Developmental Biology

Spinal neural tube formation and tail development in human embryos

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eLife Assessment

This is a **fundamental** study into human spinal neurulation, which substantially advances our understanding of human neural tube closure. Crucial unanswered questions in the field currently rely on model systems, not faithful to human development. The evidence provided is **compelling**, with a large number of specimens and the rigorous use of state-of-the-art methodology providing robustness. The work will be of broad interest to developmental biologists, embryologists, and medical professionals working on neural tube defects, and will act as a precious reference resource for future studies.

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Abstract

Formation of the nervous system in the spinal region of higher vertebrates involves primary and secondary neurulation, in which the neural tube forms by closure and canalisation respectively. These processes are incompletely understood in humans, in part due to the challenge of accessing neurulation-stage embryos (3-7 weeks post-conception). Here we present findings on completion of primary neurulation and formation of the 'secondary body' (including secondary neurulation) in 108 human embryos that span Carnegie Stages (CS) 10 to 18. Several outstanding questions on low spinal development in humans are addressed: we show that primary neurulation is completed at the human posterior neuropore with a pattern of neural plate bending similar to that in the mouse. There is no evidence of a 'transition zone' to secondary neurulation, which proceeds from CS13 with formation of a single lumen as in mouse, not coalescence of multiple lumens as has been claimed based on chick neurulation. Secondary neural tube 'splitting' is seen in the more proximal tail regions of 60% of human embryos. A somite is formed every 7 h in human, compared with 2 h in mice and a 5 h 'segmentation clock' in human organoids. Termination of axial elongation occurs after downregulation of *WNT3A* and *FGF8* in the CS15 embryonic tailbud, with a 'burst' of apoptosis that may remove the neuro-mesodermal progenitors. We conclude that low spinal neurulation and secondary body formation follow a similar pattern in humans as in mammalian model systems such as mouse and rat. Investigators are now attempting to recapitulate events of neurulation in organoids derived from human stem cells, and our findings provide 'normative data' for interpretation of such in vitro results.

Introduction

Development of the lumbosacral spinal cord is a critical period of embryogenesis. Not only does motor control and sensation in the legs and lower body depend on this event, but proper functioning of bladder, rectum and genital organs are all critically dependent on nerves arising from the low spinal cord. A major group of congenital malformations termed neural tube defects (NTDs) result when low spinal neurulation fails to be completed or is otherwise abnormal, and these can be open or closed (skin-covered) lesions.

Open spina bifida (also called myelomeningocele) results from defective closure of the primary neural tube, most often at lumbar and upper sacral levels. Subsequent neuroepithelial loss due to prolonged exposure to amniotic fluid (Stiefel et al., 2007) leads to a defect that is disabling in most individuals (Copp et al., 2015). Closed 'dysraphic' conditions arise at lower sacral and coccygeal levels of the body axis and result from disturbance of secondary neurulation, in which the neural tube forms without formation of neural folds. Dysraphic conditions involve an abnormal anatomical relationship between the secondary neural tube and surrounding tissues, often with ectopic adipose tissue, as in spinal lipoma and lipomyelomeningocele (Jones et al., 2019). Closed dysraphism may be asymptomatic, but significant disability can occur through tethering of the low spinal cord to non-neural tissues (Agarwalla et al., 2007).

In normal primary and secondary neurulation, the neural tube forms by closure and canalisation respectively (summarised in **Figure 1** of Nikolopoulou, 2017), with extensive studies of both processes in experimental animals, especially chick, mouse and rat. Despite the relative inaccessibility of neurulation-stage human embryos (3-7 weeks post-conception), a number of studies have described the anatomical, histological and ultrastructural features of secondary neurulation (Supplementary Table 1). Although limited molecular research has been performed, e.g. to determine the mode of cell death in human tail regression (Vilovic et al., 2006), a few studies have begun to address specific gene expression during human secondary body development (Olivera-Martinez et al., 2012; Yang et al., 2014). Increasingly, transcriptomic approaches are being used with organogenesis-stage human embryos (Fang et al., 2010; Yi et al., 2010; Krupp et al., 2012; Xu et al., 2023).

Caudal development comprises not only formation of the secondary neural tube, but also other tissue types within the 'secondary body' region. This part of the body axis – beyond the cloacal plate which marks the future anus – includes the secondary notochord, tail somites, caudal vessels, tailgut and surrounding surface ectoderm (future epidermis). These structures show marked tissue-to-tissue variation in development. For example, tail regression in human embryos involves loss of all tail components, whereas the rodent tail maintains the somites and notochord, but loses the secondary neural tube and tailgut. The regulation of this balance between maintenance and loss of tail structures is not understood.

A related area of interest is the molecular control of axial elongation. A population of self-renewing stem cells, termed neuro-mesodermal progenitors (NMPs), resides in the caudal-most embryonic region (the tailbud), with NMPs giving rise to neural and mesodermal derivatives, including the secondary neural tube and somites (Henrique et al., 2015). NMP maintenance is required for axial elongation, mediated via an interplay between WNT3A and FGF8 expression, which promotes NMP survival in the tailbud. Conversely, endogenous retinoic acid promotes differentiation and regulates body length (Wilson et al., 2009).

In the present study, we examined caudal development in 108 human embryos, at Carnegie Stages (CS)10-18 (3.5-6.5 weeks post-conception). The aim was to gain new information on several unanswered or controversial questions in human neurulation, including: (i) how the embryo

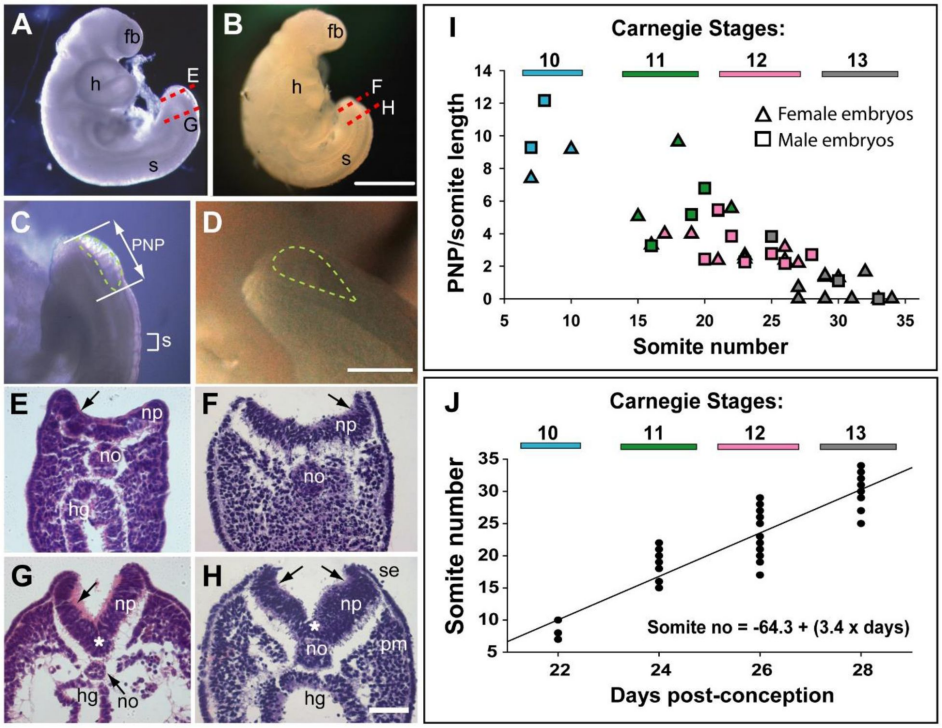

Figure 1.

Morphology and timing of human posterior neuropore (PNP) closure.

