Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant

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SUMMARY

Mechanosensory hair cells in the sensory patches of the vertebrate ear are interspersed among supporting cells, forming a fine-grained pattern of alternating cell types. Analogies with Drosophila mechanosensory bristle development suggest that this pattern could be generated through lateral inhibition mediated by Notch signalling. In the zebrafish ear rudiment, homologues of Notch are widely expressed, while the Delta homologues deltaA, deltaB and deltaD, coding for Notch ligands, are expressed in small numbers of cells in regions where hair cells are soon to differentiate. This suggests that the delta-expressing cells are nascent hair cells, in agreement with findings for Delta1 in the chick. According to the lateral inhibition hypothesis, the nascent hair cells, by expressing Delta protein, would inhibit their neighbours from becoming hair cells, forcing them to be supporting cells instead. The zebrafish mind bomb mutant has abnormalities in the central nervous system, somites, and elsewhere, diagnostic of a failure of Delta-Notch signalling: in the CNS, it shows a neurogenic phenotype accompanied by misregulated delta gene expression. Similar misregulation of delta genes is seen in the ear, along with misregulation of a Serrate homologue, serrateB, coding for an alternative Notch ligand. Most dramatically, the sensory patches in the mind bomb ear consist solely of hair cells, which are produced in great excess and prematurely; at 36 hours post fertilization, there are more than ten times as many as normal, while supporting cells are absent. A twofold increase is seen in the number of otic neurons also. The findings are strong evidence that lateral inhibition mediated by Delta-Notch signalling controls the pattern of sensory cell differentiation in the ear.

Key words: Delta, Notch, Serrate, Ear, mind bomb, Hair cell, Lateral inhibition, Zebrasfish

INTRODUCTION

The mechanosensory receptor cells of the inner ear – the hair cells – lie in specialised patches in the inner ear epithelium. Each such sensory patch consists of a fine-grained mixture of hair cells and supporting cells. An individual hair cell within a patch is generally surrounded by supporting cells, which isolate it from the next hair cell (Goodyear and Richardson, 1997); in some cases, as in the mammalian organ of Corti or the avian basilar papilla, the arrangement is remarkably regular, with the hair cells interspersed among the supporting cells in a precise periodic spacing pattern. Hair cells and supporting cells arise from a common precursor (Stone and Cotanche, 1994; Weisleder et al., 1995; Jones and Corwin, 1996). The challenge, therefore, is to explain what causes cells that are sisters or cousins, sitting side by side in the same environment, to differentiate in contrasting ways.

Lateral inhibition provides one possible mechanism: each cell that begins to differentiate as a hair cell might deliver a signal to its immediate neighbours in the developing sensory patch, inhibiting them from differentiating in the same way, with the result that they differentiate as supporting cells instead (Corwin et al., 1991; Lewis, 1991). Studies in Drosophila, in C. elegans, and more recently in vertebrates have shown that signalling of this sort controls cell differentiation in several different tissues, where it is mediated by the transmembrane protein Notch, acting as signal receptor, and the transmembrane protein Delta, acting as ligand (Artavanis-Tsakonas et al., 1995; Lewis, 1996). In the central nervous system, for example, nascent neural cells express Delta and thereby inhibit their neighbours, which express Notch, from simultaneously differentiating along a neural pathway (Campos-Ortega, 1993; Chitnis et al., 1995). The lateral inhibition is thought to operate competitively because of a feedback loop that tends to amplify any initial difference between neighbouring cells: a cell that expresses Delta more strongly activates Notch more strongly in its neighbours; and activation of Notch in these cells inhibits not only their differentiation, but also their expression of Delta, thereby reducing their ability to deliver lateral inhibition in return (Heitzler and Simpson, 1991; Ghyset al., 1993; Sternberg, 1993; Wilkinson et al., 1994; Chitnis, 1995). In vertebrates, this type of regulation occurs at a transcriptional level: blockade of Delta-Notch signalling by the dominant-negative
construct Delta^dn leads to a dramatic up-regulation of endogenous Delta expression, while forced overexpression of exogenous Delta has the opposite effect (Chitnis et al., 1995; Haddon et al., 1998). Delta-Notch-mediated cell-cell interactions with this type of feedback regulation of Delta are sufficient in principle to generate a regular spacing pattern of cell types (Collier et al., 1996).

