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Single origin of the epithelium of the human middle ear

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ABSTRACT

Objective: The epithelium lining the human middle ear and adjacent temporal bone cavity shows a varying morphological appearance throughout these cavities. Its embryologic origin has long been debated and recently got attention in a newly proposed theory of a dual embryologic origin. The epithelial morphology and its differentiating capabilities are of significance in future mucosa-targeted therapeutic agents and could affect surgical approaches of the temporal bone. This study aims to analyze reported murine histological findings that led to the theory of a dual epithelial embryological origin and immunohistochemically investigate whether such an epithelial embryological origin in the human fetal middle ear could be true.

Methods: By combining a sagittal sectioning technique and immuno-histochemical staining, a comprehensive immuno-histological overview of the fetal human middle ear during a critical stage of tympanic cavitation was provided. A critical analysis of previously reported findings leading to the theory of a dual epithelial embryological origin and a comparison of these findings to the findings in the human fetal middle ear was performed.

Results: The reported findings and critical analysis provide multiple arguments for an entirely endodermal embryonic origin of the epithelium lining the tympanic cavity.

Conclusion: Different morphological epithelial appearances throughout the tympanic and temporal bone cavities could be explained by different stages of epithelial differentiation rather than different embryologic origin and endodermal rupture does not seem to be a necessity for these cavities to form.

1. Introduction

Since the 1960's it was generally accepted that the epithelial layer lining the tympanic and mastoid cavity has an endodermal origin. The respiratory-like epithelium was considered an extension of the upper respiratory tract as it was thought to develop as a consequence of the invagination of the first pharyngeal pouch. In the 1980's a mesodermal origin was also proposed (Hentzer, 1984) and recently a possible dual embryological origin was suggested based on findings in transgenic mice (Thompson and Tucker, 2013). A dual epithelial origin might explain the varying morphologic epithelial appearances which are found throughout the tympanic cavity. In general, the floor of the Eustachian tube and the anterior hypotympanum are lined with a pseudostratified cylindrical epithelium while the roof of the Eustachian tube and the medial-posterior part of the meso- and epitympanum are lined with a cuboidal or flat epithelium (Hentzer, 1970; Shimada and Lim, 1972; Hiraide and Inouye, 1983; Bremond and Coquin, 1972). Both promontory and posterior hypotympanum seem to be transition zones (Hentzer, 1984; Hentzer, 1970; Shimada and Lim, 1972; Sade, 1966; Lim et al., 1973; Akaan-Penttila, 1982). The number of ciliated cells and goblet cells clearly differ in a similar fashion. In healthy middle ears ciliated cells and goblet cells are abundant in the floor of the Eustachian tube while their numbers decrease towards its tympanic orifice (Hiraide and Inouye, 1983; Akaan-Penttila, 1982). Their density further declines towards the antrum until ciliated cells and goblet cells are almost non-existent in the mastoid cavity (Bremond and Coquin, 1972; Bak-Pedersen and Tos, 1973). The proposed dual embryological origin hypothesis could be considered to be a paradigm shift, as the general assumption was that these morphological differences arise from a variation in epithelial differentiation rather than a completely different embryological origin.

The question how the human tympanic cavity can be entirely lined by a continuous epithelial layer while the cavity is bridged or separated by ossicles, muscles and nerves, has also been debated over the last decades. Endodermal rupture during middle ear cavitation, as proposed by Schwarzbart, has been suggested to be a necessity for such a cavity to form (Schwarzbart, 1959). However, it could be substantiated that an epithelial sac covers the ossicles during endodermal invagination, rejoins on the opposite side and continues invagination until it reaches the opposite cavity wall. This would explain the existence of the

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epitympanic mucosal folds that are found attached to the ossicles and tendons in the healthy human middle ear (Palva and Ramsay, 2007). The dual origin hypothesis would render this hypothesis mute as the ossicles would be lined by their own embryological derived epithelial layer. The hypothesis of a dual epithelial origin might also affect the recently proposed theory on the development of cholesteatoma, being the most common benign destructive tumour of the middle ear. The cholesteatoma mucosal traction theory builds on the potential of ciliary traction or mucosal migration along the middle ear ossicles towards the mastoid cavity. In order for this theory to be true, epithelium in the upper half of the middle ear needs to possess the potential to differentiate and form cilia and goblet cells, which does not seem to fit the dual epithelial origin theory (Jackler et al., 2015). Other consequences of a dual epithelial origin could be of clinical importance. The use of mucosa-targeted therapeutic agents in the treatment of diseased middle ears might have a different or no effect on the adjacent cavities in the temporal bone. Surgical approaches should also be reconsidered as an increasing number of surgeons chooses to obliterate the temporal bone cavity after temporal bone surgery in order to avoid recurrent disease. If, due to its origin, the epithelium towards the attic does not possess the capacity to form cilia and goblet cells no mucoid cysts will form after obliteration and total exenterating of the mastoid cavity would not be required.

