Cochlear epithelial of dog fetuses: a new source of multipotent stem cells

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Abstract Hearing loss caused by the damage of cochlea sensory cells or neurons is a common human disease, but also affects dogs and other animals. To test their progenitor nature as potential value for future therapies, we characterized cells derived from the cochlear epithelium in dog fetuses. In total, 8 fetuses of 35–40 days of gestation, derived from castration campaigns, were investigated. Cells were analysed by the MTT colorimetric assay and in regard to cell cycle, differentiation capacities, immunophenotypes and qPCR analysis. In culture, cells had a fibroblast-like morphology. Phenotypic immunocharacterization showed positive staining for mesenchymal stem cell and pluripotency markers and were negative for hematopoietic cell markers. Cells possessed differentiation capacity for the three main cell lineages:

osteogenic, adipogenic and chondrogenic, altogether indicating their nature as mesenchymal stem cells. Thus, cells derived from fetal cochlear tissues indeed may provide valuable sources of progenitor cells for cell therapy of canine deafness and other diseases.

Keywords Hearing loss · Stem cells · Cell therapy · Animal model

Introduction

According to the World Health Organization, more than 360 million people have hearing disability (America 2016). Dogs and other animals are also affected (Gotthelf 2007; Cunha et al. 2003; Müller and Heusinger 1994). Deafness is the partial or complete inability to sense air waves, caused by factors such as age, intensive and repetitive noise, infections, poisoning or physical injuries (White et al. 2006; Hutchin and Cortopassi 2000). There are two types: Conductive hearing loss is located in the outer or middle ear and is caused by injuries affecting the transmission of sound stimuli to the cochlea. Sensorineural deafness is characterized by abnormalities of the cochlear receptors or auditory regions of the brain (Guyton and Hall 2011). The latter results from damage or loss of hair cells of the cochlea or associated neurons. It is generally irreversible, because of replacement or regeneration

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inability of the cells (Barboza Jr et al. 2008). To nevertheless enhance cochlear regeneration, several studies attempted to use stem cells isolated from the inner ear in rodent models. In rats, cells derived from inner ears showed self-renewal properties, expression of proliferation markers and differentiation into different cell types (Li et al. 2003; Malgrange et al. 2002). Lou et al. (2007) demonstrated that such stem cells contributed in the regeneration of damaged cells in the rat. Likewise, recovery of hair cells in vitro have been demonstrated in mice by application of fetal cochlea stem cells (Shi et al. 2012; Savary et al. 2007; Oshima et al. 2007; White et al. 2006; Doetzlhofer et al. 2004). Also, embryonic and neural stem cells have been used to experimentally treat deafness in mice (Parker et al. 2007; Corrales et al. 2006). However, studies in other experimental models that are phenotypically more similar to humans seemed to be useful. For that, the dog resulted as an adequate choice, because it shares several diseases equivalent to humans (Ambrosio et al. 2007; Cunha et al. 2003). Moreover, it has a long life with complex social behavior, which allows long-term studies (Horn et al. 2004). Thus, we here characterized cells derived from the cochlear epithelium in dog fetuses of 35–40 days of gestation in order to test their progenitor nature as potential value for therapies that may support classical ones.

Materials and methods

Animals used

Eight dog fetuses of 35–40 days of gestation were used from castration campaigns in São Paulo (SP, Brazil). The study was approved by the Ethical Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science at the University of São Paulo (FMVZ-USP) (No. 2947/3013). Gestational age was determined by crown-rump-length (Evans and Sack 1973).

Isolation, morphological characterization and cryopreservation of the cells

Fragments of cochlear epithelial tissues were washed twice in Phosphate Buffered Saline Solution

(PBS) containing 10% penicillin and streptomycin (LGC Biotecnologia, Cat. BR30238-01, Cotia, São Paulo, Brazil) and mechanically separated. Explants were deposited in petri dishes of 100 mm diameter (Corning, Cat. 3296, Corning, NY, USA), emerged in fetal bovine serum (FBS) (LGC Biotecnologia, Cat. BR330110-01) for 4 h and kept in an oven at 37 °C and 5% of CO₂. Afterwards 10 mL of culture medium were added. 3 different culture media were tested: Alpha-MEM (LGC Biotecnologia, Cat. BR3007-05), DMEM-High glucose (LGC Biotecnologia, Cat. BR30003-05) and DMEM-F12 (LGC Biotecnologia, Cat. BR30004-05), supplemented with 10% FBS, 1% penicillin and streptomycin and 1% non-essential amino acids (LGC Biotecnologia, Cat. BR30238-01). Cells were subcultivated with 0.25% trypsin (LGC Biotecnologia, Cat. BR30042-01) when they reached a confluency of 80%. Morphological analyses were performed every 3 days using an inverted microscope (NIKON ECLIPSE TS-100). For freezing, cells were trypsinized and 10⁶ cells were resuspended in 1.5 mL of freezing medium containing 90% fetal bovine serum and 10% of DMSO (LGC Biotecnologia, Cat. 13-0091.01), equally distributed in microtubes (Corning, Cat. 430055), transferred to the device Mr. Frosty and kept over night in the freezer at 80 °C. After 24 h, the wells were stored in liquid nitrogen. When necessary, the cells were subjected to rapid thawing in a water bath. All experiments were conducted in passage 4. Cell cycle analyses were performed in the passages 2, 3 and 4.