The sensory bristles of Drosophila provide one of the best-characterised examples of lateral inhibition mediated by the Notch signalling pathway (Hartenstein and Posakony, 1990; Ghysen et al., 1993; Parks and Muskavitch, 1993; Jan and Jan, 1995; Zeng et al., 1998): the four cell types that form the bristle derive from a single sensory mother cell, and signalling via Notch, with Delta and the Delta-related protein Serrate acting in parallel as ligands, is necessary to cause them to adopt their different fates. In the accompanying paper (Adam et al., 1998), we point out a detailed parallel between insect bristles and the sensory patches of the vertebrate inner ear. In particular, we show in the chick embryo that Delta, Serrate and Notch homologues are expressed in the ear with a timing and pattern suggesting that they mediate lateral inhibition in the developing sensory patches: C-Delta1 is expressed in scattered cells that appear to be nascent hair cells, as expected if lateral inhibition from each hair cell serves to force its neighbours to be supporting cells rather than hair cells. C-Serrate1, meanwhile, is expressed more uniformly within the sensory patch, and its product may act there as an additional ligand for Notch.

For further evidence that lateral inhibition indeed controls the differentiation of hair cells and supporting cells, we have turned to the ear of the zebrafish (Haddon and Lewis, 1996). We have previously identified four fish homologues of Delta, called deltaA, deltaB, deltaC and deltaD, and analysed their role in governing genesis of primary neurons in the early central nervous system: deltaA, deltaB and deltaD are all expressed strongly in the nascent neurons, all deliver lateral inhibition, and all are subject to feedback regulation of the type described above (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). Because the ear develops relatively late, it is difficult to test the function of these genes in the ear by the simple method of RNA injection into an early blastomere. Zebrafish mutants, however, provide an alternative approach.

In both the Tübingen and the Boston zebrafish mutagenesis screens, mutant embryos were encountered that show a neurogenic phenotype, analogous to that seen in Drosophila embryos with mutations in the Delta-Notch signalling pathway (Jiang et al., 1996; Schier et al., 1996). The mutant gene was named white tail (wit) in Tübingen, and mind bomb (mib) in Boston, but complementation testing has shown that the mutations are allelic. mind bomb takes precedence as the official gene name. In the neural plate of mib mutants, neurons are produced in the normal regions, but in greatly excessive numbers, and contiguous with one another instead of being separated by intervening non-neuronal cells (Jiang et al., 1996): the pattern is similar to that seen in embryos where Delta-Notch signalling is blocked by injection of RNA coding for the dominant-negative Delta construct Delta^dn (Appel and Eisen, 1998; Haddon et al., 1998). mib mutations also cause abnormalities in other tissues where Delta-Notch signalling is thought to have a role, notably the somites, which likewise show disturbances of segmentation that match those seen in embryos where Delta-Notch signalling is known to be blocked or defective (Conlon et al., 1995; Hrabé de Angelis et al., 1997; Jen et al., 1997). All these observations suggest that mib codes for a component that is required for Delta-Notch signalling.

If the mib mutation corresponds to a failure of Delta-Notch signalling, and Delta-Notch signalling is needed to control the hair-cell/supporting-cell decision, we should expect to see a disturbance in the ratio of hair cells to supporting cells in the sensory patches of the mutant ear. In this paper we test this prediction.

MATERIALS AND METHODS

Fish rearing and embryo culture
Zebrafish embryos were obtained by natural spawnings and maintained at 28.5°C in system water. Several wild-type strains were used, but comparisons between mutant and wild-type phenotypes are based in all cases on sibling embryos produced from matings between heterozygotes. The allele of mind bomb described in this paper, referred to throughout simply as mib, is the ta52b (white tail) allele from Tübingen (Jiang et al., 1996). It is the strongest of the five known alleles, as judged by the severity of the defects in pigmentation and somite segmentation, and may thus be a null mutation. Embryos were staged according to Kimmel et al. (1995); embryonic ages are given in hours post fertilization (hpf) at 28.5°C

Semi-thin resin sections
Embryos were fixed in BT fix (Westerfield, 1995) at 4°C overnight, embedded in Araldite (Agar Scientific Ltd), sectioned at 2 µm and stained with toluidine blue.

