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ORIGINAL ARTICLE

Expression of Notch Receptors and Ligands in the Coccygeal Body

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Abstract

BACKGROUND: The recent discovery of neural stem cells in the sacrococcygeal end of the filum terminale, the presence of remnants of the most powerful totipotent stem cell generators and inductors, the primitive streak and node, the existence of the unique non-mutator sacrococcygeal teratomas, and the recent disclosing of neuroimmunomodulatory and hematopoietic roles of Luschka's body, indicate that the sacrococcygeal region is a distinctive anatomic environment rich in stem cells and instructive signals, and that the coccygeal body may constitute a more complex entity than a mere caudal, vascularly-derived glomic anastomosis. Ascribed as an arterial-venous shunt located at the tip of the coccyx and analog to the glomera caudalia in other vertebrates, the glomus coccygeum has recently revealed a complex organ with peculiar 3D topology, broad innervation, catecholamine-synthesizing activity, and neutrophil-formation and lymphopoietic-regulating properties.

METHODS: In the present research work, we sought to start exploring the potential cell-functional roles of the glomus coccygeum by conducting a methodical assessment of the expression of Notch pathway receptors and ligands in the human Luschka's body.

RESULTS: Our data indicates that Notch receptors are dynamically and distinctively expressed in the coccygeal body and that Notch ligands are markedly differentially expressed in newborn and adult coccygeal glomi.

CONCLUSIONS: Our observations suggest that Notch signaling may have relevant roles in glomus coccygeum function and biology.

INTRODUCTION

Discovered by the eminent anatomist Hubert von Luschka over 150 years ago and considered by him as the posterior counterpart of the pituitary gland (Luschka, 1860a, 1860b), the glomus coccygeum (coccygeal body, Luschka's body) was later ascribed by 20th century anatomists and histopathologists as a vascular anastomosis between the caudal aorta and the middle sacral vein and, therefore, analog to vertebrates' glomera caudalia and with plausible roles in thermoregulation (Hongo & Luck, 1953; Schumacher, 1908; Staubesand, 1953b, 1953c, 1953a; Wright, 1977). Notwithstanding that its biology and functional relevance have remained largely unexplored, few more recent studies have started to shed light into the organismal functions of this organ. Histologically, the coccygeal body is a complex glomus tissue formed by vascular anastomosis and epithelioid cells that are markedly positive for smooth muscular actin (Gatalica *et al.* 1999; Jin *et al.* 2017; John & Rao, 2017; Maggiani *et al.* 2011; Rahemtullah *et al.* 2005; Santos *et al.* 2002) and arranged within a connective tissue-derived capsule (Jin *et al.* 2017; Kondo, 1972; Maggiani *et al.* 2011; Santos *et al.* 2002; Woon & Stringer, 2012). The organ is formed by various lobes whose numbers vary across individuals and is, most of the times, located as a helicoidal structure in the lower and anterior side of the last coccygeal vertebrae (Kubota, 1954; Sargon *et al.* 2002; Sargon *et al.* 1998). The Luschka's body receives innervation from both the sympathetic and parasympathetic trunks and also from the spinal anococcygeal nerve (Henningsen, 1969; Jin *et al.* 2016; Jin *et al.* 2017; Kubota, 1954). While highly vascular, a plausible neuroendocrine function of the glomus coccygeum could be attributed since acetylcholine-like activity has been detected in human coccygeal glomus extracts (Luckner & Staubesand, 1951), adrenalin and noradrenalin have been found present in the epithelioid cell compartment (Wright & Wormald, 1979), and the removal of caudal glomera in rats results in decreased levels of dopamine, adrenalin and noradrenalin in the bone marrow (Conti *et al.* 2000).

Notch is a highly-conserved signaling pathway that is widely used by multicellular organisms during development and in the maintenance of adult homeostasis (Jafar-Nejad *et al.* 2010; Kopan & Ilagan, 2009; Ntziachristos *et al.* 2014), and plays crucial roles in the regulation of important biological processes including cell proliferation, differentiation, cell fate specification, compartment boundary formation, asymmetric cell division, and lateral inhibition (Carlson & Conboy, 2007; Chiba, 2006; Fortini, 2009; Kopan & Ilagan, 2009; Regan *et al.* 2013; Tien *et al.* 2009; Wang *et al.* 2009). Importantly, Notch is also crucial during gastrulation and is central in maintaining the pace and synchronicity of the molecular clock during somite formation, in instructing node's inductive and ciliary-dependent signals, and in regulating left-right asymmetry (Krebs *et al.* 2003; Liao & Oates, 2017; Lopes *et al.* 2010; Raya *et al.* 2003). Notch signaling is initiated when heterodimeric transmembrane Notch receptors (Notch1, 2, 3 and 4) on "signal-receiving cells" interact with transmembrane ligands of the Delta (Delta-like1, 3 and 4) and Serrate/Jagged (Jagged1 and 2) families on apposed "signal-sending cells" (Guruharsha *et al.* 2012; Jafar-Nejad *et al.* 2010; Kopan & Ilagan, 2009). Upon ligand binding, Notch receptors experiment a catch bond-mediated pulling force (Luca *et al.* 2017) from the signal-sending cell that causes, in the signal-receiving cell, a series of sequential proteolytical cleavages catalyzed by ADAM10/17 proteases (S2 site) and the γ-secretase complex (S3/S4 sites) that result in the release of the Notch intracellular domain (NICD) and its consecutive translocation to the cell nucleus, where, in a complex with CSL (Rbpjk) and Mastermind proteins, activates the expression of Notch downstream target genes (Guruharsha *et al.* 2012; Jafar-Nejad *et al.* 2010; Kopan & Ilagan, 2009). Notably, the Notch pathway can also be activated in a non-canonical, ligand-independent manner, which primarily occurs when there is disruption of genes that control endosomal sorting and ubiquitination, and often results in accidental and aberrant pathway activity that has been distinctively observed in human malignancies (Palmer & Deng, 2015; Siebel & Lendahl, 2017; Wu *et al.* 2007).