Cell metabolism assay (MTT)

To analyze cellular metabolic viability, we tested the 3 culture media (DMEM-High glucose, Alpha-MEM and DMEM-F12) at days 1, 4 and 7. Approximately 10³ cells were plated in 96 wells in 210 µL/well for each day. At each experimental day, the culture medium was removed and 100 µL of a new medium with 10 µL of MTT solution A (Life Technologies, Cat. V-13154, Carlsbad, CA, USA) was added followed by incubation of for 4 hours at 37 °C. Then, we added 100 µL of solution B and maintained at the same conditions. Finally, the analysis was performed in a spectrophotometer (MQuant—Bio Tek Instruments, Winooski, VT, USA).

Analysis of cell cycle phases

Cells were removed from the culture and centrifuged for 5 min at 200 g, after that the supernatant was discarded and the cells were resuspended in buffer for the cytometric analysis (FACS FLOW—BD). Then, cells were centrifuged for 3 min at 200 g and the supernatant was discarded, and resuspended with 1 mL ethanol 70% RNase, transferred to microtubes and stored at a temperature of -20°C . Cells were treated with 40 mg/mL (Propidium iodide-PI, Life Technologies), 10 mg/mL (RNaseA, Sigma-Aldrich, St. Louis, MO, USA), and samples were analyzed on a flow cytometer FACS Aria Cell Sorter (Becton–Dickinson, San Jose, CA, USA) and analyzed by the software Modfit 2.9.

Immunophenotyping by fluorescence

On passage 4, the cochlear cells (1×10^4) were grown on coverslips in DMEM-high glucose medium. After 48 h, cells were washed twice in Tris-buffered saline (20 mM Tris–HCl pH 7.4 in 0.15 M NaCl, and 0.05% Tween-20) and fixed for 24 h in 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 (Sigma). After blocking non-specific binding with 5% bovine serum albumin, cells were incubated overnight at 4°C with the primary antibodies in bovine serum albumin. Primary antibodies added were directed against vimentin (sc-73259, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -tubulin (Cell Signaling Technology, Danvers, MA, USA), CD73 (sc-14684, Santa Cruz Biotechnology, Inc, Heidelberg, Germany) Stro-1 (sc-47733, Santa Cruz Biotechnology), Nestin (sc-33677, Santa Cruz Biotechnology), Myosin VIIa (ab3481, Abcam, Cambridge, UK), CD117 (A4502, Dako Cytomation, Carpinteria, CA, USA), Oct-4 (sc-4420, Santa Cruz Biotechnology); Sox-2 (sc-17320, Santa Cruz Biotechnology), and Nanog (n-17, sc30331, Santa Cruz Biotechnology). Then they were washed three times in Tris-buffered saline, fluorescein isothiocyanate conjugated secondary antibody was added and incubated at room temperature for 1 h. The secondary antibodies were anti-goat IgG, anti-rabbit IgG (Dako Cytomation), and anti-mouse IgG (Chemicon International, Temecula, CA, USA). The slides were mounted using Vectashield with DAPI (H-1200, Vector Laboratories, Inc., Burlingame, CA, USA). Analyses were performed using a fluorescence

microscopy LSM 510 (Carl Zeiss Microscopy, Jena, Germany). For negative controls, an IgG (Clone Ci4, Merck Millipore, Billerica, MA, USA) was substituted for primary antibody.

Flow cytometry

Immunophenotypic analysis was performed by using flow cytometry. For that, 10^5 cells/mL were trypsinized, centrifuged, resuspended in PBS and incubated with the primary antibodies (dilution 1:100) for 30 min at 4°C . Primary antibodies included were directed against: markers for pluripotency: Oct-4 (sc-4420, Santa Cruz Biotechnology) and Sox-2 (sc-17320, Santa Cruz Biotechnology); against cytoskeleton markers: Cytokeratin 18 (sc-32329, Santa Cruz Biotechnology) and β -tubulin (sc-47751, Santa Cruz Biotechnology); against the mesenchymal markers: Stro-1 (sc-47733, Santa Cruz Biotechnology), CD90 (ab225, Abcam, Cambridge, UK) and CD105 (ab53321, Abcam); against the neuronal progenitor cell marker Nestin (sc-33677, Santa Cruz Biotechnology); against the vascular endothelial growth factor VEGF (ab1316, Abcam); against the marker for ciliated cells Myosin VIIa (ab3481, Abcam); against the marker for hematopoietic precursor cells CD117 (A4502, Dako Cytomation); against the marker for hematopoietic cells CD34 (BD555824, Becton Dickinson, San Jose, CA, USA). After this, the cells were incubated with the specific secondary antibodies: goat anti-mouse (1:500) (Life Technologies A11017) or donkey anti-rabbit (1:500) (Abcam 150061) for 30 min at 4°C . Finally, the cells were washed and analyzed by flow cytometer BD FACSAriaII (Becton–Dickinson).

