Delayed neurogenesis with respect to eye growth shapes the pigeon retina for high visual acuity

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ABSTRACT
The macula and fovea located at the optical centre of the retina make primate visual perception unique among mammals. Our current understanding of retina ontogenesis is primarily based on animal models having no macula and no fovea. However, the pigeon retina and the human macula share a number of structural and functional properties that justify introducing the former as a new model system for retina development. Comparative transcriptome analysis of pigeon and chicken retinas at different embryonic stages reveals that the genetic programmes underlying cell differentiation are postponed in the pigeon until the end of the period of cell proliferation. We show that the late onset of neurogenesis has a profound effect on the developmental patterning of the pigeon retina, which is at odds with the current models of retina development. The uncoupling of tissue growth and neurogenesis is shown to result from the fact that the pigeon retinal epithelium is inhibitory to cell differentiation. The sum of these developmental features allows the pigeon to build a retina that displays the structural and functional traits typical of primate macula and fovea.

KEY WORDS: Avian transcriptome, Retinal ganglion cell, Photoreceptor, Notch signalling, Atonal homolog 7, Fibroblast growth factor 3, Neurogenin 2

INTRODUCTION
Primate retinas are characterised by a specialised central region, the macula, while their periphery resembles that of other mammals. The macula is subdivided into different areas, including the fovea and the foveola. The latter is located in the centre of the fovea, is devoid of rods and contains only cones. The neuronal composition of the fovea favours neurons of the midget pathway, a circuit essential for high visual acuity and colour vision, consisting of one cone that forms synapses with two bipolar cells, which in turn make connections with two retinal ganglion cells (RGCs). About half of the 1.2×10⁶ RGCs in the human retina are located in the fovea, which represents less than 1% of the total retinal surface (Kolb, 1995). The macula and fovea make primate visual perception unique among mammals. An understanding of the genetic network underlying the development and maintenance of this highly specialised region is instrumental to addressing issues about human macula-related retinopathies, yet our current knowledge of retina ontogenesis is primarily based on animal models that have no macula and no fovea. With a RGC:rod ratio of ∼1:100, the mouse retina resembles the peripheral retina of primates. The ratio of RGCs to photoreceptors is higher in the chicken retina but the density of RGCs in the area centralis is still much lower than at the foveal rim in the human macula (∼14×10⁵ versus ∼60×10⁵ RGCs/mm² in chicken versus human).

The transcription factor (TF) atonal homolog 7 (ATOH7; also known as ATH5) is required for the production of RGCs in vertebrates (Brown et al., 2001; Del Bene et al., 2007; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Matter-Sadzinski et al., 2001; Wang et al., 2001) and the transcriptional network underlying the production of RGCs is well conserved between mouse, chicken and fish (Brown et al., 1998, 2001; Hufnagel et al., 2010; Kay et al., 2001; Matter-Sadzinski et al., 2001, 2005; Sinn et al., 2014; Skowronska-Krawczyk et al., 2004, 2009). However, whereas the majority of the ATOH7-expressing cells enter the RGC lineage in the chick, only a few percent produce RGCs in the mouse (Prasov and Glaser, 2012; Skowronska-Krawczyk et al., 2009; Yang et al., 2003). Sustained expression of ATOH7 is required during the last cell cycle for cells to enter the RGC lineage. In chick, efficient NGN2-mediated activation of Atoh7 transcription, Atoh7 autostimulation and HEVS.3-mediated lengthening of the cell cycle increase the proportion of cells that enter this lineage (Chiiodini et al., 2013; Skowronska-Krawczyk et al., 2009). However, this still does not result in numbers of RGCs as high as in the macula and fovea (Fig. 1).

The retinas of flying birds have high acuity regions, with one and sometimes two foveae. The pigeon has two regions of high RGC density and one fovea (Fig. 1) (Galifret, 1968; Querubin et al., 2009) and the visual acuity of the pigeon and human eyes are comparable (Hodos and Leibowitz, 1977; Williams, 1985). This prompted us to introduce the pigeon as a new model system for analysing retina development. Our study uncovers that by deferring neurogenesis to the end of the period of cell proliferation, the pigeon has developed an effective way to expand the pool of progenitor cells that enter the RGC lineage, thereby increasing the density of RGCs and the number of optic nerve axons in the adult eye. We further show that, although neurogenesis proceeds according to different schedules in pigeon and chick, retinas grow at a similar pace in both species. Moreover, the developmental patterning of the pigeon retina differs from the current models of retina development, which are largely based on studies performed with chick, mouse and fish embryos. Our study reveals that mechanisms exist for the eye of vertebrates to disconnect tissue growth and cell differentiation, allowing the pigeon to develop a retina that displays structural and functional features of macula and fovea.
RESULTS

