6s camera (Canon) mounted on an Axiovert 40C microscope (Carl Zeiss). The magnitude of cell migration from explants was calculated by measuring the density of cells in a 175 μ m x 175 μ m area located 270 μ m from the center of the explant. The orientation of pNC migration from explants was analyzed as previously described (Babaliari et al., 2018). Briefly, confluent regions of cells proximal to the explant were segmented and linearized, then the ImageJ Directionality plugin using local gradient orientation (Liu, 1991) was applied. The cellular features oriented to $90^{\circ} \pm 30^{\circ}$ were considered to be aligned perpendicular to the explant. Mean integrated peak areas, bounded by 60° and 120° , were used to compare the magnitude of perpendicular alignment in all conditions.

Time-lapse video microscopy

Mesenchyme explants were prepared and cultured as described above in media containing 0.1 μ g/ml Hoechst dye solution (ThermoFisher Scientific) used to label all nuclei. Chamber slides containing attached explants were imaged at 3 minutes and 27 second intervals for 17 hours using an FV1200 laser scanning microscope (Olympus) with a stage top incubator (Tokai Hit). Movies were generated using IMARIS Software (Oxford Instruments).

Quantification of Corneal Measurements

578 *Corneal thickness*

579 Differences in corneal thickness between control and knockdown samples was determined by
580 averaging measurements taken at three separate locations along the cornea. All measurements
581 were taken perpendicular to the radial curvature of the cornea. Since corneal thickness is not
582 always uniform in overexpression samples, measurements were taken at three locations across
583 the center of the thickened region.

584 *Cornea cell counts and density*

585 Nuclei labeled by the DAPI staining of corneal sections were used to count stromal cells within a
586 width of 200 μm along the entire height of that region. Cells were either counted manually or
587 using the threshold particle analysis in ImageJ Software in which nuclei from the epithelium and
588 endothelium are discounted from the final tally. Cell density was determined by dividing the
589 number of nuclei and the area, which was measured by multiplying the value of the corneal
590 thickness by the width of the selected region. Comparison of cell counts and density between the
591 anterior vs posterior corneal region was determined by taking similar areas for all corneas, which
592 was set at a value of 20% the average thickness of control corneas.

593 *Cell proliferation*

594 Cell proliferation was determined in corneal sections by first utilizing the cell count technique
595 described above. The total number of cells was represented by all the DAPI-positive nuclei, out
596 of which the BrdU-positive cells were quantified. Cell proliferation was calculated as a
597 percentage of the total DAPI-positive nuclei that were BrdU positive.

598

599 **Software and Statistics**

ImageJ Software was used to measure the staining intensity or tissue morphological features, such as cornea thickness and cell density. The number of cornea sections analyzed are summarized in each figure legend. All statistical analyses were conducted using GraphPad Software. Data are presented as scatterplot with mean values. Statistical significance was determined by two-tailed unpaired Student's t-test was used to compare differences between means. Samples with p-values < 0.05 were considered significant.

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COMPETING INTERESTS

No competing interests to declare.

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FIGURE LEGENDS

Figure 1. Npnt expression during early ocular development. Expression of Npnt mRNA and protein were examined via section in situ hybridization (A-C) or immunohistochemistry (D-L). (A-B) *Npnt* expression in the retina pigment epithelium layer of the optic cup and region of the presumptive lens fiber cells at E4 and E5. (C) Initial expression of *Npnt* by pNC is observed during the second wave of migration into the stroma (black arrows). (D-F) At E4, Npnt protein was detected in the optic cup, lens epithelium, in the periocular mesenchyme proximal to the presumptive cornea region (arrow), and in the matrix of the primary stroma (asterisk). (G-I) At E5, vivid expression of Npnt protein is localized in the primary stroma adjacent to the corneal epithelium and diffusely throughout the primary stroma (asterisk), and persists at low levels in the optic cup and lens epithelium. At E6, vivid expression of Npnt protein persists in the primary stroma adjacent to the corneal epithelium and it remains diffusely expressed throughout the primary stroma (asterisk). At this time, low expression of Npnt protein is also observed in the migratory periocular neural crest cells invading the primary stroma (arrows in L). Abbreviations: ec, ectoderm; oc, optic cup; pom, periocular mesenchyme; rpe, retinal pigment epithelium; st, stroma; en, corneal endothelium; ep, corneal epithelium. Scale bars: 100 μ m.