Total RNA isolation, cDNA synthesis and real time reverse transcription PCR (qRT-PCR)

Total RNA was isolated using Trizol manufacturer's protocol and treated with DNaseI (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized using 500 ng total RNA, amplification of the resulting cDNAs and analyses were performed as previously described by Fratini and collaborators (Fratini et al. 2016). To determine the best endogenous gene, three different genes were tested (*GAPDH*, *RPLP0*, *Myo7A*) (Table 1) and the stability analysis was performed

Table 1 List of primers sequences used for quantitative real time polymerase chain reaction

Gene name	Gene	Direction per gene	Accession number
Thy-1 cell surface antigen	<i>CD90*</i>	Forward 5' TGTGAGGGGTAGAAGGGTAGA Reverse 5' TATATGCTAGGCCCAGATGCAC	NM_001287129
Endoglin	<i>CD105*</i>	Forward 5' CACAACGGCACCCTTACCT Reverse 5' CATGCAACGGAAGGATGGTC	XM_005625330.2
Nanog homeobox	<i>Nanog</i>	Forward 5' GTGACTCAGAACAGCTCTGCC Reverse 5' CCACAGTGGGAGGTTTCCAG	XM_005642425.1
Octamer-Binding Protein 4	<i>Oct4</i>	Forward 5' TCGTGAAGCCGGACAAGGAGAAG Reverse 5' AGGAACATGTTCTCCAGTTGCCT	XM_538830.2
SRY-Box 2	<i>Sox2</i>	Forward 5' AACCCCAAGATGCACAACCTC Reverse 5' CGGGGCCCCGGTATTTATAATC	XM_005639752.2
Paired box 8	<i>Pax8</i>	Forward 5' TCAATGCCTTTCCCCATGCT Reverse 5' CCCACCATCTCTCTCCCTGA	NM_001003248.1
Myosin VIIA	<i>Myo7A</i>	Forward 5' GTAGGGAGTGCCGTGTCTTG Reverse 5' GCTACCAGTTTGTCTCCCT	XM_005633457.1
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH*</i>	Forward 5' AGTATGATTCTACCCACGGCAAA Reverse 5' CACAACATACTCAGCACCAGCAT	NM_001003142
Ribosomal Protein Lateral Stalk Subunit P0	<i>RPLP0</i>	Forward 5' GCCGCTCGGCTCACATATAA Reverse 5' AGACGATGTCACTCCCACGA	XM_005636025.1

* Fratini et al. 2016

using RefFinder (Xie et al. 2012). The most stable gene to expression normalization was *Myo7A*. Relative expression levels of *Pax8*, *Nanog*, *Sox2*, *CD90* and *CD105* were calculated according to Pfaffl's model (Pfaffl 2001). Four different cochlear cell cultures derived from cochlear epithelium of dog fetuses were analysed in qRT-PCR (Table 1).

Statistical analyses were performed using Graph-Pad Prisma 6.01 software. Anova test followed by Tukey's test for post hoc comparison were used for statistical analysis. The results were presented as mean $\pm$ SEM. A *p* value of less than 0.05 was considered statistically significant.

Differentiation assays

Following Favaron et al. (2014), 10^4 cells were plated in 24 wells for osteogenic and adipogenic differentiation and 10^6 suspensions of cells were used for chondrogenic differentiation. For osteogenic differentiation we performed Von Kossa staining, for adipogenic differentiation Oil Red staining and for chondrogenic differentiation Trichome Masson and Picrosirius staining (Tolosa et al. 2003).

Results

After 24 h of the addition of culture medium, release of the first cells from the cochlear explant fragments was observed. Cell confluence of 80% was observed from the second day, and, therefore from this period took place the trypsinization. The cells had fibroblastoid shape with an elongated cytoplasm, with a central core characteristic for this cell type. This fibroblastoid shape was prevalent in the cells from passage 1 until passage 4, and did not show changes even after thawing, growth capacity was maintained.