Cloning and sequencing of Serrate homologues
A fragment of a Serrate homologue, cloned by PCR as described by Haddon et al. (1998), was used to screen a 15- to 19-hour zebrafish λZAP II cDNA library (a gift from Bruce Appel), yielding clones corresponding to the zebrafish gene we have called serrateB. By probing the same library with a C-terminal fragment of the chick C-Serrate1 gene (Myat et al., 1996), we discovered an additional zebrafish Serrate homologue, serrateA. cDNAs were excised from the vector using Stratagene’s Rapid Excision Kit. Double-stranded sequencing was performed in both directions using an ABI PRISM system. Sequence comparisons were made using the Wisconsin GCG Gap program.

In situ hybridisation
Digoxigenin RNA antisense probes were made using Stratagene’s RNA transcription kit. Whole-mount in situ hybridisation was essentially as described by Oxtoby and Jowett (1993); in situ hybridisation on cryosections followed Strähle et al. (1994) with minor modifications. Probes for delta genes were as described by Haddon et al. (1998). serrateB was linearised with XbaI and transcribed with T7.

Whole-mount phalloidin and antibody staining
To visualise and count hair bundles, fixed intact embryos were stained with FITC-phalloidin, and in some cases counterstained with the red fluorescent nuclear dye 7AAD, and viewed by confocal microscopy, as described by Haddon and Lewis (1996).

To identify neurons, whole-mount embryos were stained with anti-islet-1 antibody (1:500) with HRP detection, as in Hammerschmidt and Nüsslein-Volhard (1993). This antibody recognises a LIM homeobox-containing nuclear protein expressed by early differentiating neurons (Korzh et al., 1993).
Mounting and imaging

For photography, live embryos were anaesthetised in 0.2-0.5 mM MS222 (Sigma) and immobilised in 1.2% low-melting point agarose (BioRad). Fixed and stained embryos were mounted in glycerol under a coverslip supported at its corners by high-vacuum grease, or were dehydrated in methanol and cleared in 2:1 benzyl benzoate: benzyl alcohol solution before mounting. Images were edited using Adobe Photoshop.

Single-cell labelling and fate mapping

Cells within the ear were labelled by iontophoretic injection. Clark 1.2 mm glass capillaries with an internal filament were pulled to make injection needles. Needles were back-filled by capillary action with lysinated rhodamine dextran (Mr 10x10^3; Molecular Probes; 5% in 200 mM KCl). Embryos were anaesthetised and mounted in low-melting point agarose, surrounded by Ringer’s saline. The needle was positioned using a micromanipulator whilst monitoring with fluorescence optics, until contact was made in the ear epithelium. An oscillating voltage was then applied to the needle using a microelectrode amplifier (BioLogic); any cell touching the needle tip became labelled. The position of labelled cells was immediately recorded using a confocal microscope (BioRad MRC600). Embryos were then released and re-mounted in agarose 24 hours later, and again after 48 hours, to determine the location of the labelled cell or cells, using the confocal microscope as before.

RESULTS

Expression of delta genes foreshadows hair-cell differentiation in the normal ear

Differentiated hair cells, identifiable by their stereociliary bundles, are first seen in the zebrafish ear at about 24 hours post fertilization (hpf); they arise in two small patches, at the anterior and posterior ends of the otocyst respectively (Haddon and Lewis, 1996; Riley et al., 1997). These patches, corresponding to the maculae of the future utricle and saccule, then enlarge by addition of new hair cells and supporting cells, a process that probably continues throughout life (Platt, 1993; Haddon and Lewis, 1996).

We have used in situ hybridisation to examine how hair-cell production is related to the expression of deltaA, deltaB and deltaD in the ear epithelium. The expression patterns are similar for all three genes. Already at the 10-somite stage (14 hpf), when the otic placode is just becoming visible, expression is seen in two small groups of cells at its anteromedial and posteromedial ends, and this pattern persists over the next few hours as the placode becomes converted into a vesicle (Fig. 1, upper panels).

