Lectins selectively label cartilage condensations and the otic neuroepithelium within the embryonic chicken head

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Abstract

Cartilage morphogenesis during endochondral ossification follows a progression of conserved developmental events. Cells are specified towards a prechondrogenic fate and subsequently undergo condensation followed by overt differentiation. Currently available molecular markers of prechondrogenic and condensing mesenchyme rely on common regulators of the chondrogenic program that are not specific to the tissue type or location. Therefore tissue-specific condensations cannot be distinguished based on known molecular markers. Here, using the chick embryo model, we utilized lectin labeling on serial sections, demonstrating that differential labeling by peanut agglutinin (PNA) and Sambucus nigra agglutinin (SNA) successfully separates adjacently located condensations in the proximal second pharyngeal arch. PNA selectively labels chick middle ear columella and basal plate condensation, whereas SNA specifically marks extracolumella and the ventrolateral part of the otic capsule. We further extended our study to examine lectin-binding properties of the different parts of the inner ear epithelium, neural tube and notochord. Our results show that SNA labels the auditory and vestibular hair cells of the inner ear, whereas PNA specifically recognizes the statoacoustic ganglion. PNA is also highly specific for the floor plate of the neural tube. Additionally, wheat germ agglutinin (WGA) labels the basement membrane of the notochord and is a marker of the apical-basal polarity of the cochlear duct. Overall, this study indicates that selective lectin labeling is a promising approach to differentiate between contiguously located mesenchymal condensations and subregions of epithelia globally during development.

Key words: endochondral ossification; glycosylation signature; lectin; peanut agglutinin; pharyngeal arch; Sambucus nigra lectin; wheat germ agglutinin.

Introduction

This study focuses on endochondral ossification of neural crest-derived and mesoderm-derived mesenchyme in the head of the developing chick embryo. There is a shared, underlying molecular patterning mechanism for mesenchyme condensation in multiple body tissues, including limbs, axial skeleton and head (pharyngeal arch) cartilages (Hall & Miyake, 1992, 2000; Shimizu et al. 2007; Wood et al. 2010; Kumar et al. 2012; Ray & Chapman, 2015). Common genes in the head pathway include BMPs, Col2a1, Collagen X, FGFs, Indian Hedgehog and Sox9 (Wood et al. 2010). However, no differential markers for cranial cartilage tissues have been identified to date despite efforts such as gene microarray analysis of these tissues (Chapman Lab, unpublished). Specific pharyngeal arch condensations cannot, therefore, be differentiated based on known genetic markers.

To solve this problem lectins, which label soluble carbohydrates and carbohydrate moieties of glycoproteins or glycolipids, were utilized. Cells display tissue-specific sugars on their surface and secrete tissue-specific glycoproteins and glycolipids into the surrounding extracellular matrix, suggesting that lectins can differentially label tissues (Poirier & Kimber, 1997; Sharon, 2007). Peanut agglutinin lectin (PNA) is a known marker of pre-chondrogenic neural crest-derived mesenchyme in condensations that labels non-sialylated galactose residues (Sasano et al. 1992; Miyake et al. 1996; Wood et al. 2010). We, and others have shown that PNA labels the columella condensation but not the otic capsule or extracolumella condensation within the second pharyngeal arch (Hall & Miyake, 1995; Wood et al. 2010).
Interestingly, upon application of neuraminidase, a sialidase that removes the terminal sialic acid of carbohydrates, PNA labeling in the otic capsule and extracolumella is observed (Chapman Lab, unpublished). These data suggested to us that individual cartilages had differential carbohydrate moieties that could be used to distinguish tissues. However, there has been no systematic examination of PNA or other lectin labeling in situ.

In this study, the binding of PNA (Arachis hypogaea), Sambucus nigra agglutinin (SNA) and wheat germ (Triticum vulgaris) agglutinin (WGA) were specifically analyzed to determine which tissues are specifically labeled by each lectin (Table 1). PNA, from Arachis hypogaea (peanut) binds galactose/N-acetylgalactosamine and is specific for non-sialylated terminal galactose with a 1-3 linkage (Lotan et al. 1975; Novogrodsky et al. 1975; Hennigar et al. 1987). SNA, the elderberry bark lectin, preferentially binds N-acetylneuraminic acid and galactose moieties in an α-2,6 linkage (Shibuya et al. 1987). A third class of lectins includes WGA that specifically recognizes free and bound N-acetylglucosamine (strong) and N-acetylgalactosamine (weak) residues (Allen et al. 1973; Lotan et al. 1973). As each lectin preferentially binds a different target, it was predicted that they could be used to differentially label tissues.