The cells derivated from cochlea of dog fetuses were cultured in different media, DMEM-High glucose, Alpha-MEM and DMEM-F12. The best one of them for cell growth was DMEM-High glucose (Fig. 1a). In this medium there was a better growth of cells on culture plates, presenting morphology characteristic of fibroblast cells. In contrast in the other media Alpha-MEM and DMEM-F12 the cells showed reduced cell growth, and a heterogeneous morphology (Fig. 1b, c).

MTT analyses were accomplished for evaluating cellular metabolic viability of cultures done in the

Fig. 1 Morphological, viability and cell cycle of dog fetal cochlea cells with 35–40 days of gestation. **A–C** Analysis of the primary cell culture using different culture media: DMEM-High glucose, Alpha-MEM, DMEM-F12, respectively, passage 4. **A** Note the high confluence of fibroblast-like cells (arrow) with satisfactory pattern of growth and homogeneous fibroblast-like morphology **B, C** note the low cell confluence (circle) with deformed shape (arrows) and cell debris. **D–F** Cell metabolism assay using MTT test, passage 4. **D** Progressive growth of cells

three media for seven days: DMEM-High glucose, Alpha-MEM and DMEM-F12. The cells cultured in DMEM-High glucose showed a progressive growth during the whole period analyzed (Fig. 1d). The cells cultured in Alpha-MEM showed a continued growth was observed until the fourth day, followed by a reduction in growth until the seventh day (Fig. 1e). Similar results were obtained with cells cultured in

cultured in DMEM-High glucose. **E** Stagnation of cell growth using Alpha-MEM, on the 4th day, followed by continuous growth thereafter. **F** Continuous cell growth until the 4th day using DMEM-F12 medium, followed by linear growth. **G–I** Cell cycle analysis in passages P2, P3 and P4, respectively. **G** Note the major part of the cells in the G0/G1 phase (78.42%), **H** cells peaked in G2/M (63.03%) and **I** note the similar values for G0/G1 (41.05%) and G2/M (45.21%)

DMEM-F12. The cells maintained a linear growth until the fourth day followed by a reduction in their growth until the end of the experiment (Fig. 1f).

The phases of the cell cycle of cells from dog fetal cochlea in P2, presented the following values: 4.69% cell debris, 78.42% phase G0/G1, 0.31% in phase S, and 21.27% in G2/M phase (Fig. 1g). In P3 the following data were obtained: 8.49% cell debris,

20.18% G0/G1, 16.79 S and 63.03 G2/M (Fig. 1h). Finally, cells were also analyzed in P4 with 4.89% cell debris, 41.05% cells in G0/G1, 13.74% cells in S, and 45.21% cells in G2/M (Fig. 1i).

Immunocytochemistry technique for analyzing different cell markers was performed. The cells expressed markers for mesenchymal stem cells: Stro-1 and CD73 (Fig. 2a and 2b), cellular structures: vimentin (Fig. 2c), neuronal cytoskeleton: β -tubulin (Fig. 2d), ciliated cells: myosin VIIa (Fig. 2e), and neuronal precursor cells: nestin (Fig. 2f). In addition, the cells expressed hematopoietic precursor cell markers: CD117 (Fig. 2g) and pluripotency markers: Oct-4, Nanog and SOX-2 (Fig. 2h, i and j, respectively).

The cochlear-derived cells of dogs fetuses were analyzed by flow cytometry for cytoskeletal markers such as: Cytokeratin 18 (42.5%) and β -tubulin (29.5%), mesenchymal stem cells markers: Stro-1 (28.6%) and CD105 (21.2%), including CD90 for which we observed a low expression (7.73%). There was high expression of vascular endothelial growth factor-VEGF (77.1%) and marker of hematopoietic precursor cells: CD117 (31.4%). However, the cells showed negative response for the hematopoietic stem cell marker-CD34 (0.027%). In addition, there was not a significant expression of Nestin, a neuronal progenitor cells marker (0.054%). In contrast to Myosin VIIa, a ciliated cells marker was expressed (39.1%). High levels of pluripotency markers such as Sox-2 (44.2%) and Oct-4 (38%) were also observed (Fig. 3).

The level of gene expression showed an increase of pluripotency *Nanog* and *Sox2* genes and *Pax8*,

expressed in the developing otic region, in the cells derived from cochlear epithelium of dog fetuses. No *Oct-4* gene expression was detected in these cells. The expression level of detection of CD105 was low, and CD90 expression was detected in these cells (Fig. 4).

After osteogenic differentiation, development of a calcified extracellular matrix surrounding the cells with points of calcification was detected using Von Kossa staining (Fig. 5a).

During adipogenic differentiation the cells showed an elongated shape with cell nuclei were located in the periphery of the cytoplasm. Inside of the cytoplasm, several lipid vesicles were observed using Oil Red staining, typical characteristics of adipocytes (Fig. 5b). Chondrogenic differentiation was reached after 21 days with the development of a typical pellet of cells. Using picosirius and trichrome masson staining cells with chondrocyte morphology were observed. These cells were round in shape with large nuclei (Fig. 5c and d). Control cells maintained the same fibroblast-like morphology during the three differentiation assays (Fig. 5).