(**A,B**) Two CS12 embryos viewed from right side, each with 22-23 somites (s), and a looped heart (h). Neural tube closure is complete along most of the body axis, including the forebrain (fb), whereas the PNP remains open caudally. (**C,D**) Magnified oblique views from upper right side of the caudal region; the open PNP is outlined with dashed lines. (**E-H**) H&E-stained transverse sections, through the PNP, with section planes as indicated by dashed lines in A, B. The most caudally located sections (E,F) show a relatively flat neural plate (np), although incipient dorsolateral hinge points (DLHPs; arrows) are visible. Note the midline notochord (no) underlying the neural plate, and hindgut (hg) beneath the notochord (in E only). More rostral sections (G,H) show elevated neural folds with DLHPs clearly visible (arrows: unilateral in G, bilateral in H), located where basal contact of the neural plate changes from surface ectoderm (se), to paraxial mesoderm (pm). A median hinge point (MHP; asterisks in G,H) overlies the notochord. (**I**) PNP length (double headed arrow in C), normalised to somite (s) length (bracketed in C), determined from photographic images of 40 human embryos (24 females; 16 males) at CS10 (n = 4), CS11 (n = 8), CS12 (n = 16) and CS13 (n = 12). Symbol colours indicate the Carnegie Stages assigned at the time of collection. The PNP shows gradual closure, with completion around the 30 somite stage. (**J**) Somite number of the 40 embryos in I, plotted against days post-conception, as reported for each Carnegie Stage by O'Rahilly and Muller (1987). The linear regression equation is shown, with $R^2 = 0.82$, and $p < 0.001$. Scale bars: 1 mm in A,B; 0.4 mm in C,D; 0.1 mm in E-H.

transitions from primary to secondary neurulation; (ii) the mode of formation of the secondary neural tube; (iii) the rate of somite formation during low spinal development; (iv) the possible roles of WNT3A and FGF8 in regulating axial elongation; (v) whether a 'burst' of apoptosis coincides with termination of axial elongation. A further aim was to gather and present findings on human spinal neurulation that can serve as 'normative data' to aid interpretation of research involving multicellular 'organoid' structures, that are being increasingly used to model various aspects of human axial development (Denham et al., 2015; Fedorova et al., 2019; Moris et al., 2020; Rifes et al., 2020; Karzbrun et al., 2021; Libby et al., 2021; Amadei et al., 2022).

Results

The study involved 108 human embryos (**Table 1**), obtained from the MRC/Wellcome Human Developmental Biology Resource (*www.hdbr.org*), with UK ethics committee approval. Embryos were donated by women undergoing termination of pregnancy for 'social' reasons, in most cases by mifepristone/misoprostol-induced (medical) delivery, with a few intact embryos obtained by ultrasound-guided vacuum aspiration (surgical). All embryos in the study were chromosomally and morphologically normal, and were assigned to Carnegie Stages (CS), as described (O'Rahilly and Muller 1987; Bullen and Wilson 1997). Comparisons to mouse were with random-bred CD1 embryos, staged by embryonic (E) day, where E0.5 is the day following overnight mating.

Morphology of human posterior neuropore (PNP) closure

Relatively few human embryos with an open PNP have been reported in the literature (Müller and O'Rahilly 1987; O'Rahilly and Müller 2002), probably owing to the early stage at which primary neurulation is completed (end of week 4, post-conception). In two intact CS12 embryos (**Figure 1A,B**; crown-rump length: 3 mm; 22-23 somites), we identified an open PNP by microscopic inspection at collection (**Figure 1C,D**). Transverse histological sections confirmed an open neural tube in the caudal region, with minimal tissue damage evident, indicating that primary neurulation was not yet complete. The neural plate is relatively flat in the most caudally located sections, although incipient dorsolateral hinge points (DLHPs) are visible (**Figure 1E,F**). The notochord underlies the neural plate midline, and the caudal end of the hindgut is visible beneath the notochord in one embryo (**Figure 1E**), but not the other (**Figure 1F**). In more rostral sections, close to the 'zippering' point of PNP closure, elevated neural folds flank a marked ventral midline bend in the neural plate, the median hinge point (MHP), which precisely overlies the notochord (**Figure 1G,H**). DLHPs are also clearly present, unilaterally in one embryo (**Figure 1G**) and bilaterally in the other (**Figure 1H**). As in the mouse (McShane et al., 2015), the DLHPs are situated where the neural plate changes from basal contact with surface ectoderm to basal contact with paraxial mesoderm. We conclude that MHP and DLHPs characterise PNP closure in human embryos at CS12, marking a direct equivalence to Mode 2 spinal neurulation in the mouse embryo (Shum and Copp 1996).

Timing of human PNP closure

PNP length data were obtained from photographic images of CS10-CS13 embryos (n = 40). To allow for differences in overall embryonic size, PNP measurements were normalised to the length of a recently formed somite in the same embryo (**Figure 1C**). The plot of PNP length/somite length against somite number shows a steady decline in the length of open neural folds in the caudal region, until 6/12 embryos at CS13 have completely closed, while most of the others show a very small PNP (**Figure 1I**). There were no obvious differences in closure rate or timing between female (n = 24) and male (n = 16) embryos. Hence, closure of the PNP is completed in human embryos around the 30-somite stage, as also reported for outbred mouse strains (Copp et al., 1982).

Analysis type	Figures in paper	Total no.	No. females	No. males	No. sex unknown	No. medical	No. surgical
PNP morphology	1A-H	2 **	0	2	0	2	0
PNP closure timing	1I,J	40	24	16	0	40	0
Tail morphology, histology, cell death	2,3	37 **	14	21	2	36	1
Serial section analysis, cell death	4,6	11	5	6	0	10	1
FGF8, WNT3A expression	5	18	9	9	0	18	0
Totals		108	52	54	2	106	2

* Medical: mifepristone- and misoprostol-induced delivery; Surgical: ultrasound-guided vacuum aspiration.

** Embryos that are included in Table 2,

Table 1.

Number of human embryos in the study, with breakdown by analysis type, sex and method of pregnancy termination *

Development and regression of the human embryonic tail

Overall caudal development was studied in 37 human embryos (CS13-CS18), which covered the period 28-45 days post-conception (**Table 2**; **Figure 2A,B**,I). Crown-rump length increased 2.5-fold during this period, from a mean value of 6.4 mm at CS13 to 15.4 mm at CS18 (**Table 2**; **Figure 2J**). Observations on the intact embryos showed that the PNP is closed in most embryos by CS13, and a developing tailbud is present which exhibits mild ventral curvature and a thick rounded tip (**Figure 2C**). Somites are visible proximal to the tailbud (arrowheads in **Figure 2C**), with an intervening region of presomitic mesoderm at CS13 (yellow bracket in **Figure 2C**). By CS16, however, the somites extend almost to the tail tip (yellow arrow in **Figure 2F**). As development progresses, striking changes occur in the tail which continues to lengthen (**Table 2**) but simultaneously narrows, particularly at the tip, to yield a sharply pointed structure by CS16 (**Figure 2D-F**). At the same time, the tail straightens and even becomes dorsally bent (**Figure 2F**). Subsequent to CS16, the tail shortens (**Figure 2G**; **Table 2**), and its distal portion becomes increasingly translucent in appearance. By CS18, only a short, curved stump remains (**Figure 2H**), and the tail is lost completely thereafter. Additional embryonic tails in the CS13-CS18 range are shown in Supplementary Figure 1.

Somite formation

Between CS10 and CS13, during PNP closure, somite number increases approximately linearly with days of gestation (**Figure 1K**): mean (± SD) somite numbers were: 8.0 ± 1.4 at CS10, 18.4 ± 2.6 at CS11, 23.5 ± 3.4 at CS12 and 30.0 ± 2.8 at CS13. The linear regression equation of this relationship (**Figure 1K**) gives an increase of 20.3 somites over a 6-day period, equating to the formation of 3.4 somites per gestational day, or a new somite every 7.1 h (95% confidence intervals: 4.8, 10.4). This compares with formation of a new somite every 2 h in rat and mouse embryos (Brown and Fabro 1981; Tam 1981), and a 5 h periodicity observed for the human 'in vitro segmentation clock' in stem cell-derived presomitic mesoderm-like cells (Diaz-Cuadros et al., 2020; Matsuda et al., 2020). Following PNP closure at CS13, the largest somite number was at CS16 (36.6 ± 1.2; **Table 2**), although there was no statistically significant increase between CS13 and CS16 (**Figure 2K**). By CS17 and 18, we could identify only 31-34 somites, a significant reduction in number (**Figure 2K**). Hence, somite formation in humans occurs at a rate that is 3.5-times slower than in rodent embryos, and ceases after CS16. Subsequent somite number reduction suggests that shortening of the tail during regression involves loss of somites (**Table 2**).

Mode of cell death during tail regression

In an initial study, transverse histological sections through human and mouse embryonic tails were processed for immuno-peroxidase staining using anti-activated caspase 3. Positive cells were readily identified in the tails of both species (**Figure 3A-N**), arguing for a role of caspase-dependent apoptosis during tail regression in human and mouse. Principal sites of apoptotic cell death include the regressing tailgut (**Figure 3D,F**) and, most abundantly, the ventral mesoderm overlying the epithelial ventral ectodermal ridge (**Figure 3C,E**). We also detected TUNEL-positive cells in both mouse and human tail sections (data not shown), further confirming the presence of apoptotic cells during tail development/regression.