Figure 2. Corneal thickness is reduced in *Npnt*^{kd} corneas. (A) Schematic of *in ovo* injection of viral constructs (green) to cover the anterior region of stage 8 chick embryo. Following 7 days of

incubation, embryos were screened for GFP expression in the anterior eye region. Knockdown was verified by section in situ hybridization, which revealed reduced expression of *Npnt* in *Npnt^{kd}* cornea compared with control. (B-E) Hematoxylin and Eosin staining showing control (B,D) and thinner *Npnt^{kd}* corneas (C,E). Statistical analysis on measurements taken from (N=5 control and N=4 *Npnt^{kd}* corneas) revealed: (F) Significant reduction in thickness of *Npnt^{kd}* corneas, ** indicates $p < 0.01$. Abbreviations: ep, corneal epithelium; st, stroma; en, corneal endothelium;. Scale bars: 100 μm (B,C), 100 μm (D,E).

Figure 3. *Itga8* is expressed pNC during cornea development and it plays a role in cell migration. (A) Expression of *Itga8* is observed in pNC prior to their migration into the cornea at E4 (arrow). (B) *Itga8* is subsequently expressed in the corneal endothelium at E5 (arrows), and (C) by the migratory pNC in the corneal stroma at E6. (D) Schematic showing the isolation of periocular mesenchyme used for generating pNC explants for *in vitro* migration on *Npnt*-coated substrate in the presence or absence of $\alpha 8\beta 1$ inhibitor. (E) Explant cultured on *Npnt* substrate showing robust cell migration after 12 hours. (F) Explant cultured on *Npnt* substrate in the presence of $\alpha 8\beta 1$ inhibitor showing fewer cell migration after 12 hours. (G) Statistical analysis performed on (N=6 explants on *Npnt* substrate) and (N=6 explants on *Npnt* substrate plus inhibitor) revealed significant reduction in cell density of migratory cells in the presence of the inhibitor. * indicates $p < 0.05$. Abbreviations: ec, ectoderm; pom, periocular mesenchyme; en, corneal endothelium; ep, corneal epithelium; st, stroma. Scale bars: 100 μm .

Figure 4. Knockdown of *Itga8* reduces pNC migration and results in reduced corneal thickness. (A-B) Whole-mount E5 anterior eyes immunostained for GFP and counterstained with phalloidin to reveal cell membranes. (A) Control eye showing robust expression of GFP by pNC cells that migrated into the corneal region to form the endothelial layer. (B) *Itga8^{kd}* eye showing that relatively fewer GFP cells migrated into the cornea (arrows). Dotted lines demark the boundary between the cornea and periocular mesenchyme. (C-D) Hematoxylin and Eosin staining of E7 corneal section showing (C) normal corneal thickness in control and (D) reduced corneal thickness in *Itga8^{kd}* embryos. Double-sided arrows indicate corneal thickness. (E) Statistical analysis of measurements taken from (N=5 control and N=6 *Itga8^{kd}* corneas) revealed significant reduction in thickness and cell count, and no difference in cell density in *Itga8^{kd}* corneas, * indicates $p < 0.05$. (F-G) BrdU immunofluorescent analysis of cell proliferation in E7 corneal sections. Quantification of BrdU-positive cells in the corneal stroma was performed by normalizing to the total number of DAPI-positive cells. (H) Statistical analysis from N=7 control and N=5 *Itga8^{kd}* revealed a no difference between control and *Itga8^{kd}* corneas. ns, not significant. Abbreviations: en, corneal endothelium; pom, periocular mesenchyme; L, lens. Scale bars: 100 μm .