Discussion

Data derived from various fields of cell culture analysis were consistent and indicated a progenitor nature of cells derived from the cochlea epithelium of dog fetuses with multipotency of in vitro differentiation.

For culture of cochlear epithelial cells derived from dog fetuses the best results and conditions were

Fig. 2 Immunocytochemistry analyses of dog fetal cochlea cells with 40 days gestation. **A, B** Expression of mesenchymal stem cell markers: Stro-1 and CD 73, respectively. **c** Expression for vimentin. **D–F** Expression of neuronal markers: β -tubulin,

myosin VIIA and nestin, respectively. **H–J** Expression of pluripotent stem cell markers: Oct4, Nanog and Sox 2, respectively

Fig. 3 Immunophenotyping by flow cytometry. Positive expression for cytoskeletal markers (cytokeratin and β -tubulin) and most mesenchymal cell markers (Stro-1, CD90 and CD105). High levels of expression for vascular endothelial growth factor marker (VEGF), hematopoietic precursor cell marker (CD117),

and negative response for hematopoietic cells (CD34) and neural precursor cells (Nestin); expression of markers for ciliated cells (Myosin VIIa) and pluripotent cells (Sox-2 and Oct-4)

Fig. 4 Level of gene expression in the fetal cochlea cells of dogs at 40 days of gestation. Graph evidencing the expression of the following genes: Pax8, Nanog, Sox2, Myosin 7A, Oct-4, CD105 and CD90

obtained using the DMEM-High glucose, which maintained satisfactory characteristics of growth and cell viability. Similar results were previously obtained by Diensthuber et al. (2009) for cultures of cochlear epithelial cells of different mice lineages (BALB/c and Math-1/nuclear green fluorescence protein (nGFP) transgenic mice) using similar culture medium.

Media containing DMEM-High glucose and DMEM-F12 (1:1) have been proven to be successful for culturing rodent cochlea cells and were suitable for the applied purposes (Chao et al. 2013; White et al. 2012; Diensthuber et al. 2009; Yerukhimovich et al. 2007; Savary et al. 2007; Martinez-Monedero and Edge 2007; Doetzlhofer et al. 2004; Li et al. 2003). In our adherent cultures, cochlear epithelial cells have typical fibroblast-like morphology, as observed for other mesenchymal stem cell cultures, including multipotentiality (Favaron et al. 2014; Chao et al. 2013; Wenceslau et al. 2012). In contrast, rodent

Fig. 5 In vitro cell differentiation into the following lineages. **A** Osteogenic lineage, von Kossa staining. Cells differ from controls which showed fibroblast-like morphology with centralized nuclei with branched appearance. Calcification points (arrow) and extracellular matrix (Em) occurred during differentiation. **B** Adipogenic differentiation, Oil Red staining.

Fibroblast-like morphology, with lipid vesicles (arrow) in the cytoplasm. Cell nuclei located in the periphery (N). **C** and **D** Chondrogenic differentiation, stained with trichrome masson and picrosirius, respectively. Cells showing rounded shape similar to chondrocytes

cochlea tissues so far were cultured in suspension with induction factors N2 and/or B27 (Yerukhimovich et al. 2007). Then, cells modified their fibroblast-like appearance into spherical shape with a differentiation potential especially towards neural cell lineages (Diensthuber et al. 2009; Yerukhimovich et al. 2007). Thus, here the adherent culture condition method was chosen since it seems to be more suitable to produce multipotent stem cell lineages and may be favourable compared to the other approaches.

The phenotypic characterization of the cochlear epithelial cells was carried out using immunocytochemistry and flow cytometry techniques. The immunocytochemistry technique is a qualitative technique (Abbas et al. 2012) in contrast to flow cytometry that is a quantitative technique (Alcantara 2010).

The applied set of markers indicated differences in cell types in the experiment. In our flow cytometry analysis nestin was absent. Although it is a marker of neural progenitor cells, as demonstrated for mice tissues (Chao et al. 2013), it is probable that these neural cell lineages likely went lost during our undifferentiated cell cultivation. In contrast, ciliated, sensory cells and pluripotent stem cells were maintained, indicated by the presence of myosin VIIa and tubulin expression (Dabdoub et al. 2008; Banerjee et al. 2008; Piatto et al. 2005; Oliveira et al. 2002). In addition, the phenotypic characterization revealed the presence of the pluripotency markers Oct-4 and Sox-2, mesenchymal stem cell markers, i.e. Stro-1 and CD105, CD117 as markers for haematopoietic progenitor cells, and VEGF as vascular endothelial growth factor that functioned as an angiogenic agent. In contrast, CD34, a marker for mature hematopoietic cells, was absent. Thus, the data indeed revealed stem cells or the progenitor nature of these cells similar to what was seen to be characteristic for mesenchymal stem cells (e.g. Wenceslau et al. 2012; Dominici et al. 2006; Picciotti et al. 2006).