Previous observations on the human middle ear are mainly based on fragments of axial histologic sections of the tympanic cavity because it is difficult to envelope the entire circumference of the tympanic cavity in regular histologic cassettes. None of the previously published observations in human material concerned immunohistochemical staining, since they dated back from the beginning of the second half of the 20th century (Hammar, 1902; Whyte et al., 2002; Clements et al., 1956). The purpose of the present study was to look for evidence that would either prove or discard the hypothesis of a dual embryonic epithelial origin by the use of immunohistochemical staining of sagittal sectioning of the fetal middle ear. We chose to investigate the human fetal tympanic cavity at a 25 week gestational age since this is a critical period of gestation in which tympanic attic cavitation is initiated (Hammar, 1902; Whyte et al., 2002; Clements et al., 1956).

2. Materials and methods

2.1. Literature search

A broad search on publications regarding histological investigation of the middle ear in the English scientific literature, published from 1900 to present (MEDLINE and Web of Science (Mesh search terms “histopathology” OR “histology” OR “ultrastructure” AND “ear, middle”)), delivered a handful of articles studying the human fetal tympanic cavity during early stages of development. Very few authors studied the tympanic cavity during the period of gestation in which tympanic cavitation is initiated, i.e. 20–29 weeks. Whyte et al. and Guggenheim et al. showed that, at this time, the fetal tympanic cavity is formed and ossification of the auditory ossicles is completed (Whyte et al., 2002; Clements et al., 1956). As was shown by Hammar et al., these ossicles are still surrounded by mesenchyme, but the cavitation process is already well advanced since parts of the attic already appear to be covered with cuboid epithelium (Hammar, 1902). These previous observations are mainly based on fragments of axial histologic sections of the tympanic cavity. A sagittal approach, encompassing the complete middle ear cavity, would allow for better understanding of the cavitation process and lead to less artefacts due to the easily rupturing of loose connective tissue found in the cavity at this stage. None of the published observations on the developing human middle ear cavity concerned immuno-histochemical staining, since they all dated back from the beginning of the second half of the 20th century.

2.2. Immunohistochemistry

We chose to investigate an ethanol preserved 25 gestational week-old fetus that was donated to science and made available by the department of Anatomy, Embryology and Physiology of the Amsterdam University Medical Centers, Amsterdam, the Netherlands. Autopsy and findings on a 7 Tesla MRI scan (Philips Medical Systems, Eindhoven, The Netherlands) showed no signs of developmental abnormalities. After initial treatment with a chelating solution (EDTA: titriplex III (250 g) in 1750 ml aqua dest and 25 g natriumhydroxide) the fetal skull was split along the midline and submerged in a patented formic and hydrochloric acid reagent (TDE™ 30 Reagent, SAK1428, Sakura Finetek Europe). It was electrolytically decalcified in a borosilicate glass beaker. Adequate decalcification was achieved after four days. Stepwise ethanol dehydration and clearing in three different xylene immersions was performed using a Shandon Pathcentre. The fetal skull was then fixated in paraffin. Serial sagittal sections, 7 μm thick, spanning the skull base from occiput to orbit, were cut on a Reichert-Jung Polycut S (LEICA) at 10 °C. An ionizer (Ultrastar Honeywell) was used to neutralize electric charges and prevent static adherence and fold over of the relatively large histologic sections. Sections were stained with hematoxylin and eosin, periodic acid–Schiff, von Gieson and Alcian Blue. Every second to fourth section was immunohistochemically stained with monoclonal antibodies against vimentin (M0725 Marker Clone V9 by Dako) as a potential mesenchymal cell marker, cytokeratine 14 (CK14) LL002 (Thermo Scientific) and E-cadherin HECD-1 (Invitrogen) as epithelial cell markers and Ciliated Cell Marker Clone LhS 28 (Sigma) which is expected to stain antigens in the sub-apical zone of ciliated epithelial cells in the upper respiratory tract (Ivaska et al., 2007; Abou-Kheir et al., 2015). Sections were examined with an Olympus BX51 light microscope (Olympus Nederland B.V., Zoeterwoude, the Netherlands). Positive tissue type controlling was carried out on every immunohistochemical stain.