Eye growth and retina development in pigeon and chick

Analysis of pigeon (*Columba livia*) and chick (*Gallus gallus*) at equivalent embryonic developmental stages (Fig. S1A) revealed no striking difference in eye development. Measuring the surface of the retina (Fig. 2A), the number of retinal cells (Fig. 2B) and the number of cell divisions per day (Fig. 2C) indicated that pigeon and chick retinas grow and expanded at a similar pace. The cell division rates were remarkably close in both species from embryonic day (E) 4 to 8. The average rate of cell division during this period (Fig. 2C) is consistent with cell cycles that last 11-15 h (Chiodini et al., 2013). By E8, the majority of cells had been produced in pigeon and chick, and the number of cells increased modestly in both species between E8 and E10 (Fig. 2B).

Despite the fact that the retinas grew at a similar pace in the two species, we noted significant differences in the histology of the retinas at equivalent embryonic ages (Fig. 2D). Whereas at E9 the outer nuclear layer (ONL) bearing the cone and rod photoreceptors, the inner nuclear layer (INL) bearing the bipolar, amacrine and horizontal cells, and the ganglion cell layer (GCL) bearing RGCs and displaced amacrine cells, were already well developed in the central and peripheral chick retina, these layers were indiscernible in the E9 pigeon retina (Fig. 2D). Retinal lamination became visible in the pigeon central retina at ~E10 and the retinas of both species looked similar at ~E12, with some notable differences. In pigeon, the nerve fibre layer (NFL) that contains RGC axons is much thicker and the ratio of GCL cells to ONL cells is ~4-fold higher than in chick (Fig. S1B, Fig. S2).

Comparative transcriptome analysis reveals delayed cell differentiation in the pigeon retina

Intrigued by the 3-day delay in the development of the retinal layers in pigeon, we wondered whether it reflected a delay in neurogenesis. In the current animal models, RGCs are the first neurons to be born and they appear shortly after the formation of the optic cup. In chick, the first *Atoh7*-expressing cells and the first RGCs with growing axons are detected at E2 and E2.5, respectively (Goldberg and Coulombre, 1972; Matter-Sadzinski et al., 2005; McCabe et al., 1999; Prada et al., 1991), whereas in pigeon they were detected at E5 and E5.5, respectively (Fig. 3A). Axon counting in the developing optic nerve is a direct and unequivocal method to monitor the dynamics of RGC differentiation. Whereas axon number peaks in chick at E10 (Rager, 1980), only ~20% of the final number of axons were counted at this stage in pigeon (Fig. 3B). The expression profile of *Atoh7* was analysed by northern blot and real-time quantitative PCR (RT-qPCR). In pigeon, *Atoh7* mRNA was first detected at E6 and transcript accumulation peaked between E8 and E10, 3 days later than in the chick (Fig. 3C,D). Consistent with the fact that NGN2 activates transcription of the *Atoh7* gene (Hufnagel et al., 2010; Matter-Sadzinski et al., 2005; Skowronska-Krawczyk et al., 2009), Ng2 expression was also delayed in pigeon (Fig. 3E). The observation that *Ng2* and *Atoh7* expression peaked at the end of the period of cell proliferation in pigeon, whereas it culminated when cells were still rapidly dividing in chick (Fig. 2C), suggests that proneural gene expression is not coordinated with the rate of cell division.

Since the timing of RGC differentiation differs between pigeon and chick, we examined the extent to which the development of the whole retina varies between the two species. We compared the diversity and dynamics of the pigeon and chick RNA-Seq transcriptomes at three critical stages: (1) E3-E3.5 (referred to as E3), encompassing the onset of neuronal differentiation in chick; (2) E5-E5.5 (referred to as E5), encompassing the onset of neuronal differentiation in pigeon and the peak of *Atoh7* expression in chick; (3) and E8-E8.5 (referred to as E8), which encompasses the upregulation of *Atoh7* in pigeon. RNA samples were isolated in triplicate at each stage and the 18 samples were processed for transcriptome analysis by RNA-Seq. In order to organise the large
amount of data, we ordered genes according to their expression profiles in chick using the three paired embryonic stage comparisons: E3-E5, E5-E8 and E3-E8. Because our aim was to identify genes that participate in early retina development, we selected among the transcripts and the corresponding genes those that displayed significant fold changes ($\geq 1$ and $\leq -1$ in log$_2$) in chick between E3 and E5. This set represents $\sim 36\%$ of the compendium of chicken genes that we selected and is referred to as 1/N/N. About 75% of these genes showed different expression profiles in pigeon and chick (Table S1). A heat map generated with clustered genes upregulated or downregulated between E3 and E5 in chick revealed that $\sim 64\%$ of the corresponding genes in pigeon did not show significant variation in expression during this early period; instead, their expression changed between E5 and E8 (Fig. S3A).