Through qPCR, the expression of Nanog, Pax8, Sox2, Myosin 7A and Cd90 genes was evaluated. The CD105 gene was expressed at low positive levels. Nanog is a specific pluripotent transcription factor (Lou et al. 2014). In addition, Sox2 and PAX8 are also transcription factors that have been suggested as the most important markers responsible for the development of the inner ear in humans (Lou et al. 2014). Studies in rats revealed that these genes are essential

for the development of the cochlea and for the development and maintenance of sensory cells (Dabdoub et al. 2008; Bouchard et al. 2010). Myosin 7A is expressed in sensory tissue of embryos and its expression has been used for detection of nerve fibers and ciliated cells (Dabdoub et al. 2008). A study in mice showed that the genes Nanog, Pax8, Sox2 and Myosin 7A were expressed in cells derived from the cochlea (Lou et al. 2014), similarly to our analyses. The cells derived from the cochlear epithelium of canine fetuses have characteristics of mesenchymal cells, this fact was confirmed by the low expression of the CD105 gene and high expression of CD90. Similar results were obtained by Cardoso (2015) for the culture of amniotic cells derived from the amniotic membrane of fetuses of dogs.

The multipotentiality of cochlear epithelial cells derived from dog was supported by the successful in vitro differentiation into the main cell lineages, i.e. osteogenic, adipogenic and chondrogenic cell lines, as preconized by several authors (Lima et al. 2012; Dominici et al. 2006).

Through these results, it is possible to classify these cells as stem cells, because they are adherent to the plastic in culture, they show expression of mesenchymal markers and absence of expression of hematopoietic cell markers. In addition, these cells, when induced, have an in vitro differentiation ability, as preconized by Dominici et al. (2006) who established these parameters for human cells for classification of stem cell lines. However, for animals nothing has been established, and following the human conditions (Dominici et al. 2006) we classified the cochlear epithelial cells derived from canine fetuses as a multipotency cell lineage.

In conclusion, the data derived from various fields of analysis consistently indicated that the cells derived from the cochlear epithelium of dog fetuses were able to be maintained undifferentiated in cell culture and resulted to be mesenchymal stem cells that may be of value for stem cell therapies in order to improve deafness and other diseases.

References

- Abbas AK, Lichtman AH, Pillai SHIV (2012) *Imunologia celular e molecular*, 7th edn. Elsevier, Rio de Janeiro