In the present research work we examine the expression of Notch pathway receptors and ligands in the human Luschka's body and, furthermore, conduct a comparative expression analysis of them between newborn and adult coccygeal glomi. Our studies are the first to explore the implication of the Notch pathway in the function of Luschka's body in vertebrates.

MATERIALS AND METHODS

Tissue specimens

Formalin-fixed and paraffin-embedded pericoccygeal tissues from two-week old newborn were obtained from the Department of Pathology at Wayne State University School of Medicine, while tissue sections from formalin-fixed and paraffin-embedded adult pericoccygeal tissues were obtained from the Department of Pathology at the University of Pittsburgh. Samples were obtained strictly following approved Institutional policies and protocols.

Antibodies and immunoreagents

The following antibodies for Notch receptors and ligands were used: anti-Notch1 (rabbit monoclonal D1E11, Cell Signaling, 3608S), anti-Notch2 (rabbit polyclonal, Santa Cruz, sc-5545), anti-Notch3 (rabbit polyclonal, abcam, ab23426), anti-Notch4 (rabbit polyclonal, SIGMA, 07-189), Jagged1 (goat polyclonal, Santa Cruz, sc-5545), Jagged2 (rabbit polyclonal, SIGMA, HPA030636), Delta-like-1 (rabbit polyclonal, abcam, ab10554), Delta-like-3 (rabbit polyclonal, Cell

Signaling, 2483S) and Delta-like-4 (rabbit polyclonal, BIORAD, ahp1274). For alpha smooth muscle actin immunodetection the 1A4 mouse monoclonal antibody (SIGMA, A2547) was used. Normal goat serum (from Vectastain ABC kit) and normal horse serum (S-2000-20, used in Jagged1 and SMA immunodetection) were used as unspecific antibody binding blockers and were obtained from Vector Labs. Biotinylated horse anti-goat (BA-9500), Biotinylated horse anti-mouse (BA-2001), Vectastain ABC kit (PK-4001, used for immunodetection of rabbit primary antibodies), and ImmPACT DAB (SK-4105) were also obtained from Vector Labs.

Immunohistochemistry

5µm tissue sections were used for immunohistochemical reactions. Tissues were deparaffinized in xylenes and hydrated through serial alcohols to water. Antigen retrieval was done through heat-induced epitope retrieval and conducted by boiling tissue samples for 20 min in 10mM sodium citrate solution pH6.0. Endogenous peroxidase blocking was performed by incubation in 3% H_2O_2 in methanol and sections were subsequently incubated for 1h in 3% normal serum in PBS for blocking unspecific antibody binding. Samples were then incubated with distinct primary antibodies overnight at 4°C in PBS containing 0.01% Tween-20. After three washes in PBS, samples were incubated with biotinylated secondary antibodies (either horse anti-goat for Jagged1 immunoreactions, horse anti-mouse for SMA immunodetection, or goat anti-rabbit from the Vectastain ABC kit for all other immunoreactions) for 1h at room temperature. Incubation with avidin DH and biotinylated horseradish peroxidase complexes (Vectastain ABC kit) were done for 30 min in PBS containing 0.01% tween-20 and following manufacturer instructions. Chromogenic reactions were conducted in time-measured manner to comparably assess the expression of Notch receptors and ligands between newborn and adult specimens. Counterstaining was performed with Mayer's hematoxylin and samples were dehydrated and mounted with Permount (Fisher, SP15-100). Images were acquired in a Leica DMLB microscope and using Spot Imaging Software 5.1 (Diagnostic Instruments).