3. Results

Our approach delivered over 800 subsequent sagittal histologic sections spanning the entire fetal tympanic cavity from external auditory canal towards the tympanic orifice of the Eustachian tube. These sections comprise all functional structures expected to be found in an adult human tympanic cavity (Fig. 1).

Throughout the subsequent sagittal histologic sections, an epithelial monolayer is found, covering the inferior, medial and lateral wall of the hypotympanum. The epithelial monolayer traverses the medial wall of the tympanic membrane and then bridges the cavity where it envelopes the ossicles, tendons and its supporting structures. The epithelial layer interconnects throughout the cavity and forms a continuous sac throughout successive histologic sections. The epithelium shows a pseudostratified appearance in the hypotympanum and becomes mainly flat throughout the rest of the tympanic cavity (Fig. 2). Towards the attic, parts of the middle ear structures are surrounded by loose fibrous connective tissue. The epithelial monolayer seems to disappear once it approaches the loose connective fibrous tissue. Yet, after immunohistochemical staining for vimentin, causing selective staining of the loose fibrous connective tissue, an extremely thin non-staining epithelial monolayer becomes visible which covers the loose connective fibrous tissue in the attic. It is stretched out and almost invisible but it sharply contrasts with the underlying vimentin stained connective tissue (Fig. 3). The loose connective tissue becomes a rather thin condensed layer close to the sites where the epithelial blanket approaches the underlying middle ear structures. The epithelium advances towards and even envelopes the supra-structure of the stapes at different sites, it also attaches to the otic capsule or medial tympanic wall cranially to the oval window niche (Fig. 4).

Immuno-histochemical staining for CK14 is seen in the epithelial layer throughout the external ear canal. The epithelial layer continues