- Alcantara D (2010) Obtenção e caracterização de linhagens celular primária de osteossarcoma canino. 2010. 97 f. Dissertação (Mestrado em Ciências)—Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo
- Ambrosio CE, Zucconi E, Martins D, Vanucchi C, Perez M, Vieira N, Valadares M, Jazedje T, Miglino MA, Zatz M (2007) Extreme clinical variability in GRMD: from neonatal death to asymptomatic carriers. *Neuromuscul Disord* 17:776. doi:[10.1016/j.nmd.2007.06.056](https://doi.org/10.1016/j.nmd.2007.06.056)
- America (2016) Deafness and hearing loss. Fact sheet. N° 300. United States: World Health Organization 20
- Banerjee A, Jensen-Smith H, Lazzell A, Prasad V, Elguezabal G, Hallworth R, Ludeña RF (2008) Localization of betav tubulin in the cochlea and cultured cells with a novel monoclonal antibody. *Cell Motil Cytoskeleton* 65:505–514. doi:[10.1002/cm.20280](https://doi.org/10.1002/cm.20280)
- Barboza LC Jr, Oiticica J, Batisso AC (2008) Perspectives in the treatment of hearing loss with stem cells. *Int Arch Otorhinolaryngol* 12:111–115
- Bouchard M, Caprona D, Busslinger M, Xu P, Fritzsche B (2010) Pax2 and Pax8 cooperate in mouse inner ear morphogenesis and innervation. *Dev Biol* 10:89. doi:[10.1186/1471-213X-10-89](https://doi.org/10.1186/1471-213X-10-89)
- Cardoso MT (2015) Comparação molecular entre células-tronco mesenquimais de membrana amniótica de cão e de gato. 66f. Tese (Doutora em Ciências)—Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo
- Chao TT, Wang CH, Chen HC, Shih CP, Sytwu HK, Huang KL, Chen SY (2013) Adherent culture conditions enrich the side population obtained from the cochlear modiolus-derived stem/progenitor cells. *Int J Pediatr Otorhinolaryngol* 77:779–784. doi:[10.1016/j.ijporl.2013.02.010](https://doi.org/10.1016/j.ijporl.2013.02.010)
- Corrales CE, Pan L, Li H, Liberman MC, Heller S, Edge ASB (2006) Engraftment and differentiation of embryonic stem cell-derived neural progenitor cells in the cochlear nerve trunk: growth of processes into the organ of Corti. *J Neurobiol* 66:1489–1500. doi:[10.1002/neu.20310](https://doi.org/10.1002/neu.20310)
- Cunha FM, Coutinho SD, Matera A, Fiorio WAB, Ramos MCC, Silveira LMG (2003) Avaliação clínica e citológica do conduto auditivo externo de cães com otite. *Rev Educ Cont Vet Med Zootec* 6:1–3
- Dabdoub A, Puligilla C, Jones JM, Fritzsche B, Cheah KSE, Pevny LH, Kelley MW (2008) Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc Natl Acad Sci USA* 105:18396–18401. doi:[10.1073/pnas.0808175105](https://doi.org/10.1073/pnas.0808175105)
- Diensthuber M, Oshima K, Heller S (2009) Stem/progenitor cells derived from the cochlear sensory epithelium give rise to spheres with distinct morphologies and features. *J Assoc Res Otolaryngol* 10:173–190. doi:[10.1007/s10162-009-0161-3](https://doi.org/10.1007/s10162-009-0161-3)
- Doetzlhofer A, White PM, Johnson E, Segil N, Groves AK (2004) In vitro growth and differentiation of mammalian sensory hair cell progenitors: a requirement for EGF and periotic mesenchyme. *Dev Biol* 272:432–447. doi:[10.1016/j.ydbio.2004.05.013](https://doi.org/10.1016/j.ydbio.2004.05.013)
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8:315–317. doi:[10.1080/14653240600855905](https://doi.org/10.1080/14653240600855905)
- Evans HE, Sack WO (1973) Prenatal development of domestic and laboratory mammals: growth curves, external features and selected references. *Anat Histol Embryol* 2:11–45. doi:[10.1111/j.1439-0264.1973.tb00253.x](https://doi.org/10.1111/j.1439-0264.1973.tb00253.x)
- Favaron PO, Mess A, Will SE, Maiorka PC, Oliveira MF, Miglino MA (2014) Yolk sac mesenchymal progenitor cells from New World mice (*Necromys lasiurus*) with multipotent differential potential. *PLoS ONE* 9:e95575. doi:[10.1371/journal.pone.0095575](https://doi.org/10.1371/journal.pone.0095575)
- Fratini P, Carreira ACO, Alcantara D, de Oliveira E Silva FM, Rodrigues MN, Miglino MA (2016) Endothelial differentiation of canine yolk sac cells transduced with VEGF. *Res Vet Sci* 104:71–76. doi:[10.1016/j.rvsc.2015.11.010](https://doi.org/10.1016/j.rvsc.2015.11.010)
- Gothelf LN (2007) Doenças do ouvido em pequenos animais. São Paulo, Roca, p 368
- Guyton AC, Hall JE (2011) Tratado de fisiologia médica, 12th edn. Elsevier, Rio de Janeiro, p 1128
- Horn PA, Morris JC, Neff T, Kiem HP (2004) Stem cell gene transfer—efficacy and safety in large animal studies. *Mol Ther* 10:417–431. doi:[10.1016/j.ymthe.2004.05.017](https://doi.org/10.1016/j.ymthe.2004.05.017)
- Hutchin TP, Cortopassi GA (2000) Mitochondrial defects and hearing loss. *Cell Mol Life Sci* 57:1927–1937
- Li H, Liu H, Heller S (2003) Pluripotent stem cells from the adult mouse inner ear. *Nat Med* 9:1293–1299. doi:[10.1038/nm925](https://doi.org/10.1038/nm925)
- Lima SAF, Wodewotzky TI, Lima-Neto JF, Beltrão-Braga PCB, Alvarenga FCL (2012) Diferenciação in vitro de células-tronco mesenquimais da medula óssea de cães em precursores osteogênicos. *Pesqui Vet Bras* 32:463–469. doi:[10.1590/S0100-736X2012000500016](https://doi.org/10.1590/S0100-736X2012000500016)
- Lou X, Zhang Y, Yuan C (2007) Multipotent stem cells from the young rat inner ear. *Neurosci Lett* 416:28–33. doi:[10.1016/j.neulet.2006.12.061](https://doi.org/10.1016/j.neulet.2006.12.061)
- Lou X, Dong Y, Xie J, Wang X, Yang L, Tokuda M, Zhang Y (2014) Comparing the cultivated cochlear cells derived from neonatal and adult mouse. *J Transl Med* 29:150. doi:[10.1186/1479-5876-12-150](https://doi.org/10.1186/1479-5876-12-150)
- Malgrange B, Belachew S, Thiry M, Nguyen L, Rogister B, Alvarez ML, Rigo JM, Vande Water TR, Moonen G, Lefebvre PP (2002) Proliferative generation of mammalian auditory hair cells in culture. *Mech Dev* 112:79–88
- Martinez-Monedero R, Edge A (2007) Stem cells for the replacement of inner ear neurons and hair cells. *Int J Dev Biol* 51:655–661. doi:[10.1016/j.neulet.2006.12.061](https://doi.org/10.1016/j.neulet.2006.12.061)
- Müller E, Heusinger A (1994) Microbiological results of ear swabs from dogs and cats. *Tierarztl Prax* 22:80–84
- Oliveira JAA, Canedo MC, Rossato M (2002) Otoproteção das células ciliadas auditivas contra a ototoxicidade da ampicilina. *Rev. Bras. Otorrinolaringol* 68:7–13. doi:[10.1590/S0034-72992002000100002](https://doi.org/10.1590/S0034-72992002000100002)
- Oshima K, Grimm CM, Corrales CE, Senn P, Martinez-Monedero R, Géléoc GSG, Edge A, Holt JR, Heller S (2007) Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. *J Assoc Res Otolaryngol* 8:18–31
- Parker MA, Corliss DA, Gray B, Anderson JK, Bobbin RP, Snyder EY, Cotanche DA (2007) Neural stem cells injected into the sound-damaged cochlea migrate throughout the