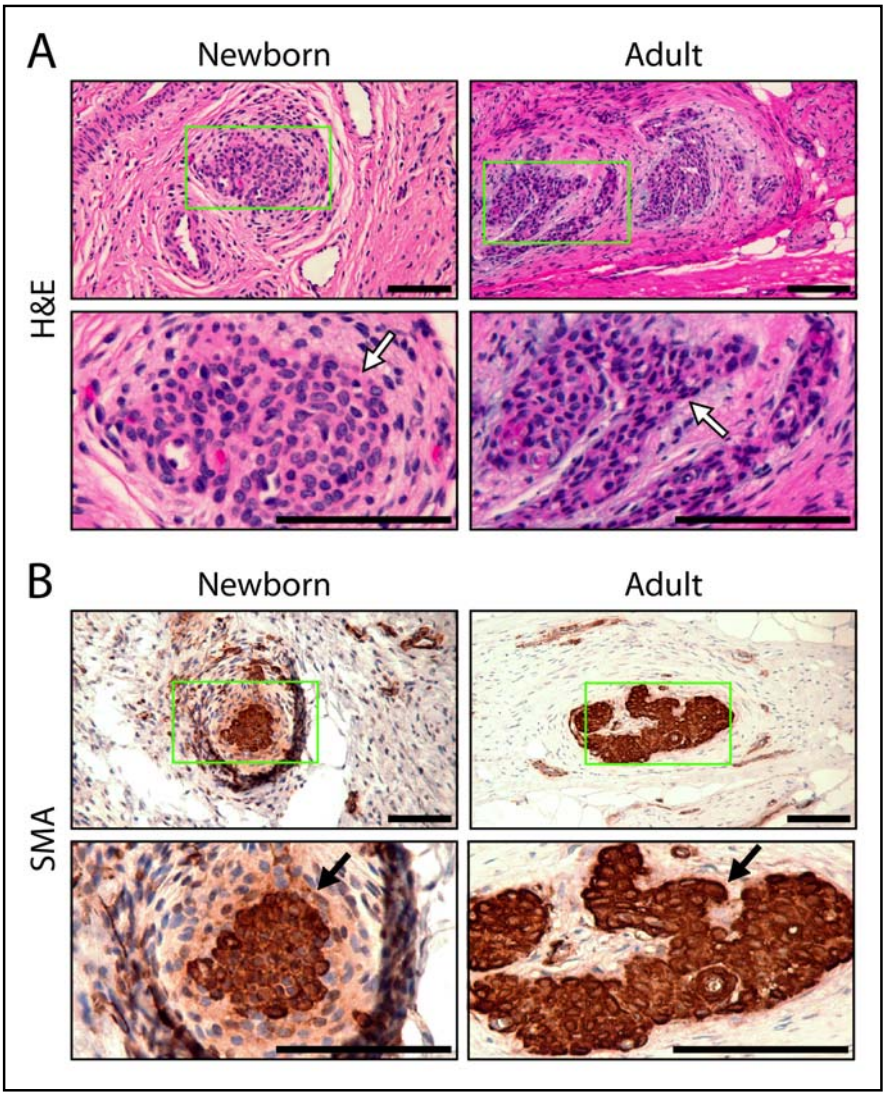

Fig. 1. Luschka's body in newborn and adult pericoccygeal tissue. (A) Representative hematoxylin and eosin (H&E) stainings in newborn (2-week old) and adult pericoccygeal specimens showing the presence of Luschka's bodies in anastomotic structures surrounded by a stromal-type capsule. The bottom, high-magnification images correspond to the regions marked by a green rectangle in the top microphotographs. Note the distinctive H&E staining showed by the epithelioid cells conforming the coccygeal bodies (white arrows in high magnification images) compared to the surrounding pericoccygeal tissue. **(B)** Smooth alpha actin (SMA) immunohistochemistry showing strong immunoreactivity (brown signal) in epithelioid cells of adult and newborn coccygeal bodies. Note that although SMA immunoreactivity is easily and readily observable in epithelioid cells in both newborn and adult coccygeal bodies (also indicated by black arrows in bottom images), the margins for SMA signals in adult specimens are better well defined, while some peripheral cells on the coccygeal body in newborn specimens display low levels of SMA. The green rectangles in top microphotographs indicate the regions showed in the bottom, high-magnification images. Bars, 100 µm.

RESULTS

Luschka's coccygeal body in newborn and adult pericoccygeal tissue. To begin exploring the role of Notch signaling in Luschka's body, we first decided to analyze specimens of adult and newborn (2-week old) pericoccygeal tissue for determining the presence of coccygeal glomi. Our analysis in hematoxylin and eosin (H&E) stainings showed that putative coccygeal glomi were readily identifiable in both types of specimens, although they were present with relatively smaller size in newborn samples (Fig 1A). Coccygeal bodies were usually presented apposed to blood vessels, had a surrounding stromal capsule, and their epithelioid cells had a distinctive H&E staining appearance that was easily recognizable. Notwithstanding however, and in order to further confirm the presence and to also determine the relative location of Luschka's bodies in the distinct specimen sections, we conducted immuhistochemical stainings for alpha smooth muscle actin (SMA), which is a very well established marker for the epithelioid cells present in coccygeal glomi (Gatalica *et al.* 1999; Jin *et al.* 2017; John & Rao, 2017; Maggiani *et al.* 2011; Rahemtullah *et al.* 2005; Santos *et al.* 2002). Our stainings for SMA indicated that the coccygeal bodies, from either newborn or adult specimens, were intensely positive for SMA (Fig. 1B), and the bodies presented in various forms from fusiform shapes to lobular and circular ones. Interestingly, our data also showed that while the immunohistochemial stainings in adult coccygeal bodies were quite well demarcated and with clean borders with respect to the neighbor stromal cells, some epithelioid cells located relatively peripheral in the cocygeal bodies in newborn specimens displayed a somewhat weak, but nonetheless, positive SMA staining (Fig. 1B).

Notch receptors are dynamically expressed in the glomus coccygeum. After having verified the presence and location of coccygeal bodies in adult and newborn pericoccygeal tissue sections, we then decided to conduct comparative immunohistochemical stainings between newborn and adult coccygeal bodies using previously-characterized antibodies against Notch receptors (Table I). Our results indicate that Notch receptors are dynamically expressed in the coccygeal glomi, particularly with respect to Notch1 and Notch2 (Fig. 2). More concretely, our data indicates that while Notch1 is expressed at moderate levels in newborn coccygeal glomi, its expression is almost completely lacking in the adult counterpart (Fig. 2A). Similarly, Notch2 protein expression seems to also be differentially regulated between newborn and adult coccygeal glomi, although in this case it is evident that, although at quite low levels,