The results demonstrate that all three lectins label specific tissues within the head, irrespective of their common molecular pathways. PNA and SNA are spatially restricted, whereas WGA labels tissues with less discrimination, likely due to the wider variety of saccharides it recognizes. PNA labels the columella, the bony portion of the single middle ear bone in the chick and the basioccipital (basal plate), whereas SNA labels the extracolumella, the persistent cartilage of the columella, and the otic capsule, effectively differentiating each of these three tissues. SNA also labels blood vessels, with SNA and WGA both distinguishing basement membranes. In the inner ear, SNA labels sensory and vestibular hair cells, whereas PNA labels the entire epithelium and is highly specific for the statoacoustic ganglion. These results show that lectins can effectively separate the identity of numerous tissues within the developing head and are candidates for differentiating other tissues throughout the embryo with common origins or shared developmental fates.

### Methods

**Chick embryos**

Clemson University Poultry Farm supplied fertilized chicken eggs that were incubated to the desired stages at 38.5 °C in a humidified incubator. Embryos were staged according to the Hamburger-Hamilton table of normal stages and embryonic day (E4-E6) and harvested in normal saline solution (Hamburger & Hamilton, 1992) and then fixed in 4% paraformaldehyde overnight at 4 °C. This study was approved by the Clemson University IACUC under protocol number 2011-041.

**Paraffin embedding and sectioning**

Paraffin embedding and sectioning were performed essentially as previously described (Wood et al. 2010). Following overnight fixation, chick embryonic heads 4-6 days old were washed twice in 1× phosphate buffered saline (PBS) and then passed through a graded series of ethanol washes. This was followed by multiple Neo-Clear washes, followed by a similar washes with Paraplast at 60 °C. Sectioning was performed at 12 μm.

**Lectin labeling**

Fluorescein-labeled peanut agglutinin (PNA-FITC, #FL-1071), fluorescein-labeled Sambucus nigra lectin (SNA-FITC, #FL-1301) and fluorescein-labeled wheat germ agglutinin (WGA-FITC, #FL-1021) were purchased from Vector Laboratories. PNA Alexa Fluor® 647 Conjugate (#FL-1301) was purchased from Life Technologies (Thermo Fisher Scientific). Each lectin was diluted in PBS/Triton-X-100 and applied to the tissue at the following dilutions: 1 : 40 PNA Alexa Fluor 647, 1 : 200 PNA FITC, 1 : 100 SNA FITC, and 1 : 50 WGA FITC. Slides were placed flat into a humidified chamber and overlaid with 200 μL of the labeling solution and incubated at 4 °C for 48-72 h. Following several PBS washes to remove the excess lectin solution, the tissue was incubated in a 1 : 5 dilution of DAPI/PBS solution (1 mg mL⁻¹) for 30 min to label the nuclei. The sections were mounted with Slowfade, coverslipped and imaged.

**Imaging**

All sections were imaged using a Nikon TIE inverted confocal microscope with a Roper Scientific HQ2 camera at the Clemson Light Imaging Facility. Image processing and analysis were performed using Nikon NIS Elements version AR 3.2 and FIJI. Images were false-colored as follows: DAPI nuclear labeling – blue, PNA – magenta, SNA – green, WGA – red.
SNA – green, and WGA – yellow. Adobe Photoshop CS6 was used to prepare composite images.

Results

To determine the suitability of selected lectins to label tissues within the developing chick embryonic head we used transverse sections at the level of second pharyngeal arch from E4–E6 to visualize lectin binding properties (Fig. 1A–J). We were interested in differentiating between the adjacent endochondral tissues of the inner and middle ear. These included the otic capsule surrounding the inner ear, the nascent bony columella that will insert into the oval window of the inner ear, and the persistent cartilage of the extracolumella that inserts three processes into the tympanic membrane (Table 2). Fluorescent labeling with PNA, SNA, and WGA (Fig. 1) demonstrated that tissues throughout the head were differentially labeled, including the inner ear epithelium, basioccipital and neural tube/notochord.