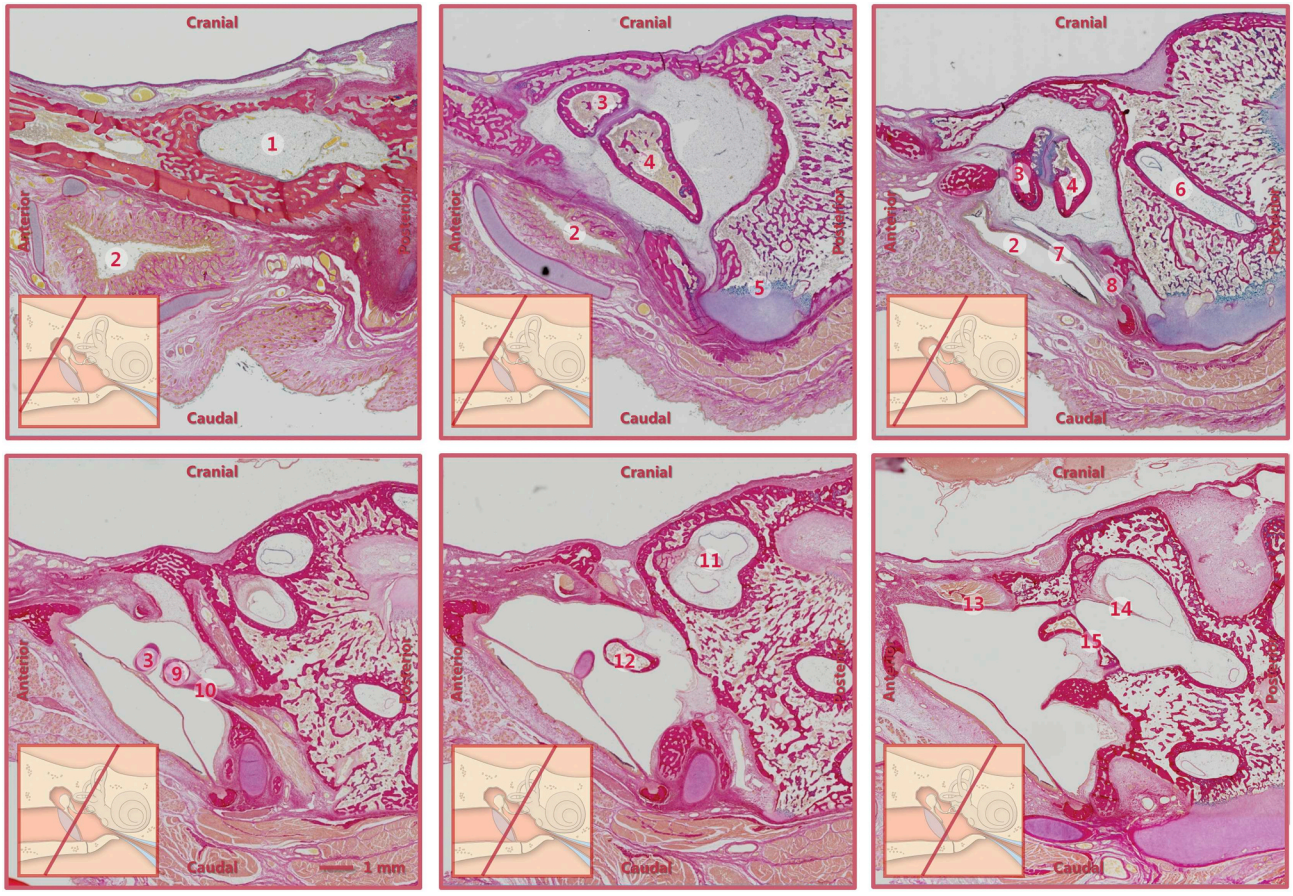

Fig. 1. Different sagittal histologic sections of the tympanic cavity of a 25-gestational week old fetus.
Sequence from lateral to medial (attic towards Eustachian tube):
1 = antrum/attic; 2 = external meatus; 3 = malleus; 4 = corpus incudis; 5 = endochondral bone formation; 6 = semicircular canal; 7 = tympanic membrane; 8 = chorda tympani; 9 = incudo-stapedial joint; 10 = stapedial muscle/tendon; 11 = cupula; 12 = stapedial crura; 13 = tensor tympani muscle; 14 = basilar membrane within the cochlea; 15 = stapes footplate.

to express CK14 as it traverses along the lateral surface of the tympanic membrane (Fig. 5). The epithelial sheet or sac which covers the medial surface of the tympanic membrane and envelopes the structures throughout the tympanic cavity does not express CK14, neither does the epithelium in the meso-, epi- and hypotympanum, not even towards the orifice of the Eustachian tube (Fig. 5). Monoclonal Anti-Ciliated Cell Marker Clone (LhS28) does not stain throughout our sections. Positive tissue type controlling showed that the staining process was done correctly. Nevertheless, islands of cilia can clearly be appreciated in our fetus without immunohistochemistry. They are abundant on the surface

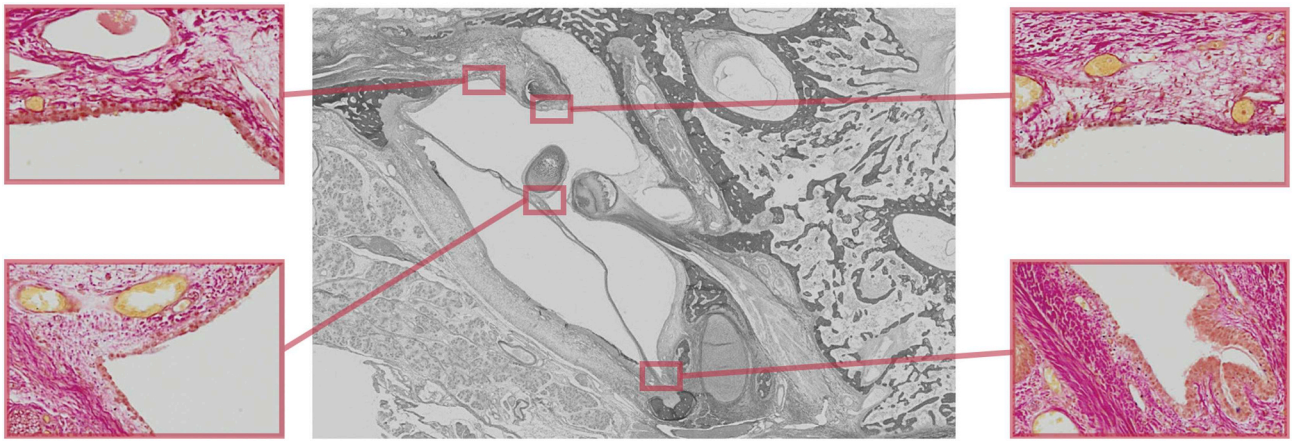

Fig. 2. Sagittal section at the level of the incudo-stapedial joint in the tympanic cavity of a 25-gestational week old fetus.
Throughout the cavity, a continuous epithelial layer covers the ossicles, tendons and middle ear cavity. In the hypotympanic space the epithelium is pseudostratified, it becomes a cuboidal monolayer at the medial surface of the tympanic membrane, pseudostratified in the anterior meso- and epitympanic spaces and again cuboidal to even flat towards the posterior epitympanic space. Primary image magnification of enlarged sections: 10×.