To determine whether this delay reflected the late onset of RGC differentiation in pigeon, we focused our analysis on genes regulated by ATOH7 (Fig. S3B). About 95% of such genes were upregulated between E3 and E5 in chick. By contrast, pigeon orthologues were activated or displayed stronger activation between E5 and E8. The delayed onset of expression of ATOH7-regulated genes in pigeon and, in particular, of the ATOH7 target genes involved in axonal outgrowth and neuronal activity (Fig. S3C; e.g. Stmn2, Chrnh3, Ptn) (Chiodini et al., 2013; Skowronska-Krawczyk et al., 2009), indicates that neuronal differentiation is delayed in pigeon. Deferred expression of TFs that exert control over photoreceptor, amacrine, horizontal and bipolar cell specification (Fig. S3C) is reflected in the delayed development of the ONL and INL (Fig. 2D). Taken together, our data suggest that cell differentiation into most types of retinal neurons is postponed in pigeon.

The genetic programme underlying retina growth unfolds at the same pace in pigeon and chick

About 25% of the selected 1/N/N set of genes displayed the same expression profiles in the three paired embryonic stage comparisons of the two species. These included the core cell cycle regulators cyclin A1 and cyclin D2, the TFs Sox2, Klf5 and c-Myc and the tumour suppressor p63 (Tp63) (Fig. 4E, Table S1), providing further evidence that cell cycle progression and the dynamics of progenitor cell expansion proceeded at the same pace in pigeon and chick up to E8. Moreover, 12 genes encoding TFs and transcriptional regulators displayed identical expression profiles in both species, suggesting that they regulate aspects of retina morphogenesis that develop independently of neurogenesis and
cell differentiation (Fig. 4E). Comparative morphological analysis (Fig. 4A-D) indicated that the curvature of the retinal epithelium, the closure of the optic fissure and the formation of the optic disc developed at a similar pace in both species.

Cell adhesion, matrix and matrix-associated proteins help establish the static aspects of epithelial architecture and are thought to participate in epithelial cell dynamics, driving the morphogenetic events that shape the retina. Genes encoding cell adhesion and matrix proteins and displaying the same expression profiles in pigeon and chick (Fig. 4F) could be driving those epithelial morphogenesis processes that are independent of cell differentiation. By contrast, other genes encoding matrix or cell adhesion components displayed early variations in expression in chick, but not in pigeon, suggesting that they might play a role in cell differentiation (Fig. 4G). In sum, our transcriptome comparison revealed that parts of the genetic programme underlying retina development progress independently of cell differentiation.

### Delaying neuronal differentiation as an adaptation for increasing RGC numbers

We asked whether the delayed neuronal differentiation had an effect on the total number of RGCs produced in pigeon. At the peak of Atoh7 expression, ~5% and ~50% of the total number of progenitor cells had been generated in chick and pigeon retinas, respectively (Fig. 2B), while the RT-qPCR analysis indicated that the accumulation of Atoh7 transcripts was similar in both species (Fig. 3D). To improve the accuracy of this estimate, we measured the number of Atoh7 transcripts per microgram of total RNA using a counter analysis system from NanoString Technologies. The counts of Atoh7 transcripts were remarkably similar in E5.5 to E6 chick and in E8 to E10 pigeon retinas (Fig. 5A), suggesting that the accumulation of Atoh7 transcripts depends neither on the number of cell divisions nor on the size of the retina. Taking into account the differences in RNA content at the peak of Atoh7 expression (Fig. 5C), the number of Atoh7 transcripts per retina was ∼3-fold higher in pigeon (Fig. 5D,E).