Figure 5. Effects of overexpression of *Npnt* during corneal development. Embryos were injected with RCAS virus expressing GFP alone (control) or GFP and the full-length *Npnt* protein, and corneas were analyzed at the developmental stages indicated. (A-F) Representative corneal sections from E9 control (A-C) and *Npnt^{oe}* corneas (D-F) showing levels of *Npnt*

transcript (A,D) and protein (B,E) expression, and H&E staining indicating corneal thickness (double sided arrows, C,F). (G,H) Measurements for corneal thickness and cell counts were taken from: E7, N=10 control, N=10 *Npnt^{oe}*; E9; N=7 control, N=12 *Npnt^{oe}*; E15; N=6 control, N=9 *Npnt^{oe}*. Bar graphs show no difference at E7, but a significant increase at E9 and E15 in corneal thickness (G), and corneal cells (H). (I) Cell densities were determined from: E7, N=8 control, N=7 *Npnt^{oe}*; E9; N=6 control, N=11 *Npnt^{oe}*; E15; N=7 control, N=6 *Npnt^{oe}*. Bar graph shows that there were no differences at E7 and anterior cell densities at E9 and E15, but the posterior cell densities were significantly decreased. (J and K) BrdU analysis and quantification of cell proliferation in corneal sections taken from E7, N=8 control, N=7 *Npnt^{oe}*; E9; N=9 control, N=9 *Npnt^{oe}*; E15; N=7 control, N=6 *Npnt^{oe}*. No significant differences were observed at E7 and E15, but there was a significant reduction at E9. ns, not significant; * indicates $p < 0.05$; ** indicates $p < 0.01$; and *** indicates $p < 0.001$. Abbreviations: ep, corneal epithelium; st, stroma; en, corneal endothelium. Scale bars: 100 μm .

Figure 6. Overexpression of versions of *Npnt* with either mutant or truncated RGD domains does not increase corneal thickness. (A) Schematic showing the full-length *Npnt*, *Npnt* with mutated RGD to RAE sequence, and the truncated version containing only the EGF domain. (B) representative sections of E9 corneas showing corneal thicknesses (double sided arrows) following overexpression of control, RGD mutant, and truncated versions of *Npnt*. (C-E) Quantification of measurements taken from N=5 control, N=9 RAE, and N=12 EGF showing: (C) No significant differences in corneal thickness. (D,E) Significant reduction in cell count and density in RGD mutant, but no difference in the truncated version. ns, not significant; * indicates $p < 0.05$. Scale bar: 100 μm .

Figure 7. Inhibition of Rho-kinase attenuates pNC migration on *Npnt*. (A-D) Comparisons of pNC migration from explants cultured for 24 hours on either (A) *Npnt* substrate alone, or in the presence of (B) the ROCK inhibitor Y27632. (C-D) Quantification of cell area taken from N=8 *Npnt* and N=8 *Npnt* plus ROCK inhibitor showing significant reduction in (C) cell area and (D) cell migration in the presence of ROCK inhibitor. (E) Quantification of cell orientation from N=6 *Npnt* and N=6 *Npnt* plus ROCK inhibitor explants showing significant difference in the fraction aligned between $90^\circ \pm 30^\circ$. (F-G) Cells stained for actin (phalloidin) and focal adhesion (pY118 paxillin) showing: (F) Formation of actin stress fibers and focal adhesions by pNC migrating on *Npnt*. (G) Substantial decrease in actin stress fibers in the presence of the ROCK inhibitor. (H) Quantification taken from N=4 *Npnt* and N=4 ROCK inhibitor explants, showing significant increase in the number of pY118 paxillin-positive puncta. * indicates $p < 0.05$; ** indicates $p < 0.01$. Scale bars: (A,B) 100 μm ; (F,G) 50 μm .

Figure 8. Dynamics of pNC response to the ECM in context of the expression of *Npnt* and Fn during corneal development. (A) Cross-section of E5 eye immunostained for *Npnt* and Fn. *Npnt* appears in an increasing gradient from the periocular region into the cornea, whereas Fn stains both the periocular mesenchyme and cornea. (B) All pNC respond to Fn via expression of $\alpha 5\beta 1$, but a subpopulation of pNC that reside in the region adjacent to the presumptive cornea express both $\alpha 5\beta 1$ and $\alpha 8\beta 1$, and become competent to also read the additional gradient of *Npnt* in the ECM, thus migrating into the corneal region. Abbreviations: pNC, periocular neural crest; oc, optic cup; ps, primary stroma; en, corneal endothelium.

Figure 3-Video 1: Migration of pNC from mesenchyme explant on Npnt-coated substrate. Time-lapse movie was taken over 17 hours with images taken every 3 minutes and 27 seconds. The cells are shown in bright-field and fluorescent Hoechst nuclear staining (blue). Relates to Figure 3E.