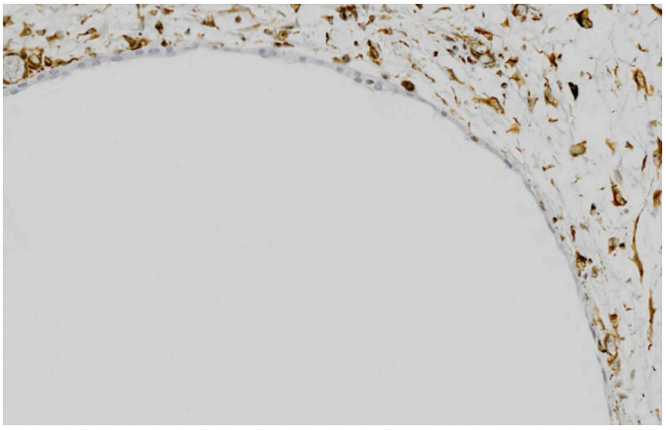

Fig. 3. Sagittal section showing the posterior epitympanic space at the level of the incudo-stapedial joint in the middle ear of a 25-gestational week old fetus. The epithelial monolayer covering the loose connective tissue on the lower right corner and further down was only noticed after immunohistochemic vimentin staining of the underlying loose connective tissue. Primary image magnification: 10×.

of the pseudostratified epithelial regions and absent at sites were the epithelial sheet has a flat monolayered configuration. Also, E-cadherin (HECD-1) staining did not show positive staining throughout our specimen although positive tissue type controlling turned out to be positive.

Histologic sections collected during a phase of accelerated microtome cutting speed, in order to reduce preparation time, show a rupture of the epithelial monolayer in regions where it bridges the loose connective fibrous tissue. In these sections fragments of acellular material are occasionally found floating along the surface of the promontory (Fig. 6). These acellular fragments appear to be torn from their underlying structures as they do not connect to the promontory. In sections acquired after decreasing the microtome cutting speed, signs of epithelial rupture or floating of acellular fragments were no longer observed.

4. Discussion

The hypothesis of a dual embryological origin of the epithelial lining of human middle ears could not necessarily be supported by our findings (Fig. 7a). The demonstrated continuous epithelial lining, enveloping the middle ear ossicles and their supporting structures, its attachment to the promontory in the attic and the presence of a vimentin negative epithelial layer covering a vimentin positive loose connective tissue in the attic, could support the hypothesis of a single endodermal origin of the middle ear epithelial lining (Fig. 7b). On different aspect our findings might not be in concordance with the results found in transgenic mice, as described by Thompson and Tucker (2013). Based on their findings Thompson and Tucker suggest that a mesenchymal-to-epithelial transformation takes place in the attic and along the promontory in the tympanic cavity. A possible programmed epithelial rupture would subsequently form a lining continuous with the endodermally derived epithelial lining of the hypotympanum and auditory tube. In our specimen the epithelial lining is already fully interconnected throughout the cavity, including the attic, while invagination or tympanic cavitation seems to be just passing the ossicles. At this stage of cavitation and ossicular encapsulation no clear signs of epithelial sac ruptures are found except for several micro ruptures in samples collected during increased microtome cutting speeds. The authors of the dual epithelial origin theory describe a stained superficial tissue layer on the promontory after staining Wnt1creR26R transgenic mice with X-gal and they suggest this concerns an epithelial layer. They hypothesize that this finding demonstrates the neural crest cell origin of the epithelial layer covering the promontory. However, histological figures presented in their publication show an apparent absence of a stained submucosal layer in the promontory region of these mice. We assume that the thin epithelial layer, which we only identified after vimentin staining of the sharply contrasting submucosa, could have been overlooked by the authors. The apparent lack of a clear submucosal layer in the promontory region in transgenic mice supports this idea and suggests that the stained layer is not the superficial layer. Strengthening this assumption is the fact that an X-gal positive staining submucosal layer does appear in the hypo-tympanic region. Another possible explanation of the found contradiction could be the occurrence of histologic artefacts due to the section cutting speed. Our results with higher section cutting speed demonstrated fragments and strips of acellular material which show morphologic similarity to the superficial non-staining layer found on the promontory in Sox17icreR26R mice. If our explanation of the described results in mice is correct this will undermine the conclusion that a neural crest cell epithelial origin is present.