A burst of apoptosis at cessation of tail elongation

We observed enhanced apoptosis in the mouse tailbud at E13.5 (**Figure 3J**), compared with E13.0 and E14.0 when relatively few dying cells were present (**Figure 3I,K**). Similarly, in sections through the caudal-most region of human embryos, apoptosis was not observed at CS13 (**Figure**

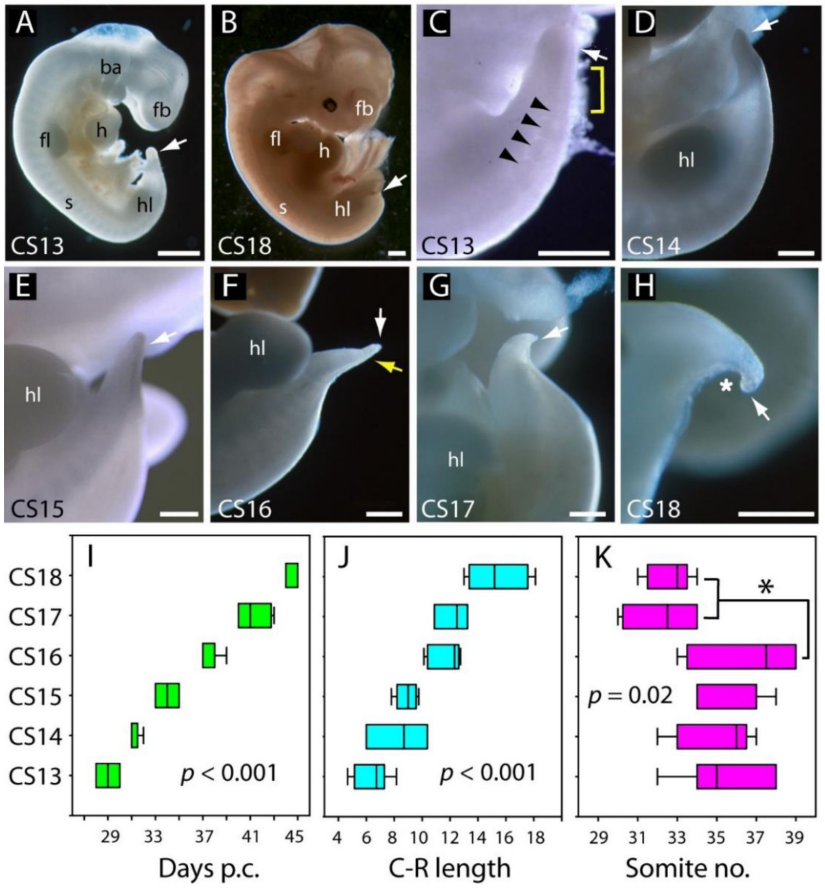

Figure 2.

Development of the tail in human embryos.

(**A,B**) Whole embryos at CS13 (A) and CS18 (B), showing the range of stages studied (4-6.5 weeks post-conception). The tail bud (arrow) is well formed at CS13 following completion of PNP closure at CS12, whereas, by CS18, tail development and regression are largely complete and only a small tail remnant remains (arrow). (**C-H**) Higher magnification views of the caudal region at CS13 to CS18. At CS13, the tail bud is relatively massive, tapering gradually and with a rounded end (arrow in C). Somites are visible rostral to the tail bud (arrowheads) with an intervening region of presomitic mesoderm (yellow bracket). At CS14 and CS15 the tail narrows progressively, with distal tapering (arrows in D-E). By CS16, this has yielded a slender structure with a narrow pointed end (white arrow in F) in which somites extend almost to the tail tip (yellow arrow in F). Thereafter, the tail shortens progressively (arrows in G,H), develops a marked flexion (asterisk in H), and becomes increasingly translucent (G,H). (**I-K**) Analysis of embryos in the range CS13-CS16 (**Table 2**), plotting CS against: (I) days post-conception (p.c., see Materials & Methods), (J) crown-rump (C-R) length in mm, and (K) somite no. One-way ANOVA on ranks shows all three parameters vary significantly with CS (*p*-values on graphs). Somite no. reduces significantly between CS16 and CS17/18 (* $p < 0.05$). Abbreviations: ba, branchial arches; fb, forebrain; fl, forelimb; h, heart; hl, hindlimb; s, somites. Scale bars: 1 mm in A,B; 0.5 mm in C-H.

Carnegie Stage (CS)	Age range (days post-fertilization)	Number of embryos	Somite number **	Crown-rump length ***	Tail length (total) ***	Tail length distal to somites ***
12	25-27	2	22, 22	3.0, 3.0	N/A	N/A
13	28-30	7	35.4 ± 2.3	6.4 ± 1.3	1.06 ± 0.44	0.49 ± 0.16
14	31-32	5	35.0 ± 2.0	8.4 ± 2.2	0.99 ± 0.21	0.56 ± 0.06
15	33-35	7	35.9 ± 1.8	8.9 ± 0.8	1.18 ± 0.55	0.67 ± 0.15
16	37-39	8	36.6 ± 2.6	11. 7 ± 1.2	1.29 ± 0.48	0.46 ± 0.13
17	40-43	4	32.3 ± 2.1	12.2 ± 1.2	1.18 ± 0.20	0.34 ± 0.01
18	44-45	6	32.6 ± 1.1	15.4 ± 2.2	1.14 ± 0.48	N/D

* Summary of embryos that underpin Figure 1A-H (CS12) and Figures 2 and 3 (CS13-18). For full data set, see Supplementary Table 1.

** Somite numbers: mean ± SD (except CS12, where actual somite numbers are shown). Somite number was available for all embryos except n = 5 at CS18.

*** Lengths (mm): mean ± SD (except CS12, where actual lengths are shown). Length measuements were available only for a sub-set of embryos. See Supplementary Table 1 for full details.

N/A: not applicable; N/D: not determined

Table 2.

Measurements of human embryos, CS12-18 *

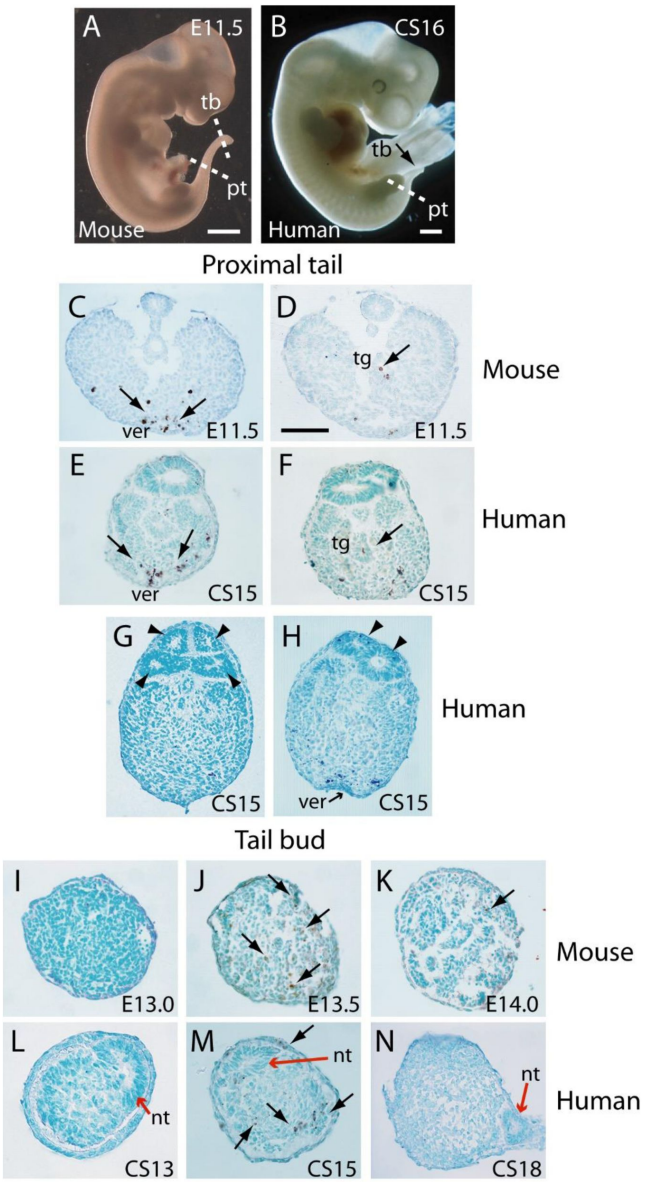

Figure 3.