Morphology of the chick embryonic head

The major structures of the head from E4 to E6 include the neural tube and underlying notochord, the inner ear epithelium, second pharyngeal arch and cartilages including the otic capsule, columella, basioccipital, quadrate, and Meckel’s cartilage. Transverse sections of the head show widespread WGA-positive expression, notably highlighting surface ectoderm and notochord (Fig. 1A–C). PNA is more...
Table 2: Differential lectin binding properties of cranial condensations.

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BP, basilar papilla; C, columella; EXC, extracolumella; FP, floor-plate; OC, otic capsule; PNA, peanut agglutinin lectin; SNA, *Sam-bucus nigra* agglutinin lectin; WGA, wheat germ agglutinin lectin.

The intensity of labeling in the cranial condensations (Fig. 1) is indicated by a minus sign for no labeling, a single plus sign for light labeling and a double plus sign for dense labeling.

regionally restricted with labeling in the neural tube, basal plate cartilage (basioccipital), columella and specific regions of the inner ear epithelium (Fig. 1A–F). The ventral pharyngeal endoderm of the oral cavity is also prominently PNA-labeled. SNA labels surface ectoderm, blood vessels and capillaries, restricted regions of the inner ear epithelium, the extracolumella, the quadrateme and Meckel’s cartilages (Fig. 1D–I). Although the quadrateme and Meckel’s cartilages are first pharyngeal arch derivatives, spatial rearrangement to integrate the various elements of the jaw and skull occur to form a functional unit. Double labeling with SNA and PNA (or DAPI) in the neural tube reveals that SNA labels the surrounding capillaries but are excluded from the floorplate itself (arrow, Fig. 1E,F).

Differential labeling by PNA and SNA distinguishes between columella, extracolumella and otic capsule condensation

The otic capsule, columella and extracolumella have different tissue origins, but all undergo endochondral ossification (Figs 2 and 3). The columella and extracolumella arise from rhombomere 4-derived neural crest cells, whereas the columella footplate has both neural crest and mesodermal contributions. Otic capsule is of triple origin – neural crest, head mesoderm and the first somite (Couly et al. 1993; Wood et al. 2010; Kumar et al. 2012; Ray & Chapman, 2015). The shared endochondral program means that, for example, prechondrogenic and chondrogenic cells express Sox9 and Col2a1, respectively, without regard for cell origin or the skeletal element in which they reside. Thus, endochondral gene markers are of little value in separating individual condensations. Cartilage cells synthesize and deposit the surrounding extracellular matrix, which are specific to the cartilage.

The nascent otic capsule cartilage surrounds the otic epithelium of the cochlear duct, the vestibule and the semicircular canals, later forming the bony labyrinth (Fig. 2). At E4, the otic epithelium is in a horizontal orientation, but repositions, turning in a more vertical position as the cochlear duct elongates and extends medio-ventrally during head growth (Fig. 2A–I). At E4, the prechondrogenic cells of the columella are selectively labeled by PNA (magenta), directly ventral to the otic mesenchyme and lateral to its future position between the blood vessels (Fig. 2A, arrowheads). PNA labels non-sialylated carbohydrates. By E5, PNA labeling displays the prechondrogenic cells present between the blood vessels (Fig. 2B). At E6, a prominent columella condensation, latero-ventral to the otic capsule, is observed (Fig. 2C,F). At this juncture, the columella footplate, the equivalent of the stapes footplate in mammals, will insert into the future oval window.

The nascent otic capsule (SNA, green) separates the columella from the otic epithelium. SNA recognizes sialylated carbohydrates. Between E4 and E5, prechondrogenic mesenchyme of the ventral part of the otic capsule, which is adjacent to the columella, is labeled by SNA (Fig. 2D–I). Similarly, the prominent otic capsule condensation is visible by SNA labeling at E6 (Fig. 2F,I). Additionally, at E4, SNA labeling highlights a number of capillaries between the two large vessels in the brain and highlights small capillaries present in the future columella region. The large vessels are the anterior cardinal vein (lateral at E4, but dorsolateral by E6) and the internal carotid (medial at E4, but ventro-medial at E6) that flank the columella (Fig. 2A–C). By E5, the small capillaries remodel and are located in the surrounding columella perichondrium, and are only occasionally observed within the condensation (Fig. 2D–I,F). WGA labels tissues in the chick embryonic head indiscriminately between E4 and E6 (Fig. 2A–C).