In the 25-gestational week-old fetus we investigated, none of the epithelium throughout the tympanic cavity (including the hypo-

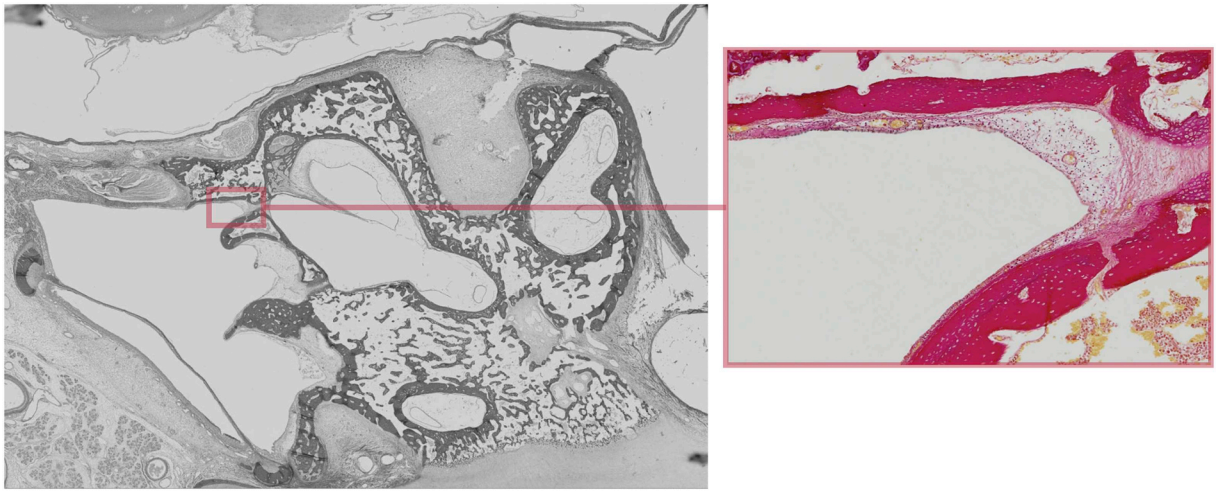

Fig. 4. Sagittal slide at the level of the stapes footplate in a 25-gestational week old fetus.
Magnification shows the thinning of the loose connective tissue as the epithelial sheet becomes adherent to the stapes and the otic capsule antero-cranially to the oval window niche. Primary image magnification of enlarged section: 10×.

Fig. 5. Sagittal section at the level of the incudo-stapedial joint in the tympanic cavity of a 25-gestational week old fetus.
Magnification shows the immuno-histochemic CK14 staining in the epithelial layer in the external ear canal and lateral surface of the tympanic membrane. Epithelium on the medial surface of the tympanic membrane, the ossicles and throughout the tympanic cavity does not express CK14. Primary image magnification of enlarged section: 10×.

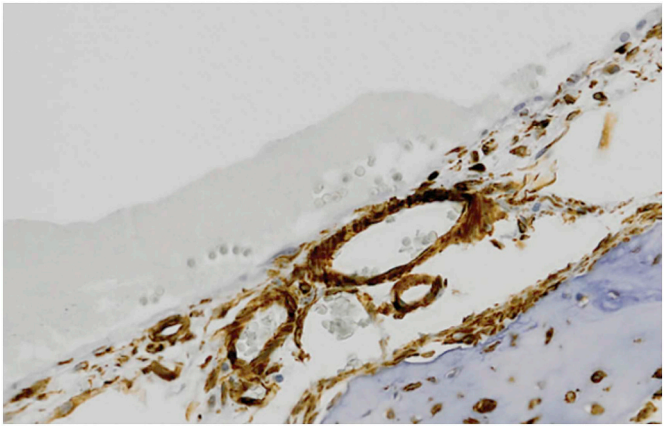

Fig. 6. Acellular material along the promontory during accelerated microtome cutting speed.
Fragments of acellular material are occasionally found floating along the surface of the promontory. Primary image magnification: 10×.

tympanum) expressed CK14. CK14, however, was expressed in the epithelium of the external ear canal. In a murine specimen, a late onset of CK14 expression was found in parts of the tympanic cavity. This CK14 expression did not become apparent prior to postnatal day 16 (Thompson and Tucker, 2013). It is conceivable that in our human fetal specimen the epithelium in the tympanic cavity is in an earlier stage of development before any expression of CK14 commences. A gradual onset of CK14 expression throughout the entire tympanic cavity could therefore be true in both human and murine specimens and would not necessarily imply a dual epithelial origin.