To compare the proportions of cells that entered the RGC lineage at the peak of Atoh7 expression, E6 chick and E9 pigeon retinal cells were transfected with the Atoh7-RFP and CMV-GFP reporters and fluorescent cells were counted 24 h later (Fig. 5B). Atoh7-RFP identifies cells expressing Atoh7 at high level and that enter the RGC lineage (Chiodini et al., 2013). We calculated the fractions of Atoh7-expressing cells, and we estimated that in pigeon three times as many cells entered the RGC lineage during this 24-h period (∼1.26×10^6 versus ∼0.43×10^6 cells/retina in pigeon versus chick; Fig. 5C,E). This difference matches the 2- to 3-fold higher cell
density in the pigeon GCL at E12 (Fig. S2) and the 3-fold difference in the number of optic nerve axons between the two species at E17 (Fig. 5E). Likewise, in the days following the peak of Atoh7 expression, the density of RGCs reached \( \sim 70 \times 10^3 \) cells/mm\(^2\) and these neurons were uniformly distributed throughout the retina, except for modest increases in density in the central area (Fig. S2). After cessation of mitotic activity, the retinal surface area increased by a factor of two (\( \sim 110 \) mm\(^2\) at E12 versus \( \sim 250 \) mm\(^2\) 1 year post hatching). We suppose that the different RGC densities (7-10\( \times \)10\(^4\) cells/mm\(^2\) in the perifoveal and red field areas versus 1-3\( \times \)10\(^4\) at the periphery; Fig. 1) are established during this period of expansion.

In pigeon, RGCs and photoreceptors have congruent periods of genesis and there is no central-to-peripheral gradient in their emergence

Both species displayed the same high rate of cell division from E4 to E8, followed by a rapid decrease in cell divisions between E8 and E10 (Fig. 2C). However, analysis of the distribution of cells pulsed with BrdU for 1 h at E9 revealed a remarkable difference in the spatial distribution of dividing cells between the pigeon and chick retinas. According to the classical model, which is mostly based on chicken and mouse studies, the retina develops in a centro-peripheral sequence; that is, cessation of cell division occurs first in the central area and then extends to the periphery (Hufnagel and Brown, 2013; Prada et al., 1991). It appears that this model does not apply to the pigeon retina, where dividing cells are evenly distributed throughout the central and peripheral areas up to E9. The fact that the total number of cells doubled in the pigeon retina between E8 and E10 (\( \sim \)8.2\( \times \)10\(^6\) at E8 versus \( \sim \)16.5\( \times \)10\(^6\) at E10; Fig. 2B) and that 40\( \pm \)5% of cells were in S phase in the central and peripheral retina at E9 (Fig. 6A; data not shown) suggest that the vast majority of cells did progress through cell cycles that lasted more than 24 h at this stage of development. In line with this idea, the majority of BrdU-labelled nuclei displayed sparse fluorescence puncta (Fig. 6A, inset), suggesting that they had a decreased number of replication foci and that cells progressed through a long S phase (Duronio, 2012). At E12, <1% of pigeon cells were in S phase (Fig. 6B) and 35\( \pm \)3\( \times \)10\(^6\) cells per retina were counted.

To determine whether the pigeon retina displays a class-specific sequence of birth dates and a central-to-peripheral gradient in genesis, BrdU was injected in ovo at E6, E8, E9 or E10 and the retinas were processed for immunohistochemistry analysis at E12. The label was detected in the nuclei of cells that were about to be born at the
time of injection, whereas it was diluted in cells that continue to divide (La Vail et al., 1991). The first heavily labelled cells were detected in the GCL and ONL at E6 (Fig. 6D,E), suggesting that the births of RGCs and photoreceptors begin at the same time. The fact that genesis of RGCs and photoreceptors peaked (Fig. 3D). Likewise, the players in the Notch signalling pathway were measured 24 h later. (C) Comparison of growth parameters of the retinas 1 day after the onset and at the peak of Atoh7 expression. (D) Atoh7 transcript counts per retina at the peak of Atoh7 expression. (E) Atoh7-expressing cells in pigeon to Atoh7-expressing cells in chick at the peak of Atoh7 expression, and (right) axon number in pigeon to axon number in chick when fully differentiated (E12 in chick, E17 in pigeon; Fig. 3B). (A-D) At each developmental stage, data are from three biological replicates and are presented as mean±s.d.