To discover the fate of cells at the early sites of delta expression in the ear, iontophoretic injections of fluorescent dextran were made into cells at these two sites, and the positions of the labelled cells were followed by confocal microscopy over the next 48 hours, as shown in Fig. 2. As the ear enlarges, cells at the labelled sites become positioned within the two sensory maculae: anteromedial cells end up in the anteroventral (utricular) macula, posteromedial cells end up in the posteromedial (saccular) macula.

In situ hybridization on sections through the ear at later stages reveals scattered delta-expressing cells in the thickened ventral areas of epithelium corresponding to the developing sensory patches (Fig. 3). Thus the pattern of expression of delta genes foreshadows both the pattern of production of the very first hair cells, and that of the hair cells that are added later, suggesting that the cells expressing the genes in the sites described above may be prospective hair cells. In other systems, delta expression is transient, switching off as overt differentiation begins (Muskavitch, 1994; Chitnis et al., 1995; Henrique et al., 1995; Adam et al., 1998). The same seems to be true here: expression is generally not seen in the mature differentiated regions of the developing sensory patches, although deltaB-expressing cells in the hair-cell layer are encountered occasionally (Fig. 3C) (similarly, deltaB persists for a little while in differentiating neurons; Haddon et al., 1998).

Expression of serrateB at first resembles that of the delta genes and then becomes restricted to the hair-cell layer

In chick and rodent embryos, the developing sensory patches in the ear are sites of expression of homologues not only of Delta, but also of the Delta-related gene Serrate (Hayashi et al., 1996; Lindsell et al., 1996; Myat et al., 1996). Is the same true in the zebrafish?

We have found two zebrafish Serrate homologues (see Materials and Methods) which we call serrateA (serA) and
serrateB (serB). We have determined the full cDNA sequence of one of these, serrateB (GenBank accession number AF090432) and analysed its expression pattern. In amino-acid sequence, SerrateB appears slightly more closely related to chick Serrate2 (63% identity) than to chick Serrate1 (55% identity). Preliminary comparisons, based on partial sequence data, indicate that SerrateA may be more closely related to Serrate1.

At early stages of ear development, before overt differentiation of hair cells, the expression pattern of serrateB appears similar to that of deltaA, deltaB and deltaD: there are two patches of expression, at the anterior and posterior ends of the ear rudiment (Fig. 1). At later stages, however, up to at least 4 days (Fig. 3), expression of serrateB, in contrast with delta gene expression, persists in the differentiated hair cells.

**Overexpression of delta genes in mib indicates a failure of Delta-Notch signalling**

If the mib mutation causes a failure of Delta-Notch signalling, as hypothesized, we might expect to see abnormalities in the pattern of delta gene expression, because of the feedback regulation of delta genes, discussed in the Introduction: we know in the central nervous system, at least, that an artificial blockade of activity in the Notch pathway by exogenous dominant-negative Delta leads to a striking increase of endogenous delta expression (Haddon et al., 1998). Fig. 1 shows that in the early mib ear rudiment the delta genes are indeed expressed more intensely and in an increased number of cells. serrateB likewise shows increased expression (Fig. 1), strongly suggesting that it too is normally subject to inhibition by Notch activity.

The same figure also shows, incidentally, a similar upregulation of deltaA and deltaB expression in the mib neural tube, and the same phenomenon is seen for all three genes at earlier stages in the neural plate, during genesis of primary neurons (Fig. 4). This is further strong evidence that mib has a defect in the Delta-Notch signalling pathway. In wild-type embryos, artificial overexpression of deltaA, B or D inhibits production of primary neurons (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998); in mib, all three genes are overexpressed, and yet primary neurons are produced in excess (Jiang et al., 1996; Schier et al., 1996). The mib cells therefore must be refractory to the effects of the delta gene products.
In *mib*, hair cells are produced in great excess at the expense of supporting cells

The *mib* ear shows several abnormalities in its gross structure, as noted by Malicki et al. (1996). At 60 hpf, the ear is smaller than normal, the semicircular canal system is imperfectly formed, and the two otoliths have failed to enlarge (Fig. 5A,B). These appear as tiny granules, similar to those seen in the 24-hpf ear in association with the first-formed hair cells. Since the otoliths overlie the sensory maculae and are thought to be created, in part at least, from the secretions of supporting cells in the maculae (Fermin and Igarashi, 1986; Riley and Grunwald, 1996), this defect hints at a defect in the sensory patches themselves.