Prolonged electrolytic decalcification and ethanol preservation may have had a negative impact on certain antigens, including LhS28 and HECD-1, causing damage to tissue antigenicity and thereby leading to false-negativity throughout the histologic sample. This negative LhS28 immunostaining could not conceal the evident abundant existence of cilia on the surface of the pseudostratified epithelial regions. Neither could it conceal the gradual transition of cilia-rich regions into undifferentiated flat epithelial regions without cilia throughout the cavity. The presence of these undifferentiated monolayered epithelial regions

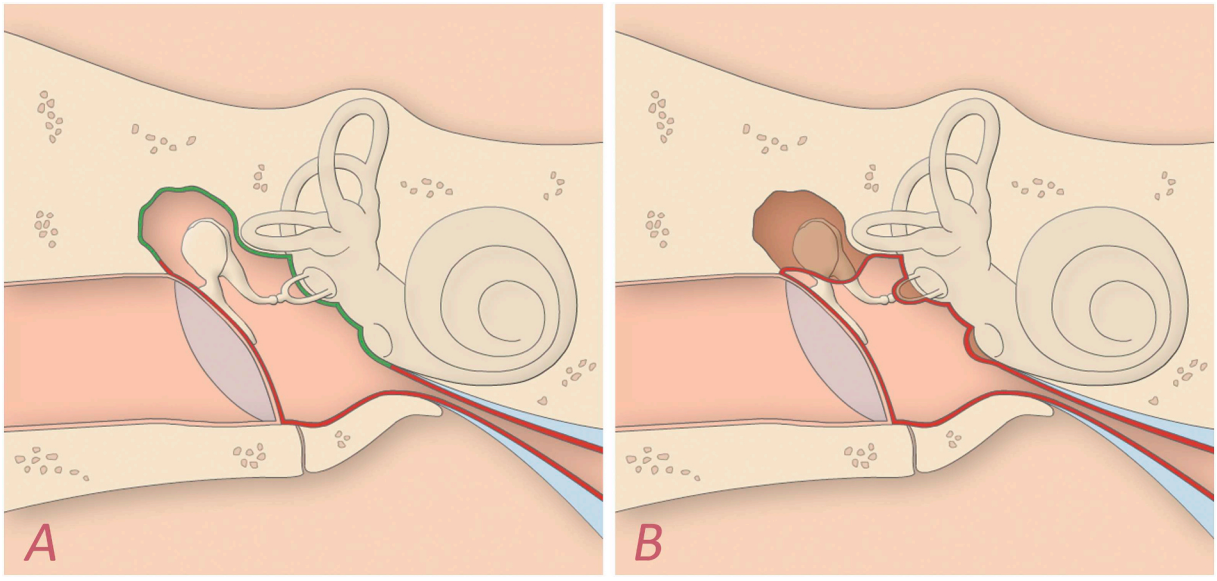

Fig. 7. Illustrations representing reported findings in murine specimen versus findings in our human fetus, ´dual versus single embryologcal epithelial origin´.
A. Figure illustrates the possible dual embryological origin of the middle ear epithelial lining based on previous findings in transgenic mice (mesodermally derived epithelium = green, endodermally derived epithelium = red).
B. Figure illustrates our findings in the human fetal specimen at 25 weeks of gestation (proposed endodermally derived epithelium = red, mesenchym = brown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

could explain the absence of α-tubulin expression in previous murine histologic samples at sites where non-endodermal tissue lays close to the surface (Thompson and Tucker, 2013). Assuming that this non-endodermal staining tissue does in fact concern a submucosal layer underneath a thin undifferentiated epithelial monolayer. This most likely represents a different stage of epithelial differentiation rather than a different embryological origin.

HECD-1 immunostaining for E-cadherin was performed in order to identify epithelial features throughout the fetal cavity. Unfortunately such features could not be demonstrated with this staining However, recent findings suggested an active role for E-cadherin as an adhesive factor in ordered migration of mesenchymal cells (Campbell and Casanova, 2015). This would discard E-cadherin expression as an exclusively epithelial feature which mitigates the argument of a non-endodermal origin of the epithelial layer on the promontory in murine specimen (Thompson and Tucker, 2013).