Tail morphology and apoptosis in mouse and human embryos.

(**A,B**) Mouse E11.5 (A) and human CS16 (B) embryos to illustrate the level of transverse sections through the proximal tail (pt) and tailbud (tb) regions of mouse (C,D,I-K) and human (E-H,L-N) embryos at the stages indicated on the panels. Immunohistochemistry was performed on paraffin wax sections for activated caspase 3 (brown stain), with counterstaining by methyl green. (**C-F**) In the proximal tail region, intense programmed cell death is observed in the ventral midline mesoderm overlying the ventral ectodermal ridge (ver) of both mouse (arrows in C) and human (arrows in E) embryos. Cell death can also be detected in the tailgut (tg) of both mouse (arrow in D) and human embryos (arrow in F). C,D are sections from a single E11.5 mouse embryo; E,F are sections from a single CS15 human embryo. (**G,H**) Multiple neural tube profiles in two human embryonic tails at CS15: four lumens are visible in one embryo (arrowheads in G) and two lumens in a second (arrowheads in H). (**I-K**) In mouse, the tail bud displays a stage-dependent burst of apoptotic cell death at E13.5 (arrows in J), with absence of caspase 3-positive cells 12 h earlier, at E13.0 (I), and only occasional dying cells 12 h later, at E14.0 (arrow in K). Note the absence of a neural tube at the mouse tail bud tip, and the sparse nature of the tail bud mesenchyme at E14.0. (**L-N**) Human embryonic tail buds show a similar developmental sequence to the mouse, with absence of cell death at CS13 (L), abundant dying cells at CS15 (arrows in M) and cessation of cell death by CS18 (N). Unlike the mouse, the secondary neural tube extends to the tail bud tip (red arrows in L-N), and this terminal neural tube portion has a single lumen in all three embryos. Scale bars in A,B, 1 mm; bar in C represents: 70 μm (C,D), 50 μm (E-K,N) and 30 μm (L,M).

3L), became intense at CS15 (**Figure 3M**), and diminished in intensity by CS18 (**Figure 3N**). Hence, in both mouse and human tails, there appears to be a 'burst' of apoptosis at the stage when tail growth ceases, and just before regression of internal structures gets underway.

Evidence for regression of the tailgut from rostral to caudal

While the human embryonic tail appears to regress from caudal to rostral (**Figure 2**), the proximal (rostral) part of the tailgut is reported to degenerate before the more distal (caudal) part in both rat (Butcher 1929; Qi et al., 2000) and mouse (Nievelstein et al., 1993a; Nievelstein et al., 1993b). To examine this question in human embryos, we performed immunofluorescence for anti-activated caspase 3, which confirmed the presence of apoptosis in both tailgut and ventral mesoderm (**Figure 4A**',B'). Using DAPI-stained sections along the secondary body axis (**Figure 4A,B**), we determined the cross-sectional area of neural tube, notochord and tailgut in two CS14 and one CS15 embryos. Total tail area served as a measure of axial position. The neural tube showed a progressive increase in area towards the proximal (rostral) end of the tail, while the notochord showed no change in area along the body axis (**Figure 4C**). Strikingly, the tailgut showed the reverse trend, with a marked caudal-to-rostral reduction in cross-sectional area (**Figure 4A,B,D**). Tailgut nuclear number also diminished from caudal to rostral (**Figure 4E**). Hence, although the rostral tailgut has not disappeared by CS15, it appears to be diminishing in size proximally, at the same time as it is being formed, as a prominent tail structure, caudally. We conclude that rostral-to-caudal loss of the tailgut may be a general phenomenon among mammalian embryos.

Expression of *FGF8* and *WNT3A* during human tailbud elongation

To begin an assessment of the mechanisms that may regulate elongation of the human embryonic tail, and its cessation, we performed whole mount in situ hybridisation for *FGF8* and *WNT3A* (n = 2 embryos minimum for each gene at each stage). These genes are developmentally regulated during axial elongation in chick and mouse embryos, with strong expression in the tailbud during elongation, and down-regulation before axial growth ceases. Direct inactivation or indirect down-regulation of the genes leads to premature axial truncation (Wilson et al., 2009).

In accordance with these findings, we observed strong expression of *FGF8* in the tailbud at CS12 and CS13, as revealed in whole embryos (**Figure 5A,B**) and longitudinal sections through hybridised caudal regions (**Figure 5E,F**). At CS14, *FGF8* expression reduced dramatically so that only a small 'dot' of expression was detected in the tailbud (**Figure 5C,G**), and by CS15 expression of *FGF8* was no longer detectable in the tail (**Figure 5D,H**). Expression of *WNT3A* followed a similar pattern with strong expression in the tailbud at CS12 (**Figure 5I,M**), reduced expression intensity at CS13 (**Figure 5J,N**), a remaining 'dot' of tailbud expression at CS14 (**Figure 5K,O**), and no detectable *WNT3A* expression in the tail at CS15 (**Figure 5L,P**). We conclude that expression of *FGF8* and *WNT3A* mirrors the relationship seen in mouse and chick, with strong tailbud expression during active axial extension, and dramatic down-regulation of both genes before the onset of axial growth cessation. It is striking that down-regulation appears complete by CS15, even though the embryonic tail does not reach its maximum length until some days later, at CS16 (**Table 2**). Down-regulation of *Fgf8* and *Wnt3a*, well in advance of cessation of axial elongation, has also been observed in mouse embryos (Cambray and Wilson 2007).

Mode of secondary neural tube formation in human embryos

In our initial study of tail morphology, 9 out of 15 human embryonic tails showed multiple lumens in some transverse sections (**Figure 3G,H**), whereas the other 6 exhibited only a single neural tube lumen. In a second group of serially sectioned tails (**Figures 4**,**6**), 6 out of 10 tails had regions of duplicated neural tube. Hence, we find a 60% (15/25) frequency of neural tube duplication, confirming previous findings of multiple neural tube lumens in many human

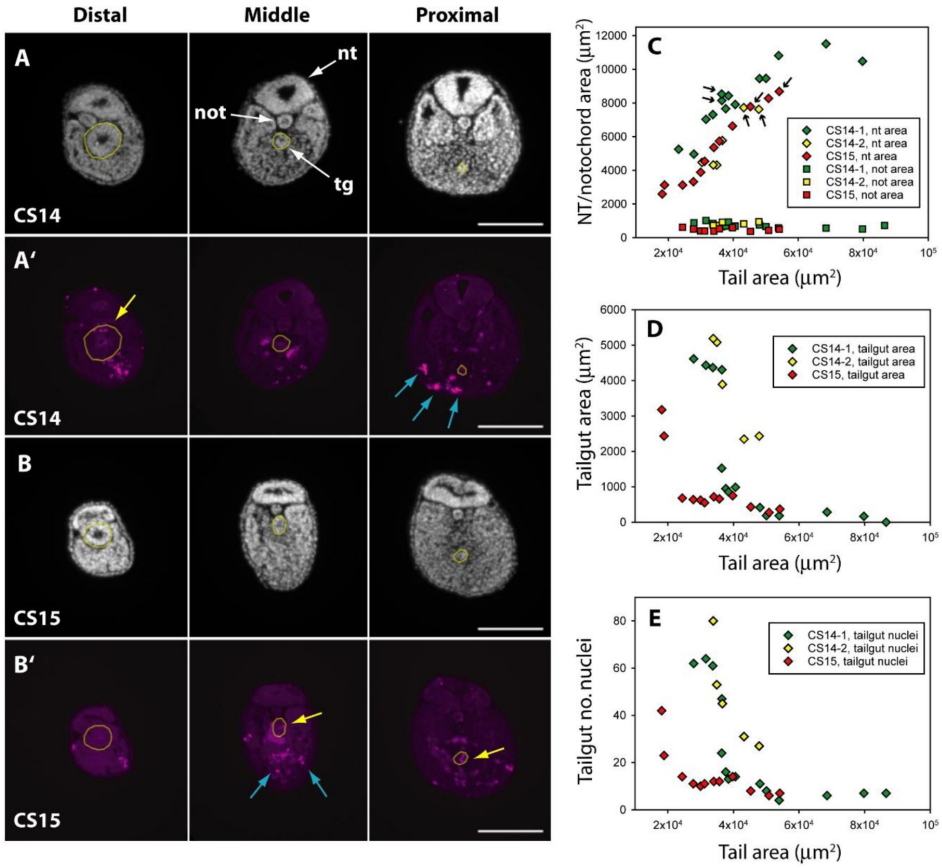

Figure 4.