Tab. 1. Antibodies used in the study

Target	Catalog number	Dilution	Key references
Notch1	3608S	1:100	Chattopadhyay *et al.* 2018, Sci Rep. **8**: 9032. Hoare *et al.* 2016, Nat Cell Biol. **18**(9): 979–992. Ding *et al.* 2018, Oncol Rep. **39**(6): 2584-2594.
Notch2	sc-5545	1:100	Stahl *et al.* 2008, J Biol Chem. **283**(20): 13638–13651. Ustunel *et al.* 2008, Acta Histochem. **110**(5): 397–407. Nickoloff *et al.* 2002, Cell Death Differ. **9**(8): 842–55.
Notch3	ab23426	1:200	Zhang *et al.* 2017, Nat Commun. **8**(1): 144. Natsuizaka *et al.* 2018, Nat Commun. **8**(1): 1758. Chen *et al.* 2016, EMBO Mol Med. **8**(7): 712–728.
Notch4	07-189	1:100	Callahan *et al.* 2011, J Cell Physiol. **226**(7): 1940–1952. Castro *et al.* 2015, Oncotarget. **6**(14): 11910–11929. Cobellis *et al.* 2013, J Anat. **213**(4): 464–472.
Jagged1	sc-6011	1:200	Lopez-Arribillaga *et al.* Nat Commun., 2018, **9**(1): 2992–3005. Massi *et al.* 2006, Mod Pathol., **19**(2): 246–254. Nickoloff *et al.* 2002, Cell Death Differ. **9**(8): 842–855.
Jagged2	HPA030636	1:100	Kousa *et al.* 2017, Dev Dyn. **246**(9): 670–681. Uhlén *et al.* 2015, Science **347**(6220): 1260419.
Delta-like1	ab10554	1:200	Xie *et al.* 2017, Cell Transplant. **26**(6): 967–982. Sörensen *et al.* 2009, Blood **113**(22): 5680–5688. Murta *et al.* 2014, PLoS One **9**(11): e113365.
Delta-like3	2483S	1:100	Song *et al.* 2018, Exp Ther Med. **16**(1): 53–60. Cardnell *et al.* 2017, Oncotarget **8**(43): 73419–73432.
Delta-like4	ahp1274	1:200	Gunin *et al.* 2014, Exp Gerontol. **55**: 143–151. Song *et al.* 2018, Exp Ther Med. **16**(1): 53–60. Hindy *et al.* 2013, Neuro Oncol. **15**(10): 1366–1378.
Smooth alpha actin (SMA)	A2547 (1A4)	1:400	Santos *et al.* 2002, Pathology. **34**(4): 339–343. Jin *et al.* 2017, Anat Rec (Hoboken). **300**(10): 1826–1837. Maggiani *et al.* 2011, Skeletal Radiol. **40**(11): 1455–1459.