**Notch signalling and patterning of the retina**

Hairy/enhancer of split 1 protein (HES1) is a Notch effector that is known to prevent premature differentiation of progenitor cells in early development, thereby allowing neuronal progenitor cell maintenance into later stages of development (reviewed by Imayoshi and Kageyama, 2014). HES1 is expressed in the developing retina and it represses Atoh7 and Ngn2 expression (Hernandez et al., 2007; Lee et al., 2005; Matter-Sadzinski et al., 2005). We examined whether prolonged expression of HES1 could account for delaying neuronal differentiation in pigeon. Both the RT-qPCR and RNA-Seq data revealed that the downregulation of *Hes1* followed similar kinetics in pigeon and chick (Fig. S4A,D). Hence, in pigeon, unlike in chick, *Hes1* was already downregulated when *Atoh7* peaked (Fig. 3D). Likewise, the players in the Notch
signalling pathway, namely Notch1, Notch2, Rbpj and Jag1 displayed similar expression profiles in pigeon and chick (Fig. S4D). By contrast, expression of the Hes5 genes was delayed in pigeon (Fig. S4B-D), consistent with the fact that they are activated by ATOH7 (Chiodini et al., 2013). The interplay between ATOH7 and HES5.3 is indeed required for cells to upregulate Atoh7 and to enter the RGC lineage. Despite the fact that Hes5.3 expression is detected in the whole subset of Atoh7-expressing progenitor cells, only 25-30% of them upregulate Atoh7 and enter this lineage in chick between E3 and E5 (Chiodini et al., 2013; Matter-Sadzinski et al., 2005). This low efficiency of RGC production at early stages is attributed to the negative effect of HES1 on Atoh7 cis-acting regulatory elements (Hernandez et al., 2007), hence keeping transcription of the Atoh7 gene at a low level in progenitor cells. Thus, we could expect that, at the onset of Atoh7 expression in pigeon, a larger subset of cells express Atoh7 at high levels. To test this idea, E6 pigeon retinas and E3 chick retinas were electroporated with Atoh7-GFP and Atoh7-RFP and fluorescent cells were counted 24 h later. The ratio of red cells to green cells was ∼3-fold higher in pigeon (Fig. 7A). Likewise, the number of Atoh7 transcripts per microgram of RNA increased ∼4-fold between E6 and E8 in pigeon, whereas it increased ∼14-fold between E3.5 and E5.5 in chick (Fig. 5A), suggesting a vigorous onset of Atoh7 expression in chick.

We wondered whether this reflected an interspecies difference in the patterning of the retina. In E2.5 chick retina, the tiny Atoh7 and Ngn2 expression domains coincide in central retina and they abut on the peripheral Hes1 domain. The concentric expression pattern of Atoh7 is maintained until E5, and Hes1 expression, on the whole, remains complementary to that of Atoh7, with transcript levels maintained high at the periphery and low in the centre (Matter-Sadzinski et al., 2005). Measurement of Hes1 expression in tissue fragments dissected in the central and peripheral retina revealed, for both areas, similar transcript levels in pigeon and chick at E6 (Fig. S4A, insets). Under these conditions, Atoh7 expression peaked throughout the central and peripheral retina in chick (Fig. 4D) (Matter-Sadzinski et al., 2005), whereas it had just started in pigeon. In pigeon, the first cells expressing Atoh7 at high level were detected throughout the central and peripheral retina at E5.5 and we did not detect any preferential labelling of the central area (Fig. 7B,C).

Moreover, in pigeon retinas electroporated with an Atoh7-lacZ reporter plasmid at E6, the β-galactosidase+ cells were scattered throughout the central and peripheral retina (Fig. 4C), whereas in chick, at the onset of RGC differentiation at E3, β-galactosidase+...
cells are confined to a tiny central domain (Matter-Sadzinski et al., 2005). Consequently, in pigeon, *Chrnb3*, which is an early marker of RGC differentiation and a target of ATOH7 (Chiodini et al., 2013; Skowronska-Krawczyk et al., 2004), is expressed in a domain that encompasses both the ventral and dorsal retina (Fig. 7D). Furthermore, fairly uniform NFL thickness at E9 suggests that, in pigeon, RGCs are produced at the same stage in central and peripheral areas, whereas in chick they emerge in a central-to-peripheral pattern (Fig. 7E). One day after the onset of *Atoh7* expression, *Atoh7* transcripts had accumulated throughout the central and peripheral retina in pigeon (Fig. 7F), whereas they are not yet detectable at the periphery in chick (Matter-Sadzinski et al., 2005). The slightly higher accumulation of *Atoh7* transcripts in the central area at E6 correlates with the increased NFL thickness and enhanced cell density in the GCL of the central retina at E12 (Fig. 7E,F, Fig. S2).

### A factor promoting neurogenesis is expressed late in pigeon

To gain insight into the mechanism underlying the late onset of neuronal differentiation in pigeon, we asked if the deferred expression of *Ngn2* and *Atoh7* resulted from a delayed expression of activators. Several members of the fibroblast growth factor (FGF) family are expressed in the developing vertebrate eye and function in optic vesicle patterning and neuronal differentiation (Guillemot and Cepko, 1992; Hyer et al., 1998; Pittack et al., 1997). RNA-Seq and RT-qPCR analysis enabled us to compare the expression profiles of six FGF genes expressed in the course of retinal development.