- cochlea and express markers of hair cells, supporting cells, and spiral ganglion cells. *Hear Res* 232:29–43. doi:[10.1016/j.heares.2007.06.007](https://doi.org/10.1016/j.heares.2007.06.007)
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45. doi:[10.1093/nar/29.9.e45](https://doi.org/10.1093/nar/29.9.e45)
- Piatto VB, Nascimento ECT, Alexandrino F, Oliveira CA, Lopes ACP, Sartorato EL, Maniglia JV (2005) Genética molecular da deficiência auditiva não-sindrômica. *Rev Bras Otorrinolaringol* 71:216–223. doi:[10.1590/S0034-72992005000200016](https://doi.org/10.1590/S0034-72992005000200016)
- Picciotti PM, Fetoni AR, Paludetti G, Wolf FI, Torsello A, Troiani D, Ferraresi A, Pola R, Sergi B (2006) Vascular endothelial growth factor (VEGF) expression in noise-induced hearing loss. *Hear Res* 214:76–83. doi:[10.1016/j.heares.2006.02.004](https://doi.org/10.1016/j.heares.2006.02.004)
- Savary E, Hugnot JP, Chassigneux Y, Travo C, Duperray C, Van de Water T, Zine A (2007) Distinct population of hair cell progenitors can be isolated from the postnatal mouse cochlea using side population analysis. *Stem Cells* 25:332–339. doi:[10.1634/stemcells.2006-0303](https://doi.org/10.1634/stemcells.2006-0303)
- Shi Y, Kirwan P, Livesey FJ (2012) Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nat Protoc* 7:1836–1846. doi:[10.1038/nprot.2012.116](https://doi.org/10.1038/nprot.2012.116)
- Tolosa EMC, Rodrigues CJ, Behmer AO, Neto AGF (2003) Manual de Técnicas para Histologia Normal e Patológica, 2nd edn. Editora Manole, Barueri, pp 19–57
- Wenceslau CV, Miglino MA, Martins DS, Ambrósio CE, Lizier NF, Pignatari GC, Kerkis I (2012) Mesenchymal progenitor cells from canine fetal tissues: yolk sac, liver, and bone marrow. *Tissue Eng A* 17:2165–2176. doi:[10.1089/ten.TEA.2010.0678](https://doi.org/10.1089/ten.TEA.2010.0678)
- White PM, Doetzlhofer A, Lee YS, Groves AK, Segil N (2006) Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature* 441:984–987. doi:[10.1038/nature04849](https://doi.org/10.1038/nature04849)
- White PM, Stone JS, Groves AK, Segil N (2012) EGFR signaling is required for regenerative proliferation in the cochlea: conservation in birds and mammals. *Dev Biol* 363:191–200. doi:[10.1016/j.ydbio.2011.12.035](https://doi.org/10.1016/j.ydbio.2011.12.035)
- Xie F, Xiao P, Chen D, Xu L, Zhang B (2012) MiRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol* 80:75–84. doi:[10.1007/s11103-012-9885-2](https://doi.org/10.1007/s11103-012-9885-2)
- Yerukhimovich MV, Bai L, Chen DHC, Miller RH, Alagramam KN (2007) Identification and characterization of mouse cochlear stem cells. *Dev Neurosci* 29:251–260. doi:[10.1159/000096415](https://doi.org/10.1159/000096415)