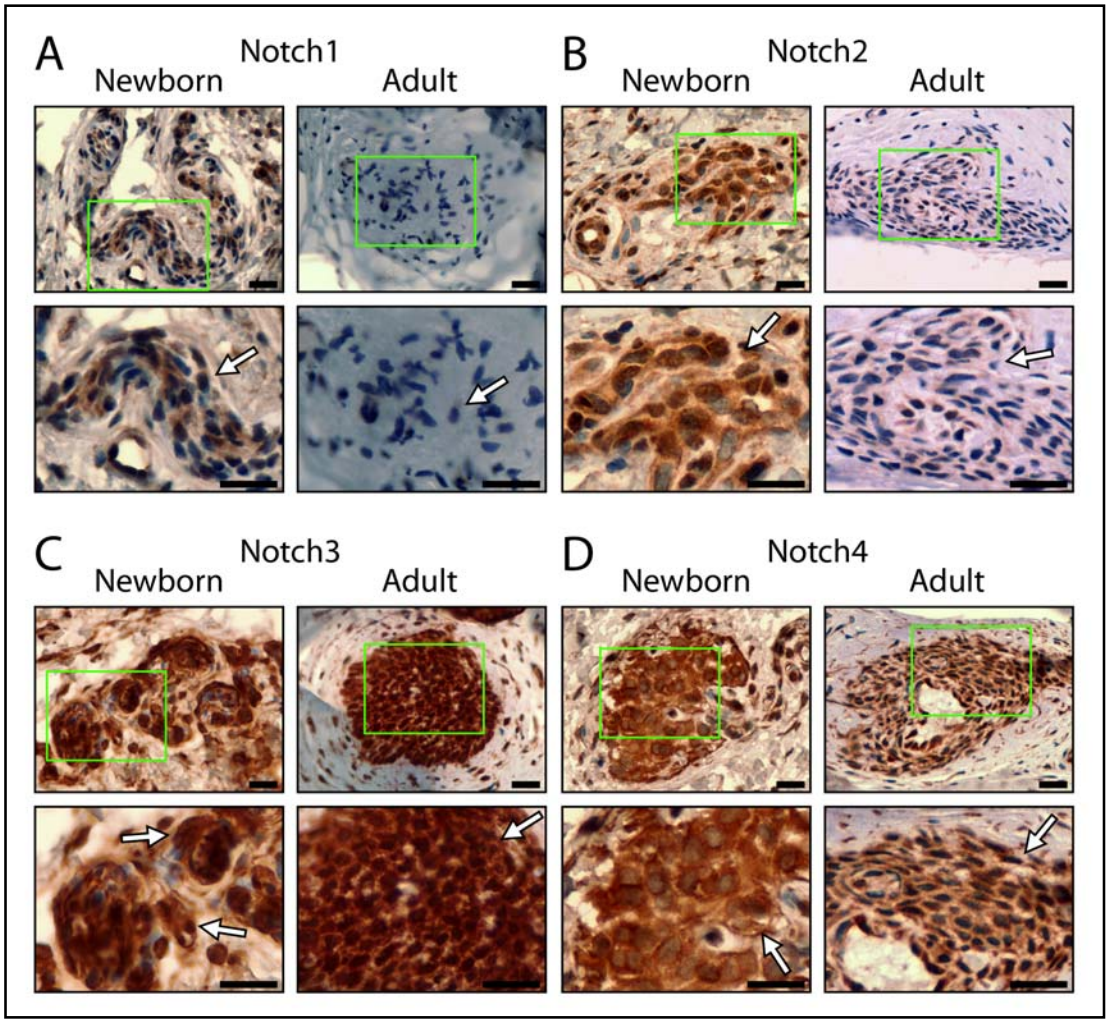

Fig. 2. Notch receptors are distinctively expressed in the glomus coccygeum. Low and high magnification immunohistochemical stainings for Notch Receptors (Notch1, Notch2, Notch3, Notch4) showing that they are differentially expressed in the glomus coccygeum in newborn and adult samples. **(A)** Notch1 receptor staining showing that Notch1 is expressed at moderate levels in newborn glomus coccygeum and is contrastingly absent in adult Luschka's body. **(B)** Notch2 receptor immunostaining demonstrating that while Notch2 is expressed at moderate levels in the newborn coccygeal body, its expression levels are markedly lesser in adult coccygeal glomus cells. **(C)** Notch3 receptor is expressed at very high levels in epithelioid cells and with comparable intensity in the newborn and adult coccygeal body. **(D)** Immunostaining for Notch4 receptor indicating that while it is expressed at high levels in newborn and adult samples, its expression is somewhat lower in the latter. Note that the stained portions represent the glomus coccygeal epithelioid cells while the surrounding unstained tissue represents smooth muscle cells and connective tissue. The green rectangles in top microphotographs correspond to the regions showed in the bottom, high-magnification images, and the arrows, in the bottom microphotographs point the epithelioid cells in the distinct specimens. Bars, 20 μm.

Notch2 is present in the adult coccygeal gland (Fig. 2B). On the contrary to these findings however, the expression levels of Notch3 and Notch4 receptors are rather comparable between adult and newborn coccgygeal glands (Fig. 2C, D), although Notch4 expression levels might be slightly decreased in adult glomi (Fig. 2D). Importantly, it should be noted that the negative and/or low-signal staining for Notch1 and Notch2 in adult glomi was not due to technical limitations since our positive-controls clearly detected Notch1 and Notch2 proteins in skin (Fig. S1), which is a tissue known for displaying high levels of expression of these two receptors (Kim *et al.* 2016; Nickoloff *et al.* 2002; Pan *et al.* 2004), and, thus, this possibility is ruled out. Together, these data indicate that the expression of Notch receptors is distinctively regulated in the Luschka's body, and that the expression of some of them (Notch1 and Notch2) is markedly developmentally-controlled.

Notch ligands of the Jagged and Delta-like families are differentially regulated in the glomus coccygeum. Once demonstrated Notch receptors expression in the coccygeal glomi, we then centered our attention in determining whether Notch ligands were also expressed in the coccygeal body and on whether their expression may be distinctively regulated between newborn and adult glomi. Like with our studies on Notch receptors, we also used previously-characterized antibodies against ligands of the Jagged and Delta-like families (Table I).

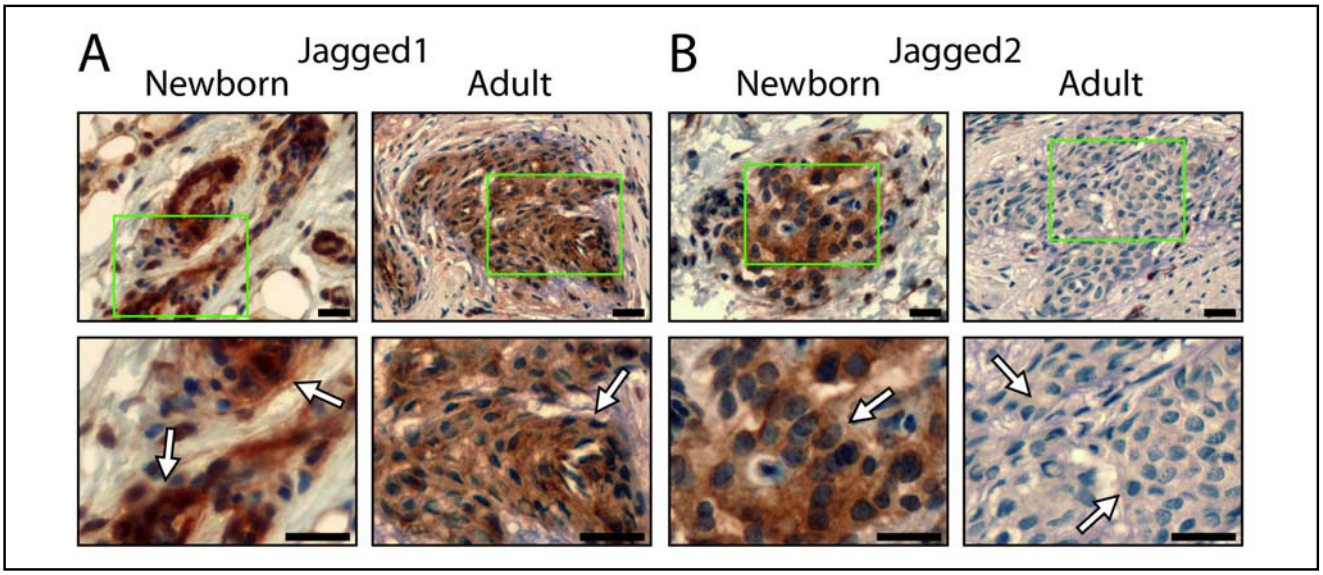

Fig. 3. Expression of Jagged ligands in the coccygeal body. Immunohistochemical stainings for Notch ligands Jagged1 and Jagged2 demonstrating a varying expression pattern in the coccygeal body of newborn and adult samples (brown staining). Low (top) and high (bottom) magnification images are presented. **(A)** Jagged1 immunostaining showing that Jagged1 is highly expressed in newborn and adult glomus coccygeum samples, although its expression in adult specimens is somewhat lower compared to newborn. **(B)** Jagged2 expression is high in the newborn glomus coccygeum, whereas, although it may still be considered positive in the adult sample (see against surrounding stroma), its levels are dramatically lower. The high magnification microphotographs correspond to the regions marked with green rectangles in top images, and the epithelioid cells are indicated with white arrows. Bars, 20 μm.