In each species, *Fgf3* displayed a transient expression profile that coincided with that of *Atoh7* (Fig. S5A). Martinez-Morales et al. (2005) have shown that the concerted activity of FGF3 and FGF8 induces retinal differentiation in chick. In pigeon, *Fgf8* expression peaked ~2 days before the onset of *Atoh7* expression and it was already downregulated when *Fgf3* was activated (Fig. S5A,B), suggesting that FGF8 was not capable of triggering RGC differentiation by itself. Likewise, *Fgf1* was downregulated at the stages when *Atoh7* was activated and RGCs produced in pigeon (Fig. S5B). Thus, despite the fact that application of exogenous FGF1 induces RGC differentiation in chick (McCabe et al., 1999), FGF3 could well be the best candidate among FGFs for activating neurogenesis in the avian retina (Martinez-Morales et al., 2005). In pigeon, *Fgf3* transcripts accumulated at similar levels in the central and peripheral retina (Fig. 7F). The broad expression domain of *Fgf3* at the time when the first RGCs differentiate could explain, at least in part, the absence of a central-to-peripheral gradient in RGC genesis (Fig. 6C-E).
The pigeon retinal epithelium is inhibitory to RGC differentiation

In pigeon, RGCs are produced, much like in the primate perifoveal area, at the end of the period of cell proliferation, whereas in the chick retina and in the primate peripheral retina, RGCs are produced days and weeks, respectively, ahead of the cessation of cell division (La Vail et al., 1991; Provits et al., 1985). In this context, we examined whether the pigeon retinal epithelium develops special features during the period that precedes the onset of neurogenesis. To address this issue, we selected 49 genes that were activated in pigeon (≥1/N/N) but not in chick (<1/N/N), between E3 and E5. This group includes 13 genes that were upregulated between E3 and E5 and downregulated between E5 and E8 (set 1; Fig. 8A, Table S2). We wondered whether their downregulation was related to the onset of neurogenesis. A ChIP-Chip analysis revealed that ATOH7 protein was bound to sequences upstream of fibulin 7 (Fbln7) in E3.5 chick retina (Fig. 8B, top). Electroporation of Atoh7 siRNAs in chick E3.5 retinas led to the upregulation of Fbln7 (Fig. 8B, bottom), suggesting that, in pigeon, ATOH7 could repress Fbln7 between E5 and E8.

FBLN7 is a cell adhesion protein that interacts with extracellular matrix molecules (de Vega et al., 2007). Several other genes encoding matrix, cell adhesion or cytoskeleton components belong to set 1 and we asked if cell-cell interactions in the pigeon retinal epithelium could inhibit cell differentiation. E3.5 pigeon and chick retinas were enzymatically disaggregated, and acutely dissociated cells were co-transfected with Atoh7-RFP and Atoh7-GFP and plated at low density on plastic slides. Similar proportions of pigeon and chick double-labelled cells were detected 24 h after plating. (Right) A double-labelled pigeon cell. (D) The central and mid-peripheral pigeon retina was co-electroporated with Atoh7-GFP and Atoh7-RFP at E4. No RFP+ cells were detected 24 h later. (E) E3.5 pigeon and chick retinas were dissociated into single cells that were plated on plastic dishes; RNA was isolated 24 h later and the levels of Atoh7, Ngn2 and Fgf3 transcripts measured by RT-qPCR. (C,E) Data are from two biological replicates and are presented as the mean. Scale bars: 200 µm.

Among the 49 genes that we selected, 23 were activated in pigeon between E3 and E8 (set 2; Fig. 8A, Table S2). None of these genes is involved in retina growth; rather, they might set the ground for the development of retinas of increased complexity resulting from high RGC density, i.e. that bear structural and functional similarities to the primate macula. The fact that genes associated with human retinopathies, such as Leber congenital amaurosis (Rd3, Chrb1) or retinitis pigmentosa (Cerkl), belong to this set strengthens the idea that development of the primate macula and pigeon retina share specificities.
DISCUSSION
In chick and mouse embryos, the onset of retinal neurogenesis starts 1 day after the formation of the optic cup. Neurogenesis is initiated in a tiny domain and proceeds in a wave from the central to the peripheral retina that correlates with the central-to-peripheral appearance of postmitotic cells (reviewed by Agathocleous and Harris, 2009). Here we show that neurogenesis in pigeon starts 4 days after the formation of the optic cup and that cells entering the RGC lineage are scattered throughout the central and peripheral retina. Contrary to what has been observed in animal species studied so far, the onset of neuronal differentiation in pigeon is not restricted to the central domain and the genesis of RGCs and photoreceptors does not propagate to the periphery in a wave-like manner. HES1 controls aspects of the patterning of progenitors in chick and mouse retinas and is required for the closure of the optic cup and stalk (Lee et al., 2005; Matter-Sadzinski et al., 2005; Tomita et al., 1996), a landmark in eye morphogenesis that develops at a similar pace in pigeon and chick, suggesting that late neurogenesis in pigeon retina does not result from the prolonged inhibition of Ngn2 and Atoh7 by HES1, but rather from a time lag in the expression of activators and, in particular, of FGF3 (Martinez-Morales et al., 2005).