Programmed cell death and tissue size along the developing human tail.

(**A,A',B,B'**) Transverse sections at distal (left), middle (centre) and proximal (right) levels of the tail. Panels show DAPI (A,B) and anti-cleaved caspase 3 immunostaining (A',B') of the same sections at CS14 (A,A') and CS15 (B,B'). Yellow dotted lines outline the tailgut. Apoptotic cell death occurs mainly in tailgut (tg, yellow arrows) and ventral mesoderm (blue arrows). Note the diminishing diameter of the tailgut from distal to proximal. (**C**) Change in transverse sectional areas of neural tube (nt, diamonds) and notochord (not, squares) along the body axis in CS14 (x 2; green and yellow symbols) and CS15 (red symbols) embryos. Embryos CS14-1 and CS15 are shown in (A,B). Tissue-specific areas (y-axis) are plotted against total tail area (x-axis), which increases from left (distal sections) to right (proximal sections). In all embryos, neural tube area increases in a proximal direction, whereas notochord area is relatively constant along the axis. Arrows: sections in which neural tube shows multiple lumens (see **Figure 6**). (**D,E**) Similar analysis for tailgut area (D) and tailgut nuclear number (E). Both show a dramatic reduction in a distal-to-proximal direction, in contrast to neural tube and notochord. Scale bars: 50 µm.

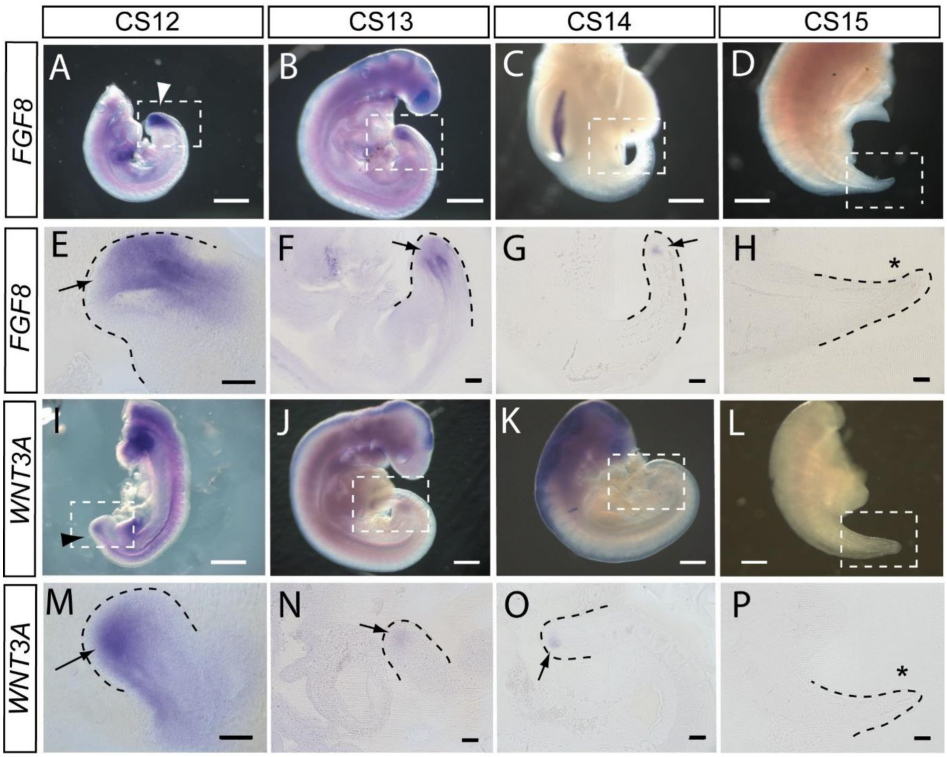

Figure 5.

***FGF8* and *WNT3A* expression in the elongating caudal region of human embryos.**

Whole mount *in situ* hybridisation (A-D, I-L) and sagittal vibratome sections through the caudal region (E-H, M-P) for *FGF8* (A-H) and *WNT3A* (I-P) in embryos at CS12 (A,E,I,M), CS13 (B,F,J,N), CS14 (C,G,K,O) and CS15 (D,H,L,P). Both genes show prominent expression domains in the tail bud at CS12 (arrows in E,M) when axial elongation is underway and the posterior neuropore (PNP) is closing (arrowheads in A,I). At CS13, following PNP closure, expression of *FGF8* and *WNT3A* remains prominent although less intense and more localised to the terminal tail bud than at CS12 (arrows in F,N). By CS14, both genes exhibit much smaller, highly localised expression domains that each appears as a 'dot' within the tail bud region (arrows in G,O). By CS15, axial elongation has ceased, the tail tip has narrowed and is increasingly transparent. At this stage, expression of neither gene can be detected (asterisks in H,P). Whole embryos shown in B,J,K; isolated trunk/caudal regions shown in A,C,D,I,L. No. embryos analysed: FGF8, n = 2 for each stage; WNT3A, n = 2 for each stage except n = 3 for CS13. Scale bars: A-D, I-L, 1 mm; E-H, M-P, 100 µm.

embryonic tails (Bolli 1966; Lemire 1969; Saitsu et al., 2004). We most often identified two neural tube profiles in a single transverse section, but in some cases more were observed (e.g. at CS15; **Figure 3G**).

These initial findings of neural tube duplication prompted a more detailed examination of the suggestion that multiple neural tube lumens represent a mode of human secondary neurulation similar to that seen in the avian embryo (Lemire 1969; Saitsu et al., 2004; Pang 2020). In chick, multiple lumens form distally in the tail bud, and coalesce more rostrally to form the secondary neural tube (Criley 1969; Schoenwolf and De Longo 1980; Yang et al., 2003; Gonzalez-Gobartt et al., 2021). An alternative view would be that multiple lumens arise only later in human secondary body formation (Catala 2021), perhaps representing splitting of the already formed secondary neural tube (**Figure 6A**).

To help resolve this issue, we asked two questions: (i) what is the status of the secondary neural tube where it is first formed, close to the tailbud tip? (ii) at what level of the tail axis is neural tube duplication most often seen? In our initial series of human tails, a single lumen was invariably noted in the distal-most portion of the neural tube, just rostral to the tailbud tip (CS13-18; **Figure 3L-N**). This was confirmed in the second series of ten serially sectioned tails (CS14-15; **Figure 6B,C,D**), thus demonstrating that secondary neurulation in human produces a neural tube with a single lumen. When multiple secondary neural tube lumens were visible in the embryonic tail, this was invariably located at more rostral levels of the tail (**Figure 3G,H**; **Figure 4C**; **Figure 6B**',C",D"), with variation in location between embryos. We conclude that multiple secondary neural tube lumens in human embryonic tails likely represent splitting, which tends to occur rostrally and does not represent a secondary neurulation mechanism involving coalescence of multiple lumens, as in chick. Humans therefore resemble other mammals, including rat and mouse, in initially forming a single secondary neural tube lumen in the tail-bud.

Discussion

The development and later disappearance of the human tail has been of interest to embryologists for more than a century (Catala 2021). To better appreciate the knowledge base for human embryonic caudal development, we conducted a systematic literature review using several search terms (see Materials and Methods). This generated a long-list of publications that was filtered to include only those with primary data relating to caudal development in human embryos. The final list comprises 28 papers (Supplementary Table 1) that span over 100 years of research, from the early 1900s (Kunitomo 1918; Streeter 1919) to recent times (Xu et al., 2023). This work was based on at least 925 human embryos obtained from varying sources (Supplementary Table 1) including induced termination of pregnancy for 'social' reasons (where most embryos are expected to be normal), as well as spontaneous abortion (miscarriage) and ectopic pregnancy, where embryonic abnormalities are likely to be frequent. Hence, interpretation of embryo morphology in these studies needs to take into account the mode of procurement of the human specimens.