Figure 3-Video 2: Migration of pNC from mesenchyme explant on Npnt-coated substrate in the presence of $\alpha 8\beta 1$ inhibitor. Time-lapse movie was taken over 17 hours with images taken every 3 minutes and 27 seconds. The cells are shown in bright-field and fluorescent Hoechst nuclear staining (blue). Relates to Figure 3F.

Source Data 1: Contains statistical analysis reported in Figures 2-7.

Source Data 2: Contains gel images for data reported in Figure 2-figure supplement 1C.

SUPPLEMENTARY MATERIAL

Supplementary File 1. Table showing the primer sequences used for riboprobe synthesis.

Supplementary File 2. Table showing the shRNA target sequences used for knockdown studies.

Supplementary File 3. Table showing the primers used to validate *Npnt* and *Itga8* knockdown efficiency.

Figure 1-figure supplement 1. Expression of Npnt transcripts and protein during late stages of development of the chick cornea. (A-C) Section in situ hybridization showing localization of Npnt in the stroma of E7, E9, and E12 corneas. (D-F) Immunohistochemistry showing that Npnt (red) is localized in the ECM directly adjacent to the corneal epithelium at E7 and E9 (D and E, arrows), and in the corneal epithelium at E9 (F, arrows). Sections are counterstained with DAPI (blue). Scale bars represent 100 μ m. Abbreviations: ep, epithelium; st, stroma; en, corneal endothelium.

Figure 2-figure supplement 1. Diagram of RCAS vectors used for expression of either: (A) GFP alone (control), or GFP together with inserts of shRNA or full-length Npnt. (B) The full-length or mutated and truncated versions of Npnt driven by the viral LTR promoter and GFP driven by the IRES promoter. (C) For RTPCR, cells plated in 30 mm dishes were homogenized in 1 mL TRIzol and RNA was isolated following manufacture's protocol. RNA samples were

1124 treated with Turbo DNA-free kit (Invitrogen) to remove residual genomic DNA and cDNA pools
1125 were generated using SuperScript First Strand System (Invitrogen). Semi-quantitative PCR was
1126 conducted using HotStart-IT Taq polymerase (Affymetrix). Knockdown efficiency was
1127 measured on 2% agarose gel using GAPDH (glyceralde-hyde-3-phosphate dehydrogenase) as a
1128 loading control. Red rectangles indicate the constructs that were chosen for the knockdown and
1129 overexpression experiments. Primers for RT-PCR are located in Table 1.

1130
1131 **Figure 4-figure supplement 1. Validation of *Itga8* knockdown in vivo.** Stage 8 chick embryos
1132 were injected in the cranial region with control and *Itga8*-shRNA viral constructs, and re-
1133 incubated until E7. Section in situ hybridization was performed using riboprobes for *Itga8*. (A)
1134 Section of control cornea showing *Itga8* expression in the corneal stroma, iris and lens
1135 epithelium. (B) Section of *Itga8^{kd}* cornea showing reduced expression of *Itga8* in the thin
1136 cornea, iris and lens epithelium. The double sided arrows indicate cornea thickness.
1137 Abbreviation: st, corneal stroma. Scale bar, 200 μ m.

1138
1139 **Figure 7-figure supplement 1. Analysis of pNC migration on Npnt substrate in the presence**
1140 **of inhibitors of components of the migratory signaling pathway.** pNC explants were
1141 examined for cell migration after 24 hour culture on Npnt substrate with or without inhibitors.
1142 (A, B) Control showing robust migration of pNC on Npnt substrate and strong phalloidin
1143 staining. (C, D) Migration of pNC is attenuated in the presence of $\alpha 8\beta 1$ inhibitor. (E, F)
1144 Inhibition of the focal adhesion kinase (FAK) also attenuated pNC migration and in addition,
1145 only a few cells remained attached to the Npnt substrate. (G, H) The ROCK inhibitor
1146 substantially decreased the formation of actin stress fibers as indicated by the low level of
1147 phalloidin staining compared to B. Scale bar, 100 μ m.

Screen eyes for GFP expression

Screen corneas for *Npnt* expression

D.

A.

B.

C.