The fact that the pigeon eye is already well developed when ATOH7 induces RGC differentiation indicates that the onset of cell differentiation does not keep pace with overall retinal growth. That retinal growth and cell differentiation may proceed according to separate schedules has not been observed previously because of the early onset of neurogenesis in the retinas of the animal models studied so far. This finding challenges the idea that an increase in the number of cells or in the length of the cell cycle during the course of retinal development may act as a biological clock that coordinates the timing of cell differentiation with tissue growth (reviewed by Cepko, 2014). We note that, although the lengthening of the cell cycle is essential for the production of RGCs in chick, the mechanism underlying this process is initiated by ATOH7 and therefore depends on neurogenesis (Chiiodini et al., 2013). By postponing neurogenesis to the end of the period of cell proliferation, while maintaining the peak of Atoh7 expression at the same level as in chick, the pigeon has developed an effective way of expanding the pool of progenitors out of which cells are selected to enter the RGC lineage.

The development of the pigeon retina might represent the rule, rather than the exception, among birds. Phylogenomic studies indicate that one node at the base of the avian tree splits the neognaths between the Galloanserae (e.g. chicken) and the Neoaves, which regroup 95% of avian species and include the orders Columbiformes (e.g. pigeon) and Passeriformes (Hackett et al., 2008; Prum et al., 2015). Although more than half of all bird species are classified in these two orders, only a handful of passerine retinas have been studied so far. It appears that these birds have a fovea and two rod-free areas with high densities of RGCs, much like in pigeon (Coimbra et al., 2009, 2015). Heterochrony between neurogenesis and growth has a profound effect on the patterning of the retina and on the ratios of cell types. In pigeon, it results in a high ratio of RGCs to cones (Querubin et al., 2009) and high visual acuity (Coletta et al., 2003; Hodos and Leibowitz, 1977). Delaying neurogenesis in the developing CNS might serve different purposes. For instance, the onset of neurogenesis in the telencephalon is delayed in primates compared with rodents, allowing for greater expansion of the founder neural cell pool before neurogenesis begins (reviewed by Florio and Hutten, 2014). In the same manner, most of the post-hatching brain growth in parrots and songbirds is due to a late expansion of the telencephalon, which is associated with a general delay of neurogenesis (Charvet and Striedter, 2011).

Cell proliferation, the expansion and folding of the retinal epithelium, the closure of the optic fissures and the formation of the optic disc all proceed at similar paces in pigeon and chick, in sharp contrast with the respective timings of neuronal differentiation. Twelve TFs and transcriptional regulators that we examined display the same expression profiles in pigeon and chick, suggesting that their role in retinal growth and morphogenesis is independent of cell differentiation. The fact that Pax2 and Hes1 belong to this set strengthens the idea that the closure of the optic fissure and optic disc formation are regulated independently of cell differentiation. In humans, the loss of ALX1 or mutations in SOX2 lead to craniofacial dysplasia characterised, among other defects, by microphthalmia (Fantes et al., 2003; Taranova et al., 2006; Uz et al., 2010), while in birds the Alx1 haplotype contributes to diversification of beak shapes among Darwin’s finches (Lamichhaney et al., 2015). Similar expression dynamics of Alx1 and Sox2 in pigeon and chick highlights how the growth of the retina is coordinated with the increase in eye size and face development irrespective of the time of onset of neurogenesis.

Our study raises two intriguing questions. First, the nature of the signal that lies upstream of the earliest known activators of neurogenesis and how this signal is acting independently of the retinal growth stage. We show that in pigeon the retinal epithelium exerts an inhibitory effect on cell differentiation until the end of the period of cell proliferation and we identified genes encoding matrix, cell adhesion or cytoskeleton components that may participate in this inhibition. Future studies should clarify how these components affect signalling and how they influence the onset of neurogenesis. In optic cups developing in vitro from mouse or human embryonic stem cells, RGCs have been identified after 9 and 24 days, respectively, in culture (Eiraku et al., 2011; Nakano et al., 2012). The late onset of cell differentiation in the human optic cup is consistent with the fact that in the primate embryonic retina, RGCs differentiate in the incipient perifoveal area at the end of the period of cell proliferation (Hendrickson, 2016; La Vail et al., 1991; Provis et al., 1985). It remains to be seen whether, in the central area of the primate retina, the epithelium exerts, as in pigeon, an inhibitory effect on neuronal differentiation.