We confirmed our findings and the distinctive lectin-binding properties of these condensations by double labeling of PNA and SNA (Fig. 2D–F). As expected, the common chondrogenic precursor cells of the columella and possibly more distal hyoid skeletal elements were marked by PNA at E4 (Fig. 2D). By E5, a condensed extracolumella is noticeable by SNA labeling and the columella and the otic capsule cells are visible at their respective positions by PNA and SNA labeling, respectively (Fig. 2E). At E6, it is evident that columella condensation is labeled by PNA and the otic capsule and the extracolumella condensation are labeled by SNA labeling (Fig. 2F). Note that the medial and dorsolateral parts of the otic capsule are not labeled by either SNA or PNA.

Next, we focused on the developmental progression of the extracolumella prechondrogenic cells (Fig. 3). WGA labels non-specifically throughout the head tissue. The extracolumella is seen as yellow WGA labeling, but this is not specific, rather it is due to the lack of PNA (Fig. 3A–C). SNA labels the extracolumella condensation, evident at E5 and E6 (Fig. 3E,F,H,I). The extracolumella condensation is not specifically labeled by PNA at E4; there appears to be a general PNA labeling of a common precursor pool of the second arch skeletal elements (Fig. 3A,D). It is likely that
when the extracolumella condenses at E5, the secreted carbohydrates have sialic acid modifications, which are now recognized by SNA, but not PNA labeling. Further, it is noteworthy that the future joint region between columella and the extracolumella does not have a sharp boundary at E6, rather there is some intermixing of PNA- and SNA-labeled cells (Fig. 2F’).

The otic epithelium shows differential lectin labeling

Finally, we analyzed lectin labeling within the otic epithelium (Fig. 4, Table 3). The inner ear arises from the otic placode situated on either side of the hindbrain and is innervated by neurons of the acoustic-vestibular ganglion. It comprises vestibular and auditory components that are required for coordinated balance and hearing. In the vestibular system, the sensory epithelium comprises several regions – the superior, lateral and posterior cristae within the semicircular canals and, in the vestibule, the maculae of the saccule and the utricle. The utricle houses the macula neglecta and the lagenar resides in the most distal region of the cochlea. In the auditory system, the sensory epithelium consists of basilar papilla in the cochlear duct. Non-sensory regions include tegmentum vasculosum (lateral cochlear wall), the border regions (distal, proximal and intersensory), endolymphatic duct, and three semicircular canals (Wu & Oh, 1996; Sienknecht & Fekete, 2008). Characteristic polarity and spatial organization of cells and ECM differentiate between sensory, non-sensory and inter-sensory regions in the inner ear (Brigande et al. 2000; Lewis & Davies, 2002; Barald & Kelley, 2004; Kelly & Chen, 2007). Therefore we hypothesized that differential lectin binding to the surface glycans will successfully distinguish between the different subregions of the otic epithelium.
Wheat germ agglutinin is non-specific within the inner ear epithelium between E4 and E6 (Fig. 4A–C); however, it does specifically label the basal lamina that separates the otic epithelium from the endolymph-filled lumen of the cochlear duct and semicircular canals. The basal lamina on the outer surface of the otic epithelium is both WGA- and SNA-positive (Fig. 4). PNA widely labels the inner ear epithelium between E4 and E6 (Fig. 4A–F). SNA is a marker of blood vessels and capillaries (Fig. 4D–I) and highlights the plentiful capillaries lining the endolymphatic duct (Fig. 4I). Interestingly, the medial otic capsule is PNA-positive, in contrast to the dorsolateral otic capsule, which is SNA-positive (Fig. 4D–F). Within the otic epithelium, SNA marks the basilar papilla, lagena macula, utricular macula and lateral crista (Fig. 4F,F’,I) but is excluded from the lateral cochlear wall and intersensory regions proximal and distal to the basilar papilla (Fig. 4F’). SNA labeling is also absent from the acoustic-vestibular ganglion (Fig. 4F’).