To reveal the underlying pattern of cell differentiation in the sensory patches, we examined the distribution of hair cells and supporting cells by labelling with fluorescent phalloidin (to reveal the actin-rich stereocilia) and anti-acetylated-tubulin (to reveal both the kinocilium and the hair cell cytoplasm; data not shown). In addition, some specimens were fixed, embedded in resin and analysed by conventional semi-thin sectioning.

In the wild-type zebrafish, hair-cell nuclei are found in the apical half of the thick sensory epithelium, sitting above a layer of supporting-cell nuclei, as shown in Fig. 5C,E,G for specimens at 28-48 hpf. The stereociliary bundles of the hair cells can be seen projecting into the ear lumen towards the overlying otolith. In *mib* mutants, the pattern is radically altered (Fig. 5D,F,H). All the cell nuclei in the sensory patch lie in the same layer, and all of them belong to hair cells, clearly identifiable by their hair bundles. Supporting cells are absent, and the number of hair cells is very much larger than normal. Thus hair cells appear to have been produced at the expense of supporting cells, just as primary neurons are overproduced at the expense of other cell types in the *mib* CNS.

To assess precisely how many extra hair cells develop in the mutant and over what time period, the numbers of hair bundles (seen by phalloidin staining) were counted (Fig. 6). In the wild
type, hair cell numbers increase rapidly at first, reaching a combined total for the two maculae of 30-40 at 48 hpf, and then rise more slowly, at a mean rate of about 11 hair cells per day. In mib, the early production is dramatically increased, so that by 36 hpf there are already an average of 110 – fifteen times as many as in the wild type at the same stage. By 48 hpf, however, the numbers in mib have begun to drop, and by 60 hpf there are no hair cells left in the ear epithelium. The process leading to their loss is shown in Fig. 5I,J: the mutant patches of hair cells bulge out from the ear epithelium into the underlying mesenchyme, pinch off from the ear vesicle and eventually disappear, presumably being devoured by macrophages. Hair cells without supporting cells evidently cannot survive for long.

In a normal embryo, additional patches of hair cells appear later, at about 60 hpf, forming the cristae. These may originate from the same ventral regions of thickened prospective sensory epithelium that contain the early rudiments of the utricular and saccular maculae (Iwasaki, 1937; Becerra and Anadón, 1993; Haddon and Lewis, 1996). In the mib ear, no such late-developing hair cells are seen: from 60 hpf onwards, hair cells are completely lacking, suggesting that the initial burst of overproduction has exhausted the supply of precursors for production of hair cells at later stages.

The number of neurons in the statoacoustic ganglion is doubled in mib

Our studies in the chick have suggested that Delta-Notch signalling may regulate not only the hair-cell/supporting-cell decision, but also the segregation of neuroblasts from the otic epithelium and the production of neurons from these neuroblasts in the statoacoustic ganglion. In normal zebrafish embryos, neuroblasts appear to delaminate from the ventral wall of the otocyst between 22 and 36 hours after fertilization, and differentiated neurons begin to be visible in the ganglion by 24-30 hours (Haddon and Lewis, 1996). Thus, in contrast with the chick, neurogenesis and sensory-patch differentiation occur concurrently in the zebrafish. The neurogenic region of the epithelium seems to overlap partially with the sites of the sensory patches, although the precise relationship remains to be determined. As in the chick, neurogenesis is associated with delta gene expression, which is visible both in regions of the otocyst that may correspond to sites of neurogenesis (see Fig. 1, legend) and in the developing statoacoustic ganglion (see Fig. 3A).

To see whether this pattern of gene expression reflects a role for Delta-Notch signalling in regulating otic neurogenesis, we have compared the numbers of neurons produced in wild-type and mib ears, using islet1 antibody staining as a neuronal marker (Fig. 7). For wild-type embryos at 26 hpf, we estimate from whole mounts that the number of islet-1-positive cells in the statoacoustic ganglion is 19.7±6.5 (mean ± s.d., n=22 ears); for mib embryos at that stage, it is 40.3±6.2 (mean ± s.d., n=18 ears). This twofold early excess in mib strongly suggests that normal otic neurogenesis is regulated at an early step by lateral inhibition, in agreement with the hypothesis.