Several aspects of caudal development are addressed by the studies in Supplementary Table 1. These include: completion of primary neurulation with PNP closure, the transition into secondary neurulation, the mode of formation and regression of the secondary neural tube, the observation of multiple neural tube lumens, the formation and regression of somites, notochord and gut in the tail region, the role of programmed cell death in tail regression, and initial studies of gene expression in human embryos. Taken together with the findings of the present study, this accumulated literature provides a strong morphological evidence base for 'in vivo' human caudal development, against which in vitro studies can be judged in the emerging field of stem cell-derived organoid differentiation. The latter is producing multicellular structures that may

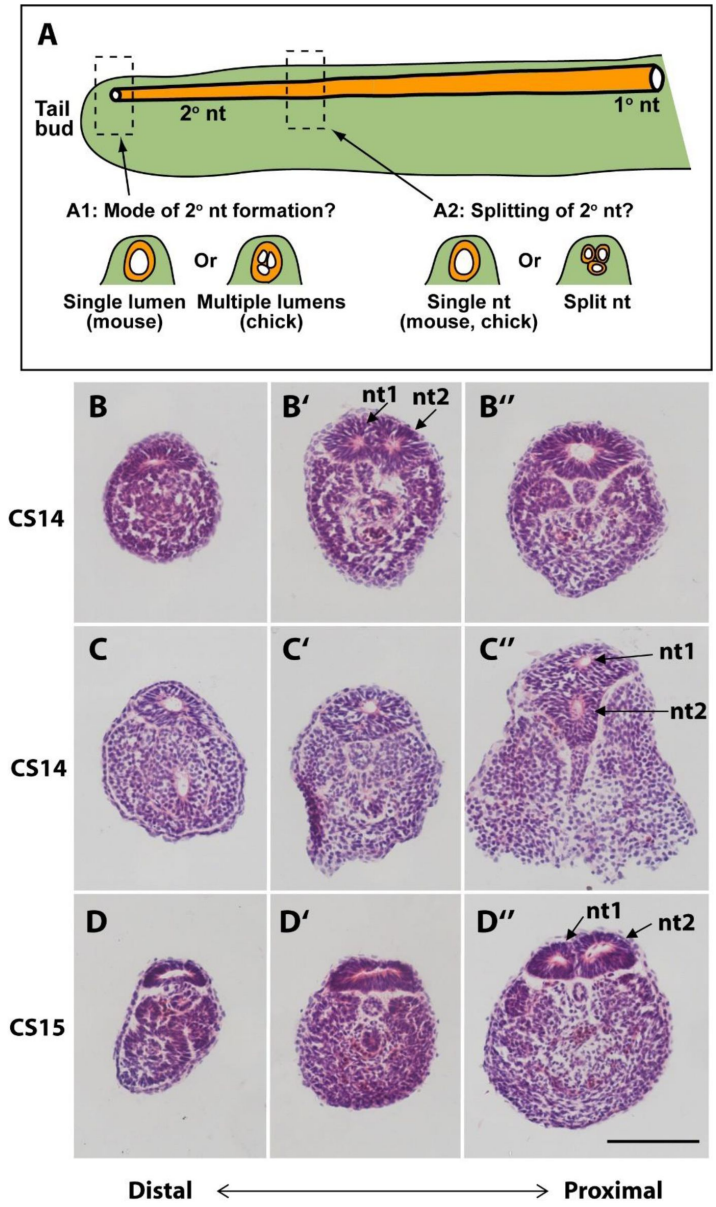

Figure 6.

Mode of formation and proximal splitting of the human secondary neural tube.

(**A**) Hypotheses on mode of formation of the human secondary neural tube (2° nt). A single lumen may be formed in the tail bud as in mouse or, by analogy to chick, multiple lumens may form initially, which then coalesce to form the secondary neural tube (A1). Alternatively, the finding of multiple neural tube lumens in sections of some human tails may reflect splitting of the secondary neural tube at more rostral levels (A2). (**B-D''**) Representative serial transverse sections (haematoxylin and eosin) through three human embryonic tails at CS14 (x 2; B,C) and CS15 (D). Sections close to the tail bud tip (left side: B,C,D) show a broad, dorsoventrally flattened neural tube with a single lumen. There is no evidence of multiple lumens coalescing in the tail bud of any embryos. Further rostrally (middle and right side panels: B',B'',C',C'',D',D''), some sections show a neural tube with single lumen (C',D'), whereas others show evidence of secondary neural tube splitting, with two lumens (nt1, nt2 in B',C'',D''). One CS14 embryo shows re-establishment of a single lumen in more proximal sections (B''), after splitting more distally (B'). These findings support a mouse-like formation of the human secondary neural tube with, additionally, splitting at various rostro-caudal levels along the tail. Scale bar: 50 μm.

ultimately provide experimentally tractable models for some aspects of human axial development (Denham et al., 2015; Fedorova et al., 2019; Moris et al., 2020; Rifes et al., 2020; Karzbrun et al., 2021; Libby et al., 2021; Amadei et al., 2022).

Concept of a human 'tail'

The human tail develops and then regresses during weeks 4-7 post-conception and is composed of a secondary neural tube, notochord, somites and tailgut, with undifferentiated mesenchyme at its tip (the tailbud or 'caudal eminence'), all within a surface ectoderm covering. Since it never becomes vertebrated, in contrast to the tails of most other mammals, some authors consider the human caudal appendage does not qualify as a 'tail' (Müller and O'Rahilly 2004). On the other hand, the presence of caudal somites with vertebra-forming potential are considered by other authors to endow the human caudal appendage with all the hallmarks of a mammalian tail (Kunitomo 1918). In keeping with common usage, we have referred to the transient human caudal appendage as a tail in this paper. Moreover, we note that the recent claim of 'tail-loss' during human evolution, due to a genetic change in the *TBXT* gene (Xia et al., 2024), ignores the fact that tail development persists in humans, albeit during a specific stage of embryonic development.

Transition from primary to secondary neurulation

Primary neurulation ends with completion of PNP closure, which we detect at the 30-somite stage in humans, similar to mice (Van Straaten et al., 1992). In mouse embryos, three sequential Modes of spinal neurulation occur (Shum and Copp 1996): in Mode 1, the upper spinal neural plate bends only at the midline (the median hinge point; MHP); in Mode 2, at intermediate spinal levels, bending is at both MHP and paired dorsolateral hinge points (DLHPs); in Mode 3, immediately before final PNP closure, the MHP is lost and bending occurs only at DLHPs. We found that PNP closure is complete in most CS13 human embryos, and that two CS12 embryos exhibit both MHP and DLHPs in their PNPs. This raises the possibility that 'Mode 2' neural plate bending is typical of the late stages of human spinal neurulation, and that 'Mode 3' is mouse-specific and not seen in humans. Alternatively, our CS12 embryos may have been developmentally too early to show Mode 3 closure. Analysis of further human embryos with open PNPs should resolve this question.

Our sectioning analysis along the body axis of CS14-CS15 human embryos revealed continuity of the neural tube from proximal (primary neurulation) to distal (secondary neurulation) levels. Indeed, it was not possible to locate with any certainty where the transition from primary to secondary neural tube had occurred. This closely resembles the mouse (Shum and Copp 1996), but contrasts with the chick where a 'transition zone' occurs between primary and secondary neurulation. The chick primary (dorsal) and secondary (ventral) neural tubes overlap for a short length of the spinal axis (Dryden 1980) and cells ingressing at the node-streak border participate in a distinct 'junctional neurulation' process with essential function of the *Prickle-1* gene (Dady et al., 2014).

Human 'junctional neurulation' has also been invoked as an explanation for an unusual form of spinal dysraphism where primary and secondary neural tubes are physically and functionally separated from each other, with no intervening neural tissue (Eibach et al., 2016). However, the lack of any evidence for 'junctional neurulation' in the present study of human embryos casts doubt on the proposed developmental origin of this rare dysraphic defect.

Mode of development of the human secondary neural tube

Secondary neurulation in mouse and rat involves formation of a single 'rosette' structure caudally, in which cells aggregate ('condense') from the dorsal tailbud mesenchyme, with subsequent (more rostral) organisation of the cells around a single lumen. This process is driven by apical junction

formation, not by cell death (Butcher 1929; Schoenwolf 1984; Kostovic-Knezevic et al., 1991; Nievelstein et al., 1993b). In chick, by contrast, a caudal-to-rostral sequence of events occurs in which several independent lumens arise in the dorsal tailbud mesenchyme and, at more rostral levels, these coalesce to form the single lumen of the secondary neural tube (Criley 1969; Schoenwolf and De Longo 1980; Yang et al., 2003). Coalescence is a cell intercalation process driven by TGFβ/SMAD3 signalling (Gonzalez-Gobartt et al., 2021).