Discussion

Condensation is a crucial event during endochondral ossification characterized by dynamic cytoskeletal reorganization and ECM modulation. Mesenchymal condensations with distinct cellular origins and divergent developmental fates express a common subset of transcriptional markers, which makes it difficult to identify and characterize individual condensations (Hall & Miyake, 2000; Eames & Helms, 2004; Szabo-Rogers et al. 2010; Wood et al. 2010; Ray & Chapman, 2015). Here, we show that differential labeling by lectins successfully distinguishes between the unique glycosylation-signature of different cartilage condensations in the chick embryonic head.

Glycosylation is a critical process during development that regulates cell–cell and cell–ECM (extracellular matrix) adhesion, cell migration, cell recognition, proliferation and apoptosis. The cell surface of each tissue has a unique glycosignature, which occurs as a result of posttranslational or co-translational modification based on phenotype and transcriptional profile. It is also dependent on interaction with the local ECM that carries its own glycosignature (Poirier & Kimber, 1997; Collin et al. 2016). Evidence suggests that sialylation of carbohydrates is a universal mechanism by which adjacent tissues sort out and remodel during hierarchical tissue organization in embryogenesis and postnatal development. Several pathological conditions and mouse mutant models such as undulated (Pax-1 mutant), splotch (Pax-3 mutant), and others...
Fig. 4 The inner ear neuroepithelia is distinguished by lectin labeling. (A–I) 20x magnification images of the inner ear epithelium in transverse section from E4 to E6. (D') 60x (F') 40x. (A–C) WGA/PNA, (D–F) SNA/PNA, (G–I) SNA/DAPI. (A–C) WGA (yellow) labels the basal lamina separating the otic epithelium from the endolymph-filled cavity of the inner ear and externally from the otic capsule. (D–F, F') SNA similarly labels the basal lamina, but only on the exterior of the epithelium. Dual PNA and SNA labeling show differential labeling with SNA highlighting the saccular macula, sensory basilar papilla of the cochlear duct and lagena macula. Note the particularly strong labeling of the vessels lining the endolymphatic duct. PNA labels the nerve fibers of the acoustic-vestibular ganglion and the medial otic capsule. (D') 60x magnification showing the basilar papilla and lagena macula separation at E4. (D'', D''') Enlargements of the basilar papilla and lagena macula showing different cellular arrangements with and without nerve fibers, respectively. (G–I) SNA differentiates the sensory utricular macula, basilar papilla and lagena macula from the endolymphatic duct, interboundary regions, lateral pouch and lateral wall of the cochlear duct. (J) Illustration depicting three-dimensional form of middle ear, otic capsule and inner ear region in chick embryo at E6 with regional SNA and PNA labeling. ap, axonal projections; avg, acoustic-vestibular ganglion; bp, basal papilla; c, columella; D, dorsal; hp, horizontal canal pouches; ib, intersensory basal papilla; lc, lateral cristae; M, medial; nslag, non-sensory lagena; oc, otic capsule; se, surface ectoderm; sm, saccular macula; tv, tegmentum vasculosum; um, utricular macula; v, blood vessel; vp, vertical canal pouches.
Lectin tissue labeling, P. Ray et al.

Table 3 Lectin labeling of the inner ear epithelium.

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BP, basilar papilla; CD, cochlear duct; ED, endolymphatic duct; LC, lateral canal; LM, lagena macula; PNA, peanut agglutinin lectin; SAG, statoacoustic ganglion; SM, saccular macula; SNA, Sambucus nigra agglutinin lectin; UM, utricular macula; WGA, wheat germ agglutinin lectin.

The intensity of labeling in the inner ear epithelium (Fig. 4) is indicated by a minus sign for no labeling, a single plus sign for light labeling and a double plus sign for dense labeling.

mutant) and brachypod exhibit altered glycosylation and lectin-binding properties in developing cartilages and other tissues (Quondamatteo et al. 2000). A key finding of our study is that adjacent mesenchymal condensations during endochondral ossification are discriminated by sialic acid modifications of glycoconjugates. Interestingly, this is reminiscent of complementary binding patterns of PNA and LFA (Limus flavus lectin) in the stratified adult epidermis where PNA recognizes the suprabasal keratinocytes, whereas LFA recognizes the adjacent basal keratinocytes based on the presence of sialic acid residues (Watt et al. 1989).