A theory of a non-endodermal or dual epithelial origin was supported by previously reported findings of ruptures in the endodermal sac. Such ruptures are consistent with our own experience. Yet, we consider these ruptures to be artefacts in fragile histologic samples filled with vulnerable thin mesenchyme, rather than an active embryonic process. If a rupture of the sac were of developmental necessity for the cavity to be bridged by other structures, we would have expected it to occur prior to the stage we investigated since the epithelial sac in our specimen already clearly folds around and connects to all three ossicles while they bridge the air-filled cavity. The process of middle ear cavitation and thinning or resorption of the mesenchyme already seems well advanced at 25 weeks of gestation; even towards the attic the epithelial sac connects to the otic capsule which is thereby exposed to the air-filled tympanic cavity (Fig. 4). These findings support our presumption that endodermal invagination might be critical to form an air-filled tympanic cavity and that endodermal rupture is not required to have the different tympanic compartments interconnect. Whether the mesenchyme actively allows migration of the epithelial sac by gradually regressing or whether the epithelium plays an active role in migration and pneumatisation itself remains debatable (Hammar, 1902). At 25 gestational weeks, mastoid pneumatisation still needs to be initiated, but it is conceivable that, once the epithelial sac has passed the ossicles and reached the attic, it could easily advance and initiate mastoid pneumatisation.

Our findings could therefore support the concept of a complete endodermal epithelial invagination around the ossicles towards the mastoid. An epithelial sac can easily cover the ossicles during endodermal invagination, rejoin on the opposite side and continue invagination until it reaches the opposite cavity wall. This would also be in concordance with the existence of the epitympanic mucosal folds that are found attached to the ossicles and tendons in the healthy human middle ear (Palva and Ramsay, 2007). Also, the fact that the ossicles are covered by a respiratory like epithelial layer is easily explained with our supported invagination theory and is less likely with the competing endodermal rupture theory. This respiratory like epithelium recently received attention in regard to the mucosal traction theory which assumes that the cilia bearing mucosa on the ossicles and in the attic plays an essential role in cholesteatoma formation (Jackler et al., 2015). We do realize that, mainly because of understandable ethical concerns, our work relies on findings on just a single human fetus. Nevertheless, we chose a fetus which was preserved during an ideal critical stage of middle ear cavitation and precise histopathological preparation delivered valuable information on the histochemical architecture during cavitation. We believe these histological findings could partly be used to substantiate the critical analysis of previously reported finding supporting a possible dual embryological epithelial origin.

5. Conclusion

This is the first immuno-histopathological investigation encompassing the entire human fetal middle ear in a critical period of gestation in which tympanic attic cavitation is initiated (Hammar, 1902; Whyte et al., 2002; Clements et al., 1956), supplying valuable information regarding the origin of the middle ear epithelium. Our findings can be used to substantiate the critical analysis of previous findings and provide multiple arguments for the hypothesis of an entirely endodermal embryonic origin of the epithelium lining the tympanic cavity. We do realize that the heterogeneity between different mammals makes it difficult to compare findings between human and murine specimens but we clearly showed that an endodermal rupture or a dual epithelial origin is not of necessity for an epithelial lined tympanic cavity bridged by other structures to form. We conclude that the different morphological epithelial appearances throughout the tympanic and mastoid cavity could be explained by different stages of epithelial differentiation and not necessarily by a different embryological origin. The endodermal embryonic origin of the epithelium lining the tympanic cavity would arise due to complete epithelial invagination and envelopment of the middle ear structures without the need of endodermal ruptures to occur. A complete endodermal epithelial embryologic origin would explain the existence of epitympanic mucosal folds (Palva and Ramsay, 2007) and it would fit the recently proposed mucosal traction theory for cholesteatoma formation (Jackler et al., 2015). It would place both the middle ear and temporal bone cavity in an embryologic and functional continuum with the upper respiratory tract. As mucosal targeted therapies might redefine the treatment of the diseased airway system this could be quite relevant in the development of middle ear and temporal bone targeted therapies.

Declaration of Competing Interest

All authors declare they have no financial or non-financial competing interests or other interests that might be perceived to influence the interpretation of the article.

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Author contributions

All authors contributed extensively to this work. H.F. van Waegeningh conducted the microscopic observations and wrote the main paper. F.A. Ebbens, R.J. Oostra and E. van Spronsen have reviewed all stages and have substantially contributed to writing the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages. No permission for re-use of material such as figures is required since all materials were created for this article by the authors.

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