Our results in these studies indicate that Jagged1 is markedly expressed in the epithelioid cells of the glomus coccygeum both in newborn and in adults although its levels are somewhat lower in the adult glomus (Fig 3A). Interestingly, our immunohistochemical stainings for Jagged2 showed that the expression of this ligand was dramatically downregulated in the adult coccygeal body (Fig 3B). With respect to ligands of the Delta-like family, our data indicates that the expression of Delta-like1 was particularly high in both newborn and adult coccygeal bodies and with even relatively slightly higher levels in the adult glomus (Fig. 4A). Like with Jagged2, our results for Delta-like3 showed that the expression of this ligand was quite robust in the epithelioid cells of newborn cocygeal bodies while it became dramatically downregulated in adult coccygeal glomi (Fig. 4B). Somewhat similarly, but to a lesser extent, we also saw that Delta-like4 expression was lower in the adult coccygeal gland compared to the newborn, although its levels in the adult glomus could be readily noticeable (Fig 4C). Notably, while relatively lower signals for Jagged1, Delta-like1 and Delta-like 4 expression could be observed in the surrounding stroma and connective tissue, the expression of Jagged2 and Delta-like3 in the newborn coccygeal glomus was remarkably well-confined to the epithelioid cell compartment (Figs. 3, 4), indicating that the expression of these ligands could be considered more restricted to coccygeal glomi. Our results indicate that although Notch ligands are patently and highly expressed of newborn coccygeal glomi, their expression is under a markedly distinctively developmental control.

DISCUSSION

Notch is an important developmental signaling system that has recently drawn significant attention due to its involvement in a number of congenital and acquired diseases including human cancers like T-cell lymphoblastic leukemia (Ellisen *et al.* 1991; Weng *et al.* 2004), breast cancer (Jiao *et al.* 2012; Robinson *et al.* 2011), glioblastoma (Hulleman *et al.* 2009; Kanamori *et al.* 2007; Shih & Holland, 2006), and lung cancer (Chammaa *et al.* 2018; Westhoff *et al.* 2009; Xie *et al.* 2013; Yuan *et al.* 2015). Notably, Notch regulates stemness and self-renewal of normal and cancer stem cells (Koch *et al.* 2013; Venkatesh *et al.* 2018) and, during gastrulation, markedly contributes to the allocation and number of progenitor cells derived from the node (Gray & Dale, 2010; Souilhol *et al.* 2015). Importantly, while Notch activity ultimately depends on the nuclear translocation of Notch receptors' intracellular domain—either as result or ligand-dependent or -independent activation—the expression analysis of its components is an essential step towards initiating studies aimed to identifying putative functional roles, determining cellular domains for the expression of the distinct pathway components, and initiating promoters' activity characterization. Furthermore, expression analysis of Notch pathway components has demonstrated quite useful as tool for predicting outcomes in a number of human cancers (Chammaa *et al.* 2018; Sun *et al.* 2017; Yuan *et al.* 2015). In our study we have conducted a concise but methodical expression analysis of Notch receptors and ligands in the human

Luschka's body with the scope of beginning to study Notch pathway contribution in coccygeal body's biology. Our results clearly show that Notch receptors and ligands are expressed in the human coccygeal body, and with particularly high levels in newborn coccygeal glomi. Furthermore, our data reveals that a developmental control is in place since the expression of some of the receptors and ligands is downregulated in the adult coccygeal body. Interestingly, Delta-like1 expression seems to show an inverse trend since its levels are, although slightly, somewhat higher in the adult coccygeal body. Also important, it should be noted that Delta-like3 expression seems to be the most confined since the surrounding myocytes and stromal cells were markedly negative in newborn specimens. Although our studies are in principle exploratory and cannot establish Notch's functional contributions to Luschka's body, they have, nonetheless, revealed a functional developmental regulation on the expression of Notch receptors and ligands, and, therefore, do warrant further investigation into the transcriptional and/or translational mechanisms involved in this differential expression regulation. Obviously, future studies with loss- and gain-of-function analyses will also be necessary to define the functional roles that Notch receptors and ligands may have in the coccygeal body.

The glomus coccygeum is considered an arteriovenous anastomosis (Rahemtullah *et al.* 2005; Santos *et al.* 2002; Sargon *et al.* 1998; Woon & Stringer, 2012) and its involvement in coccygeal glomus tumor development and coccydinia is well documented (Duncan *et al.* 1991; Maggiani *et al.* 2011; Pambakian & Smith, 1981). Certainly, the glomus coccygeum is a frequent incidental finding in pathology examination of sacrococcygeal specimens (Albrecht & Zbieranowski, 1990; John & Rao, 2017), and is often misdiagnosed as sacrococcygeal glomus tumors (Bell *et al.* 1982; Gatalica *et al.* 1999). Notwithstanding however, previous observations on the identification of vasoactive molecules in homogenates of glomera caudalia (Luckner & Staubesand, 1951; Wright & Wormald, 1979), and the reported lymphocyte-promoting, neutrophil-modulating, and metabolic energy-balancing roles (Conti *et al.* 2000), indicate that this organ may possess functions more important than what previously thought. Notably, it should also be considered that although this organ scores negative for the mesoderm formation regulator, brachyury (Maggiani *et al.* 2011), it does score positive for the EMT (epithelial-to-mesenchymal transition) inductor, vimentin, and neuron-specific enolase (Gatalica *et al.* 1999; Rahemtullah *et al.* 2005), and, furthermore, lies within a microanatomical environment from where malignant sacrococcygeal teratomas—which don't seem to bear genetic alterations (Weinberg, 2014) and, therefore, may represent a paradigm-changing paradox to the gene mutation cancer theory—arise, and where neural stem cells associated to the filum terminale and totipotent stem cells derived from the