Sialic acids are negatively charged carbohydrate molecules typically attached to the terminal positions of N- and O-linked oligosaccharides by α-2,6 linkage or α-2,3 linkage on cell surfaces or secreted glycoproteins (Morgenthaler et al. 1990; Wang, 2012). Glycation and sialic acid modification are key regulators of chondrogenesis. The α-2,6 sialic acid is commonly found in healthy primary chondrocytes found in the mature hyaline and articular cartilages and is characterized by strong SNA binding. In contrast α-2,3 sialic acid is associated with immature mesenchymal stem cells, osteoclastogenesis, cartilage matrix breakdown, inflammation, dedifferentiation induced by interleukin (IL)-1β, tumor necrosis factor (TNF)-α and pathophysiological conditions such as osteoarthritis (Toegel et al. 2010; Talaei-Khozani et al. 2011; Collin et al. 2016). The α-2,6 sialic acid promotes stability of adhesion molecules such as E-cadherin, NCAM and integrin β1, and strengthens cell–cell and cell–ECM adhesion and decreases cell migration. Conversion of α-2,6 to α-2,3 linkage is often associated with increased cell migration not only in chondrocytes but also in highly invasive cancer types such as pancreatic adenocarcinoma (Basaganas et al. 2014). Sialic acid modification also regulates the migration of mesenchymal stem cells in the neighboring synovium during the repair of articular cartilage (Tan et al. 2015). Therefore, distinct carbohydrate signatures, especially in terms of sialic acid modification, could provide insight into remodeling of prechondrogenic regions into distinct condensations during endochondral ossification. Complementary labeling by SNA and PNA suggests that adjacent prechondrogenic condensations could have a distinct glycosignature due to differential expression of cell adhesion molecules or different ECM. This conjecture remains to be verified. Differential glycosignature possibly leads to differential sorting or migration of prechondrogenic cells resulting in tissue reorganization, which will then undergo progressive cell shape changes in order to form condensations. The origin of differential glycosignatures is unknown, but could be due to the inherent heterogeneity of neural crest cells or local patterning.

It has been argued that differential lectin labeling is related to the developmental origin, fate and functionality of the cartilage. This idea is supported by the fact that PNA preferentially binds to limb condensation in mouse embryos, but not the nasal septum or Meckel’s cartilage with the mandible (Sasano et al. 1992). Our study in closely apposed tissues shows that lectin labeling and sialic acid modification are independent of cell fate or cell origins. For example, extracolumella (persistent cartilage) and otic capsule (replacement cartilage) are both recognized by SNA. Similarly, columella and extracolumella condensations, both of which primarily originate from neural crest cells, are differentially labeled by PNA and SNA, respectively. Surprisingly, the dorsolateral part of the otic capsule is not recognized by SNA and is weakly labeled by PNA, in contrast to the medial and ventro-lateral parts, which are strongly labeled by PNA and SNA, respectively. We speculate that those cells possibly have very different glycoprofiles due to local patterning (Wood et al. 2010; Kumar et al. 2012). We propose that differential lectin labeling could be used universally within the embryo to distinguish between distinct cartilage condensations.

Moreover, it is important to note that lectin labeling and the corresponding glycosignature of a particular cartilaginous element remains consistent between the condensation and overt chondrogenic differentiation phases (Table 4). We have examined the temporal variation in lectin labeling between the prechondrogenic, condensation and overt chondrogenic stages of columella, extracolumella and otic capsule, respectively (Table 4). Our results demonstrate that the glycosylation profile of each element remains constant throughout the early phases of endochondral ossification. Therefore, it appears that the unique glycosignature is a fundamental property of cells fated to become chondrocytes in a particular spatial location irrespective of the temporal regulation of the early chondrogenic program.