The mode of formation of the human secondary neural tube is controversial. Multiple neural tube lumens have often been observed in the developing or regressing tail (Bolli 1966; Lemire 1969; Hughes and Freeman 1974; Fallon and Simandl 1978; Saitsu et al., 2004; Pytel et al., 2007; Yang et al., 2014), whereas other studies identify only a single lumen (Müller and O'Rahilly 1987; Nievelstein et al., 1993b). Here, we found multiple secondary neural tube lumens in 60% of human embryos. An important question is whether such multiple lumens are part of the normal secondary neurulation process in humans – thus making the human more similar to chick than mouse, as has been claimed (Pang 2020). Alternatively, multiple lumens could arise through later 'splitting' of the previously formed neural tube. In mice, neural tube duplication is part of several mutant phenotypes and, when present, is a sign of pathology (Cogliatti 1986).

A limitation of previous studies is the paucity of information on the morphology of the secondary neural tube at specific rostro-caudal axial levels. To shed light on this question, we examined human embryonic tails with the aim of determining the axial sequence of secondary neurulation events. Our findings show that multiple lumens, if present, feature at relatively rostral (mature) levels of the secondary neural tube and are absent from the most caudal (immature) levels, close to the tailbud. This applies to embryos throughout the secondary neurulation process (CS13-17), and argues strongly against coalescence of chick-like multiple lumens as a feature of normal human secondary neurulation. A recent review has drawn the same conclusion (Catala 2021). Hence, 'splitting' of the human secondary neural tube appears a common but not obligatory phenomenon, perhaps reflecting changes related to tail regression.

Tailgut: origin and mode of regression

The tailgut is an extension of the hindgut, beginning caudal to the level of the cloacal plate (future anus), which is located ventral to somite 29 in the mouse (Nievelstein et al., 1993a). The tailgut forms and then regresses in both tailed (mouse, rat) and non-tailed (chick, human) animals. Interestingly, human tailgut loss has been described as involving rostral-to-caudal degeneration rather than a more intuitive caudal-to-rostral loss (Kunitomo 1918; Fallon and Simandl 1978). Consistent with this, the tailgut lumen persists longest at the tail tip in rat (Butcher 1929; Qi et al., 2000) and mouse (Nievelstein et al., 1993a). Our finding of a rostral-to-caudal diminution in tailgut size and cell number is consistent with tailgut loss at rostral before caudal levels also in human embryos.

In contrast to the consensus on tailgut regression, there is disagreement over the developmental origin of the tailgut. Anatomical and histological studies in rat, mouse and human often conclude that the tailgut originates by mesenchyme-to-epithelium transition of tailbud cells, in a manner similar to the origin of the secondary neural tube (Svajger et al., 1985; Gajovic et al., 1989; 1993; Nievelstein et al., 1993b). However, others consider the tailgut to arise by caudally directed extension of the hindgut (Jolly and Ferester-Tadie 1936). Grafting of the E10.5 mouse tailbud beneath the kidney capsule produced no evidence of gut epithelial differentiation, in contrast to primitive streak/tailbud fragments at E8.5 and E9.5 which regularly produced this derivative. This finding is consistent with loss of gut-forming potential in the later stage tailbud (Tam 1984).

The question of tailgut origin can also be considered in light of the identification of NMPs: the stem cell population for tissues of the caudal embryonic region (Wilson et al., 2009; Wymeersch et al., 2021). A retrospective clonal analysis found gut endoderm only as part of rostrally-derived clones, unlike neural tube and paraxial mesoderm that were represented in clones extending into the tailbud at E10.5 (Tzouanacou et al., 2009). This led to the idea that NMPs are bipotential, forming neural and paraxial mesodermal derivatives, whereas the endodermal lineage is set aside separately, early in gastrulation. These findings are consistent with results of DiI-based lineage tracing and tissue grafting experiments (Cambray and Wilson 2002; 2007) which show that the NMP population at the chordoneural hinge region (CNH) of the tailbud is fated to form neural and mesodermal derivatives, but not tailgut. Further support for this concept comes from the finding of a proliferative zone at the hindgut tip, which is required to generate the colon by caudally directed gut extension (Garriock et al., 2020). It will be interesting to determine whether a similar mechanism underlies tailgut development.

Mechanism of cessation of tail elongation

Termination of axial elongation is highly species-specific, occurring in embryos with fewer than 40 somites in human (**Table 2**), at ~ 52-somite stage in chick, and in embryos with 65 somites in rat and mouse (Olivera-Martinez et al., 2012). One question is whether the underlying molecular and cellular mechanisms are shared, despite these variations in timing, or are fundamentally different between species. Our findings with human embryos support a shared mechanism, as we find that expression of *FGF8* and *WNT3A* are developmentally regulated in close relationship to the time-course of axial elongation, similar to that in rodent embryos. Moreover, cessation of tail growth in the mouse has been linked to a burst of apoptosis in the tailbud around E13.5 (Wilson et al., 2009), and we detected an analogous burst of apoptosis in the CS15 human tailbud. Hence, a similar mechanism may underlie growth termination of the much shorter human embryonic tail.

Type and timing of cell death during tail regression

While programmed cell death is recognised to participate in tail regression, the precise mode of cell death has been debated. In immunohistochemistry studies, it was concluded that apoptosis occurs only in the human cranial embryonic region, and non-apoptotic ('necrotic-like') death of tail structures was identified in the regressing human tail (Sapunar et al., 2001; Vilovic et al., 2006). In contrast, cell death during chick tail regression was shown to involve caspase-dependent apoptosis (Miller and Briglin 1996). Using anti-caspase 3 and TUNEL methods, we identified apoptosis in the human tail, with patterns of cell death occurring in a closely similar way between human and mouse embryos. We conclude that caspase-dependent apoptosis is the predominant mode of cell loss during tissue regression in the tails of both mouse and human.

Conclusions

The findings of this study show a close parallel between human and rodent embryos in several features of low spinal development: completion of primary neurulation, transition to secondary neurulation, cellular mechanism of secondary neural tube formation and molecular basis of cessation of tail elongation. In contrast, some aspects of chick low spinal development – often cited as an accurate model for human – are not represented in the human embryos, indicating the need for caution in extrapolating findings from birds and lower vertebrates to humans. The main differences between human and mouse/rat tail development relate to timing with, for example, formation of a new somite every 7 h in humans, compared with 2 h in mouse/rat, and termination of tail elongation at the 36 to 37-somite stage in human, compared with the 65-somite stage in tailed rodents. While tail regression occurs completely in human embryos, it is noteworthy that the tail of mouse/rat embryos also regresses partially, with loss of secondary neural tube and tailgut, despite maintenance of an overall tail structure. An intriguing observation is the presence

of secondary neural tube splitting in apparently normal human embryos, whereas this is seen only under pathological conditions in rodents. Future work in human embryos and organoids may shed light on the mechanisms(s) of this phenomenon.

Materials and methods

Human embryos

All embryos were obtained from the MRC/Wellcome Human Developmental Biology Resource (HDBR; *www.hdbr.org*) with UK ethics committee approval and written consent of donors. Embryos were collected on ice in L-15 medium, rinsed in phosphate buffered saline (PBS) and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Embryos were assigned to Carnegie Stages (CS) using morphological criteria (O'Rahilly and Muller 1987; Bullen and Wilson 1997) and to 2-day post-conception intervals for regression analysis based on timings in Table 0-1 of O'Rahilly and Muller (1987). Only embryos that had normal external morphology and a normal karyotype were included in the study. Screening for aneuploidy was performed on all embryos, either by conventional karyotyping or by quantitative fluorescent polymerase chain reaction (QF-PCR) (Badenas et al., 2010).

Mouse embryos

Mouse studies were conducted under auspices of the UK Animals (Scientific Procedures) Act 1986 and the National Centre for the 3Rs' *Responsibility in the Use of Animals for Medical Research* (2019). Random-bred CD1 embryos were collected from pregnant females between embryonic days (E) 10.5 and 14.5 (E0.5 is the day of finding a copulation plug). Embryos were dissected in Dulbecco's modified Eagle's Medium (DMEM), rinsed in PBS, and fixed in 4% PFA overnight.

Embryo measurements

Measurements on human embryos were made post-fixation using an eyepiece graticule on a Zeiss SV6 stereo microscope. Crown-rump length was measured as the maximum distance from the top of the head to the base of the spine. Tail length was measured along the ventral surface, from the tail tip to the point where the tail joined the trunk. The distance from the tail tip to the caudal edge of the caudal-most somite was also measured. Somites were counted in total or, where indistinct more rostrally, the total number was estimated by considering the somite immediately rostral to the hindlimb bud as somite 24. Analysis of PNP closure by somite stage (**Figure 1I,J**) was performed using archival embryo images, with PNP length measurements normalised to caudal somite length in the same embryo (both measured in pixels on photomicrographs).