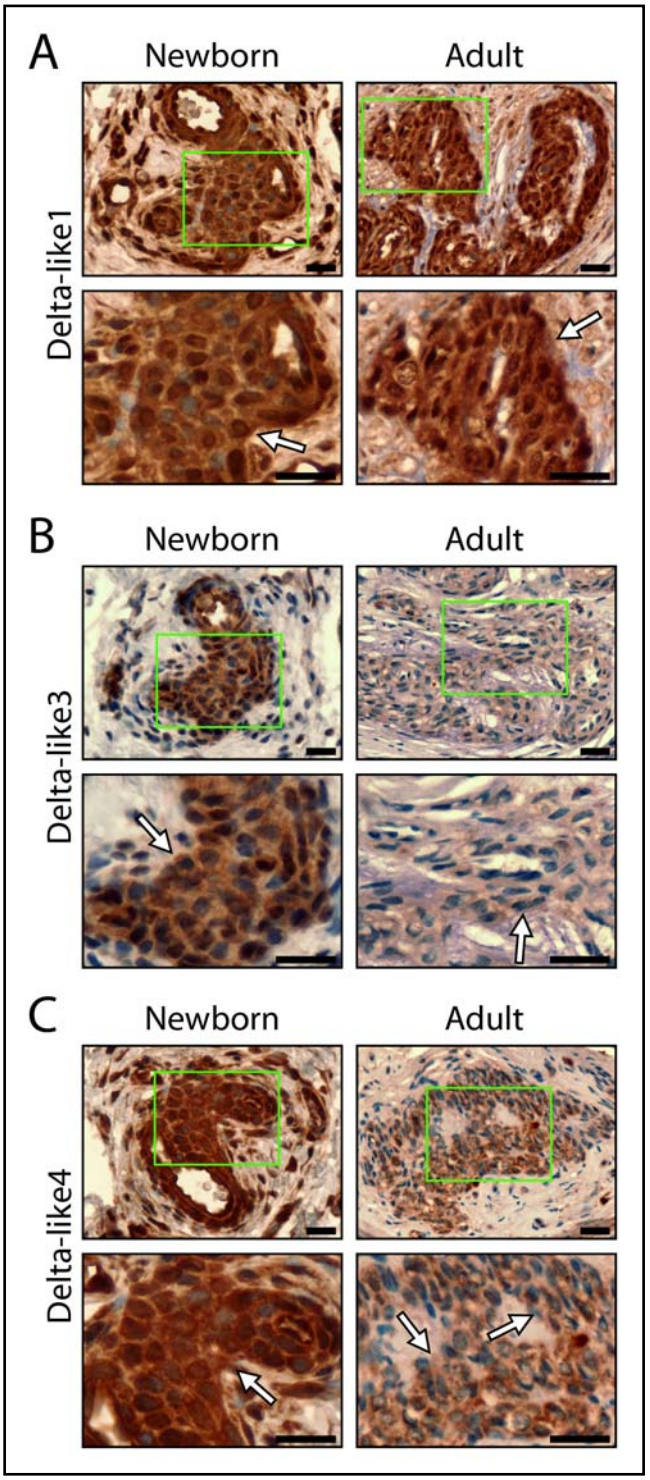

Fig. 4. Expression of Delta-like ligands in the Luschka's body. Immunohistochemical stainings demonstrating that Notch ligands of the Delta-like family (Dll1, 3, and 4) are expressed variably in newborn and adult samples of glomus coccygeum. **(A)** Dll1 is highly expressed in both the newborn and adult coccygeal body, although with slightly higher expression in adult specimens. **(B)** Dll3 immunohistochemical stainings show that Dll3 is highly expressed in the newborn coccygeal body while its expression is dramatically downregulated in adult samples. **(C)** Dll4 is highly expressed in the newborn coccygeal body while its expression is noticeably lower in adult samples of the glomus coccygeum. The green rectangles in top images mark the areas displayed in bottom images, and epithelioid cells are indicated with white arrows. Bars, 20 μm.

node and primitive streak rest (Arvidsson *et al.* 2011; Chrenek *et al.* 2017; Ferrer-Vaquer & Hadjantonakis, 2013). Interestingly, gene expression regulation seems to be quite dynamic in the Luschka's body and, thus, while it has been broadly reported to be positive for SMA in adult specimens, SMA expression has not been detected in embryonic coccygeal glomi (Jin *et al.* 2016). In this respect, we would also like to highlight that our observations indicate that newborn coccygeal bodies are markedly positive for SMA, although with less demarcated borders compared to adult glomi. While the studies on SMA expression in embryonic Luschka's bodies were carried out with human fetuses at 12-, 16- and 18-weeks of development (Jin *et al.* 2016), these contrasting observations do, however, indicate a dynamic developmental control in the gene expression profiles of SMA in the coccygeal glomi. Furthermore, these observations on developmental gene expression regulation are also in line with our current observations on the expression patterns of Notch ligands and receptors in adult and newborn coccygeal bodies. Certainly, further elucidation of the molecular programs and signals controlling gene expression in developing and adult coccygeal glomi is necessary. Finally, while anlages of coccygeal bodies have been observed as early as in 6-8 week human embryos (Jin *et al.* 2016; Vallois, 1920), the cellular ontogenesis of this organ remains obscure, as it also remains unknown whether this organ may contribute to the development of sacrococcygeal teratomas. Interestingly, the initial observations made by the very same Luschka indicating that some coccygeal glomus cells contained cilia (Banks, 1867; Luschka, 1860b) have been confirmed by more recent electron microscopy studies (Kondo, 1972). Clearly, future genetic labeling and lineage tracing studies in the node and/or primitive streak as well as in coccygeal glomi will be necessary to determine, respectively, whether the node and primitive streak contribute to coccygeal glomus ontogenesis and whether sacrococcygeal teratomas and neoplasias may derive from this hitherto forgotten organ of the human body.