DISCUSSION

We have argued that the mib mutation blocks Delta-Notch signalling, making cells deaf to lateral inhibition. The molecular nature of the mib gene remains to be discovered: genetic linkage experiments have so far allowed us only to exclude a number of possibilities, including the known zebrafish Notch homologues (Y.-J. J., unpublished). Meanwhile, the mutant provides a way to test the role of the Notch signalling pathway in the various tissues of the body. In
particular, it allows us to test the hypothesis that lateral inhibition mediated by Delta-Notch signalling controls production of the fine-grained pattern of hair cells and supporting cells in the sensory patches of the ear (Corwin et al., 1991; Lewis, 1991; Adam et al., 1998).

In mib mutants, all the cells in each patch differentiate as hair cells, indicating that Delta-Notch signalling is needed in normal development to force the neighbours of each hair cell to be supporting cells rather than hair cells. The absence of late-developing hair cells in the mutant suggests that Delta-Notch signalling is also needed to keep some cells competent to act as precursors for future hair cells, i.e. to maintain the sensory-patch stem-cell population that normally would persist to generate hair cells throughout life. This role is similar to the one we have demonstrated for Delta-Notch signalling in the chick retina (Henrique et al., 1997).

A caveat must, however, be stated. Homologues of the alternative Notch ligand Serrate are also expressed in the developing sensory patches of the ear, and the mib mutation may well affect their action as well as that of Delta. The misregulation of serrateB in mib (see Fig. 1) strengthens the suggestion that it may have a role in the control of hair cell differentiation in conjunction with the delta genes. Its persistence in the differentiated hair cells raises the possibility that it might play a part in stabilising the mature pattern of differentiation by keeping the supporting cells inhibited after delta expression has faded. Although the individual functions of the various Delta and Serrate homologues remain to be disentangled, the present findings further strengthen the parallel with bristle development in Drosophila, where signalling via Notch is required for cell diversification and Delta and Serrate act in parallel to deliver the signal (Zeng et al., 1998). It seems that information from Drosophila can indeed give insights into the development of the ear.

The finding that Notch signalling is necessary for genesis of the mixture of hair cells and supporting cells does not, of course, exclude a role for other factors in controlling the pattern. Molecules such as Numb, distributed unequally through asymmetric cell division, can bias the outcome of Delta-Notch-mediated interactions (Guo et al., 1996); cell movements can refine the spatial arrangement of the two cell types once they have been generated (Goodyear and Richardson, 1997).

The abnormalities in the gross structure of the mib ear may reflect additional roles for Notch signalling in the ear, or may be secondary to the defect in the sensory patches. The sensory patches are, for example, sites of expression of signalling molecules of the BMP family, which may be required for normal growth and global patterning (Oh et al., 1996; Takemura et al., 1996) It is also possible that the early reduction in the size of the otic vesicle in mib could reflect overproduction of delaminating neuroblasts at the expense of epithelial cells.

We have found a twofold increase in neuron production in the mib ear (see Fig. 7), but the precise role of Delta-Notch signalling in zebrafish ear neurogenesis remains to be clarified. In Drosophila sensory bristle development, extreme loss-of-function mutations in the Notch signalling pathway cause all the progeny of each sensory mother cell to develop as neurons, and no bristle shaft cells are produced (Hartenstein and Posakony, 1990). If neurons and hair cells in the fish ear derive in a similar way from a common ancestor, one might expect that in mib there should be a massive overproduction of neurons and no production of hair cells. There are many possible reasons why we do not see this. Neuroblasts and sensory epithelial cells may, for example, have separate origins in the otocyst; or their segregation, though dependent on Delta-Notch signalling, may be only mildly affected by the mib mutation, because of genetic redundancy.

Our findings in the ear are not only of interest from the point of view of ear development. Intimate mixtures of cells of contrasting types are produced from the progeny of common precursors in a wide range of vertebrate tissues, from the gut lining to the bone marrow, and it is still a mystery how such mixtures are generated. Homologues of Notch and its ligands are, however, known to be expressed in many of these sites (Gridley, 1997). The sensory patches of the ear provide one of the few examples in the vertebrate body where we can now point with reasonable confidence to a mechanism for driving such fine-grained patterns of cell diversification. The insights we gain from the ear may thus help us to understand other tissues.

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