That genes associated with human retinopathies are activated before the onset of cell differentiation in pigeon is intriguing. Very little is known about the function of the proteins that these genes encode, nor do we know whether they are expressed early in foetal human retina. The fact that variants of ATOH7 could be associated with glaucoma (reviewed by Sakurada and Mabuchi, 2015) highlights how early variations in retina development may eventually lead to retinal diseases later in life. In this context, the pigeon retina could prove to be a useful new model system with which to uncover some of the intricate mechanisms underlying growth and neurogenesis in the macula of primates.

MATERIALS AND METHODS
Animals
Fertilised chicken eggs were from a White Leghorn strain (UNIGE Animal Resources Centre). Fertilised pigeon eggs were supplied by Philippe Delaunay (Pigeonneau de la Suisse Normande, Croisilles, France).
Experimental procedures were carried out in accordance with Federal Swiss Veterinary Regulations.

**Molecular cloning**

*Atoh7* (Atoh7-RFP, Atoh7-GFP, Atoh7-lacZ), *Chrm3* (Chrm3-GFP) and CMV-GFP reporter plasmids are described in the supplementary Materials and Methods.

**RNA-seq**

RNA samples were isolated in triplicate from pigeon and chick retinas at E3-E3.5, E5-E5.5 and E8-E8.5 and the 18 samples were processed for transcriptome analysis by RNA-seq. mRNA library preparation and sequencing were performed under contract with Fasteris (Plan-les-Ouates, Switzerland). Stranded-mRNA libraries were prepared from 1 μg total RNA using the TruSeq Stranded mRNA Library Preparation Kit (Illumina) following the manufacturer’s instructions. Libraries were sequenced on an Illumina HiSeq 2500 using the TruSeq Rapid SBS Kit (Illumina) and HiSeq Rapid Run (RR) mode, with 150 bp single-end reads. The runs generated 14-24 million reads per sample, of which 69-88% were mappable to reference genomes. Mapping raw reads to reference chick and pigeon genomes was performed using TopHat 2/ Bowtie 2 (Kim et al., 2013). Chicken genome reference (WASHUC2 assembly) was obtained from Ensembl.org. Pigeon genome and transcript annotation reference files (assemblies from 2014-02-18) were obtained from the (GIGA) database (gigadb.org). Scoring transcript abundances and identification of differentially expressed transcripts was performed using Cufflinks and Cuffdiff scripts from the Cufflinks package (Trapnell et al., 2012). For differential expression, each comparison included data from three biological replicates of either developmental stage.

**Northern blot and RT-qPCR**

Retinas isolated from pigeon and chicken whole and dissected retinas was subject to northern blot analysis with *Hes5.3* and *Atoh7* riboprobes. RT-qPCR was performed using the primers listed in Table S3. For details, see the supplementary Materials and Methods.

**NanoString nCounter**

To estimate *Atoh7* transcript number, total RNA was subject to NanoString analysis using the probes listed in Table S3. Details are provided in the supplementary Materials and Methods.

**Retina morphometric measurements**

Retinas from E4 to E17 embryos were dissected, unfixed flat-mounts were photographed and surfaces were measured with ImageJ (NIH). For cell counting, retinas were dissociated as described by Matter-Sadzinski et al. (2005). Cells were counted in a Neubauer chamber. For histology, the retinas and optic nerves were fixed overnight in Trump’s 4F:1G fixative (McDowell and Trump, 1976) and embedded in an epoxy resin. Semi-thin sections (1 μm) were stained with Toluidine Blue. Ultra-thin sections (McDowell and Trump, 1976) and embedded in an epoxy resin. Semi-thin sections (1 μm) were stained with Toluidine Blue. Ultra-thin sections (100 nm) at a distance of 0.5-1.0 mm from the optic disc were processed for counting, retinas were dissociated as described by Matter-Sadzinski et al. (2011). Developmental modes and retinal ganglion cell and optic nerve formation.

**Retina electroporation and lipofection assay**

The electroporation of pigeon and chick retinas with reporters or siRNA, and the transfection of single cells dissociated from chick and pigeon retinas, were performed as detailed in the supplementary Materials and Methods.

**BrdU and lacZ staining**

Cell proliferation was assessed by in ovo injection or pulse labelling with BrdU as described in the supplementary Materials and Methods. Atoh7-lacZ reporter activity was assessed as described in the supplementary Materials and Methods.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Data availability**

Unpublished histological data concerning the pigeon retina and optic nerve are available at http://www.genes-vision.ch/pigeon. RNA-seq data have been deposited at Gene Expression Omnibus under accession number GSE89541.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.138719.supplemental

**References**