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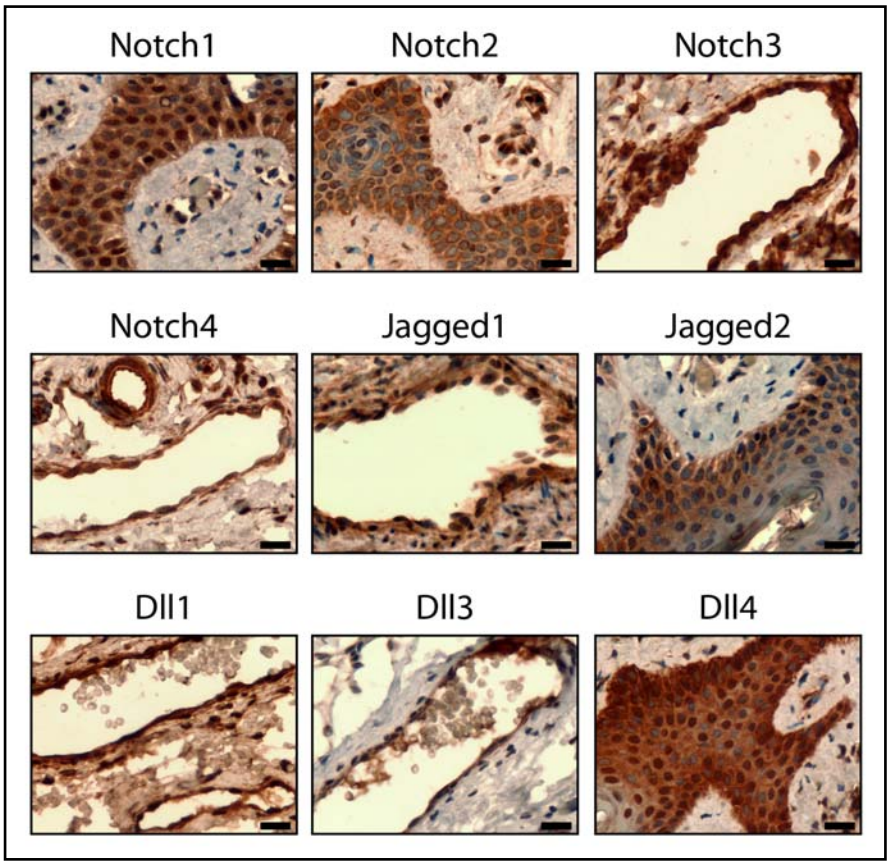
SUPPLEMENTAL FIGURE



Suppl. Fig. 1. Positive control immunostainings for Notch receptors and ligands. Positive controls are demonstrated for Notch1, Notch2, Jagged2, and Dll4 in skin tissue, and for Notch3, Notch4, Jagged1, Dll1, and Dll3 in endothelial and vascular cells. The data presented was obtained from the distinct immunostainings done in newborn coccygeal tissue and using the same antibody solutions and experimental conditions as in adult samples. Note that while negative immunoreactivity is easily observable in the distinct immunostainings for Notch receptors and ligands in the main Figures (e.g. surrounding connective tissue, absence of immunoreactivity for Notch1 in adult specimens), these immunostaining controls, in tissues widely demonstrated positive for the various Notch ligands and receptors, are necessary to rule out any technical-based lack of immunoreactivity. Bars, 20 µm.

SUPPLEMENTAL TABLE

Suppl. Tab. 1. Tissue and references for positive control immunostainings

Target	Tissue of reference	Study of reference
Notch1	Skin	Kim *et al.* 2016, Interaction of Wnt5a with Notch1 is Critical for the Pathogenesis of Psoriasis. Ann Dermatol. **28**(1): 45–54. doi: 10.5021/ad.2016.28.1.45.
Notch2	Skin	Nickoloff *et al.* 2002, Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. Cell Death Differ. **9**(8): 842–55. doi: 10.1038/sj.cdd.4401036.
Notch3	Blood vessels and vascular cells	Chen *et al.* 2016, Smooth muscle FGF/TGFβ cross talk regulates atherosclerosis progression. EMBO Mol Med. **8**(7): 712–28. doi: 10.15252/emmm.201506181.
Notch4	Blood vessels and vascular cells	Cobellis *et al.* 2008, The pattern of expression of Notch protein members in normal and pathological endometrium. J Anat. **213**(4): 464–72. doi: 10.1111/j.1469-7580.2008.00963.x.
Jagged1	Blood vessels and vascular cells	Verginelli *et al.* 2015, Activation of an endothelial Notch1-Jagged1 circuit induces VCAM1 expression, an effect amplified by interleukin-1β. Oncotarget **6**(41): 43216–29. doi: 10.18632/oncotarget.6456.
Jagged2	Skin	Uhlén *et al.* 2015, Proteomics. Tissue-based map of the human proteome. Science **347**(6220): 1260419. doi: 10.1126/science.1260419.
Delta-like1	Blood vessels and vascular cells	Sörensen *et al.* 2009, DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. Blood **113**(22): 5680–8. doi: 10.1182/blood-2008-08-174508.
Delta-like3	Blood vessels and vascular cells	Zhang *et al.* 2016, Chemotherapy enhances tumor vascularization via Notch signaling-mediated formation of tumor-derived endothelium in breast cancer. Biochem Pharmacol. **118**: 18–30. doi: 10.1016/j.bcp.2016.08.008.
Delta-like4	Skin	Gunin *et al.* 2014, Age-related changes in angiogenesis in human dermis. Exp Gerontol. **55**: 143–51. doi: 10.1016/j.exger.2014.04.010.