Previous studies have suggested that the removal of blood vessels is essential to the formation of cartilage condensations, as cartilage is devoid of blood vessels, unlike other connective tissues (Maini & Solursh, 1991). We observe small capillaries intermixed with the prechondrogenic cells that gradually disappear as condensation progresses and are absent in mature condensations. How this happens is unknown, but two studies indicate that tensile forces originating in the condensing mesenchyme restrict
and eventually cut off the thin-walled undifferentiated vessels within condensation centers, generating avascular cartilage rudiments (Wilson, 1986; Hallmann et al. 1987).

The neural tube floorplate consists of non-neuronal cells located in the ventral midline of the neural tube and is essential for dorsoventral patterning of the neural tube and axonal guidance. The floorplate is the source for chemotactic signaling molecules such as R-spondin and Netrin-1, which coordinate combinatorial guidance of commissural axons. R-Spondin is an ECM protein that recognizes and binds to glycosaminoglycan/proteoglycan residues on cell surface (Zisman et al. 2007). Therefore, preferential labeling of the footplate by PNA indicates an abundance of non-sialylated GalNAc residues on the cell surface and ECM of floorplate, which helps in the signal transduction of the secreted chemotactic proteins such as R-Spondin during neural development. This is supported by the fact that GalNAc-specific lectins such as *Wisteria floribunda* lectin (WFA) recognize the floorplate strongly in the developing spinal cord of mouse embryos (Vojoudi et al. 2015). Interestingly, SNA only marks the capillaries on the outside margin of the floorplate and recognizes the outer border of the floorplate weakly. This may signify differential posttranslational modification of the GAGs/proteoglycans on the periphery of the floorplate, which helps in the signal transduction of the secreted chemotactic proteins such as R-Spondin during neural development. This is supported by the fact that GalNAc-specific lectins such as *Wisteria floribunda* lectin (WFA) recognize the floorplate strongly in the developing spinal cord of mouse embryos (Vojoudi et al. 2015). Interestingly, SNA only marks the capillaries on the outside margin of the floorplate and recognizes the outer border of the floorplate weakly. This may signify differential posttranslational modification of the GAGs/proteoglycans on the periphery of the floorplate, which helps in the signal transduction of the secreted chemotactic proteins such as R-Spondin during neural development.

We found that lectins are successful in defining specific regions in the otic epithelium. There are seven distinct sensory regions that originate from common prosensory domains sharing common markers, including *BMP4*, *Ser1* and *Msx1* (Wu & Oh, 1996; Adam et al. 1998; Brigande et al. 2000; Cole et al. 2000; Sanchez-Calderon et al. 2004; Neves et al. 2011; Magarinos et al. 2012; Olaya-Sanchez et al. 2016). SNA labeling is an effective marker of nascent sensory regions of the otic epithelium and closely resembles the *Msx1* expression domain, which marks not only the sensory organs but also the early endolymphatic duct. PNA is a selective stereocilia bundle marker in the postnatal inner ear (Zheng & Gao, 2000). PNA also highlights the nerve fibers of the statoacoustic ganglion (SAG). Peripheral growth cones from the SAG sense molecular signals to identify and navigate to their sensory targets. In the chicken, bipolar neurons located within the SAG enervate the mechanosensory hair cells of the otic epithelium (Fantetti et al. 2011). Sensory regions secrete diffusible chemo-attractants and repellants for axon guidance. Therefore, it makes sense that the SAG and the sensory regions have complementary glycosignatures, essential for neuronal navigation. Our study also demonstrates that lectin, especially WGA, is a marker of apico-basal polarity of the otic epithelium. This is in agreement with previous findings that lectins such as SNA differentiate between apical and basal region bronchiolar epithelium in mice (Quondamatteo et al. 2000; Lewis & Davies, 2002; Barald & Kelley, 2004).

In conclusion, lectin labeling successfully distinguishes between adjacently located cartilage condensations in the embryonic head and is an effective method for universal recognition of tissues with unique glycosignatures despite common molecular pathways during development.

## Acknowledgements

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**Table 4** Lectin labeling during different stages of early endochondral ossification of the otic cartilages.

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<td>Prechondrogenic progenitors positive for PNA in a more ventrolateral position</td>
<td>PNA Positive (Condensation)</td>
<td>PNA Positive (Overt Chondrogenesis)</td>
<td>PNA Positive (Overt Chondrogenesis)</td>
</tr>
<tr>
<td>Extracolumella</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Overt Chondrogenesis)</td>
</tr>
<tr>
<td>Otic Capsule</td>
<td>–</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Condensation)</td>
<td>SNA Positive (Condensation)</td>
</tr>
</tbody>
</table>

HH, Hamburger and Hamilton stage series; PNA, peanut agglutinin lectin; SNA, *Sambucus nigra* agglutinin lectin; WGA, wheat germ agglutinin lectin.

Bold font indicates the beginning of the condensation or the overt chondrogenesis stage in the columella, otic capsule and extracolumella cartilages.

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and eventually cut off the thin-walled undifferentiated vessels within condensation centers, generating avascular cartilage rudiments (Wilson, 1986; Hallmann et al. 1987).

The neural tube floorplate consists of non-neuronal cells located in the ventral midline of the neural tube and is essential for dorsoventral patterning of the neural tube and axonal guidance. The floorplate is the source for chemotactic signaling molecules such as R-spondin and Netrin-1, which coordinate combinatorial guidance of commissural axons. R-Spondin is an ECM protein that recognizes and binds to glycosaminoglycan/proteoglycan residues on cell surface (Zisman et al. 2007). Therefore, preferential labeling of the footplate by PNA indicates an abundance of non-sialylated GalNAc residues on the cell surface and ECM of floorplate, which helps in the signal transduction of the secreted chemotactic proteins such as R-Spondin during neural development. This is supported by the fact that GalNAc-specific lectins such as *Wisteria floribunda* lectin (WFA) recognize the floorplate strongly in the developing spinal cord of mouse embryos (Vojoudi et al. 2015). Interestingly, SNA only marks the capillaries on the outside margin of the floorplate and recognizes the outer border of the floorplate weakly. This may signify differential posttranslational modification of the GAGs/proteoglycans on the periphery of the floorplate, creating a sharp boundary between the floorplate and the adjacent neuroepithelial cells. Chondrocytes in the adult vertebral column are labeled by PNA, indicating that the same glycosignature is conserved between embryonic basal plate and postnatal mature chondrocytes of the vertebral column (Collin et al. 2016).

We found that lectins are successful in defining specific regions in the otic epithelium. There are seven distinct sensory regions that originate from common prosensory domains sharing common markers, including *BMP4*, *Ser1* and *Msx1* (Wu & Oh, 1996; Adam et al. 1998; Brigande et al. 2000; Cole et al. 2000; Sanchez-Calderon et al. 2004; Neves et al. 2011; Magarinos et al. 2012; Olaya-Sanchez et al. 2016). SNA labeling is an effective marker of nascent sensory regions of the otic epithelium and closely resembles the *Msx1* expression domain, which marks not only the sensory organs but also the early endolymphatic duct. PNA is a selective stereocilia bundle marker in the postnatal inner ear (Zheng & Gao, 2000). PNA also highlights the nerve fibers of the statoacoustic ganglion (SAG). Peripheral growth cones from the SAG sense molecular signals to identify and navigate to their sensory targets. In the chicken, bipolar neurons located within the SAG enervate the mechanosensory hair cells of the otic epithelium (Fantetti et al. 2011). Sensory regions secrete diffusible chemo-attractants and repellants for axon guidance. Therefore, it makes sense that the SAG and the sensory regions have complementary glycosignatures, essential for neuronal navigation. Our study also demonstrates that lectin, especially WGA, is a marker of apico-basal polarity of the otic epithelium. This is in agreement with previous findings that lectins such as SNA differentiate between apical and basal region bronchiolar epithelium in mice (Quondamatteo et al. 2000; Lewis & Davies, 2002; Barald & Kelley, 2004).

In conclusion, lectin labeling successfully distinguishes between adjacently located cartilage condensations in the embryonic head and is an effective method for universal recognition of tissues with unique glycosignatures despite common molecular pathways during development.

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Author contributions
S.C.C. conceived the project, oversaw data acquisition and analysis and drafted the manuscript. P.R. acquired and interpreted the data and drafted the manuscript. A.J.H. and M.S. acquired and interpreted the data.

References