H&E histology

PFA-fixed caudal embryonic regions were dissected away from the remainder of the embryo and dehydrated through an ascending alcohol series to Histoclear (National Diagnostics), embedded in 56°C paraffin wax, and sectioned transversely at 7 µm thickness on a rotary microtome. For haematoxylin and eosin (H&E) staining, slides were dewaxed in Histoclear, and rehydrated through a descending alcohol series from 100% ethanol to water, then placed sequentially in: filtered Harris's haematoxylin (3 min); running tap water (1 min); Scott's Tap Water substitute (20 g sodium hydrogen carbonate + 3.5 g magnesium sulphate in 1L distilled water; ~3 sec); running tap water (1 min); 95% ethanol (1 min);eosin (3 min); running tap water (1 min); 95% ethanol (1 min); 100% ethanol (2 x 1 min); Histoclear (2x 5 min). Slides were mounted with DPX.

Immunohistochemistry

Initial studies used wax sections (**Figure 3**), and subsequently cryosections were used (**Figure 4**). For the latter, PFA-fixed tissue was dehydrated to 30% sucrose in PBS and stored at 4°C. Tissues were incubated for ~ 6 h in 30% sucrose + 7.5% gelatine in PBS at 56°C, positioned in the gelatine mix at room temperature and allowed to set, and stored at −80°C. Gelatine-embedded samples were sectioned on a cryostat at 15 µm thickness with a sample temperature of −23°C and an ambient temperature of −25°C. For staining, wax sections were rehydrated as for H&E, then blocked and incubated with antibodies as below. Cryosections were incubated in PBS at 37°C to melt the gelatine, and antigen retrieval was performed using a decloaking chamber. Slides were incubated at 110°C for 2 minutes in 10 mM sodium citrate + 0.05% Tween 20 in water (pH 6), and then returned to room temperature. Slides were rinsed in PBS + 0.1% Triton (PBST) and blocked for 1 h in PBST + 0.15% glycine + 10% sheep serum. Blocking solution was removed, followed by incubation overnight at 4°C in primary antibody solution: anti-cleaved caspase-3 (Asp175; Cell Signalling, Cat. No. 9661) diluted in PBST + 1% sheep serum at 1:1000 for wax sections or 1:250 for cryosections. The next day, slides were washed 3 x for 5 min each in PBST, then incubated in a humidity chamber for 1 h in secondary antibody solution: 1:250 Alexa Fluor donkey anti-rabbit 568 (Thermo Fisher Scientific, Cat. No. A-11011). Slides were washed 3 x for 5 min in PBST, counterstained for 5 min in 1:5000 DAPI, washed a final 2 x 5 min in PBS, and mounted using ProLong Gold mountant (Thermo Fisher Scientific, Cat. No. P36930).

TUNEL staining

TdT-mediated dUTP-biotin nick end labelling (TUNEL; Apoptag) was performed according to manufacturer's instructions. Sections were counterstained with methyl green (Vector Labs, H-3402).

Morphometric analysis of embryo sections

All image analysis was carried out using Fiji Is Just ImageJ (FIJI) software (Schindelin et al., 2012). Area was calculated using the polygon tool. Nuclei were counted using the multi-point tool. Neural tube, notochord and tailgut areas, and tailgut nuclear number, were plotted against total area of the tail section, as a measure of position along the rostro-caudal axis.

Whole mount in situ hybridisation

Digoxygenin (DIG)-labelled mRNA probes for human *FGF8* (reference sequence NM_033165.5) and *WNT3A* (reference sequence NM_033131.4) were designed for *in situ* hybridisation. Human embryos or isolated caudal regions were fixed in 10% formalin, washed in PBS with 0.1% Tween (PBT), and processed for whole mount *in situ* hybridisation. Samples were bleached in 6% hydrogen peroxide, digested in a 5 µg/ml proteinase K - PBT solution, followed by a wash in 2 mg/ml glycine, and subsequently fixed in 0.2% glutaraldehyde made up in 4% paraformaldehyde. Samples were then incubated in pre-hybridisation mix (50% formamide, 1% sodium dodecyl sulfate (SDS), 5 x saline sodium citrate (SSC), 50 µg/ml yeast tRNA and 50 µg/ml heparin), and hybridised with the corresponding digoxigenin (DIG)-labelled mRNA probes overnight at 70 °C. Hybridised probes were fixed using fixative wash solutions (solution 1: 50% formamide, 5x SSC and 1% SDS at 70 °C; and solution 2: 50% formamide, 2x SSC and 1% SDS at 65 °C). The samples were then blocked in 10% heat inactivated sheep serum, and incubated with anti-DIG-alkaline phosphatase antibody (Roche) solution. Developmental of the colour signal was carried out in nitrotetrazolium blue (NBT) and 5-bromo-4-chloro-3-indole (BCIP) solution. Whole mount images were taken using a DFC490 camera (Leica) connected to a Stemi SV11 stereomicroscope (Zeiss), and then embedded in gelatin-albumin for vibratome sectioning at a thickness of 40 µm. Sections were imaged using AxioVision v4.8.2 software on an Axioplan 2 microscope (Zeiss).

Systematic literature review (Supplementary Table 1)

PubMed (*https://pubmed.ncbi.nlm.nih.gov/*) was searched for a variety of term combinations that included: human; embryo; embryonic development; neuropore; primary neurulation; secondary neurulation; neural tube; organogenesis; tail; transcrptomics; gene expression. Retrieved papers were scanned for relevance sequentially using the title, abstract and full text, with non-qualifying papers dismissed at each stage. Additional relevant papers were identified from the bibliographies of the retrieved papers. Qualifying papers (n = 28) were those that presented original data on human low spinal/tail development, using embryo samples not described in other studies.

Statistical analysis

Linear regression analysis (**Figure 1**) and One-way ANOVA on ranks (**Figure 2**) were performed using Sigmaplot v14.5.

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Reviewer #1 (Public review):

Summary:

The authors analyzed 108 human embryos in order to address outstanding questions about human lower spinal development and secondary neural tube formation. Through whole

embryo imaging and histologic analysis, they have provided exceptional quantification of the timing of posterior neuropore closure, rate of lower spinal somite formation, and formation and regression of the human tail. Their analysis has also provided convincing qualitative evidence of the cellular and molecular mechanisms at play during lower spinal development, by identifying the presence of caspase-dependent programmed cell death and the dynamic expression of FGF8/WNT3A within the elongating embryo. Interestingly, they identified multiple polarized lumens within the site of secondary neural tube formation, and added a solid argument for the mode of formation of this structure; however, the evidence for a conclusive morphogenetic mechanism remains elusive. Finally, the authors provided a substantial review of the existing publications related to human lower spinal development, creating an excellent reference and demonstrating the importance of continuing to use each of these precious samples to further advance our understanding of human development.

Strengths:

This manuscript provides an excellent window into the key morphogenetic events of human caudal neural tube formation. Figures 1 and 2 provide beautiful images and quantification of the developmental events, enabling comparison to models that are currently in use, including model organisms and the developing spinal organoid field. The characterization of somite development and later regression is particularly important.

In Figures 3 and 4, the authors use immunohistochemistry to examine the cellular death mechanisms and spatiotemporal organization of tissue regression within the tail. They demonstrate a proximal to distal tapering of the overall tail and neural tube areas that is not present for the notochord and reveal a proximal to distal degeneration of the tailgut, similar to what is observed in rodents. The identification of caspase-dependent cell death within the human tail provides an explanation for the mechanism of this regression, especially given the notable lack of presence of any gross necrosis.

Next, the authors have addressed current questions regarding the molecular pathways present during elongation of the embryo and later regression of the tail structure. The in situ hybridization experiments in Figure 5 show important evidence for a maintained neuromesodermal progenitor pool of stem cells that promote axial elongation. Additionally, the authors have conducted serial transverse sections of the tail to better understand the formation of the secondary neural tube in humans. They found a rodent-like formation involving a singular rosette caudally at the tailbud tip, and that multiple lumens, if present, were located more rostrally. This clearly differs from chick secondary neurulation. Finally, as mentioned above, the non-trivial collection and review of the existing human secondary neural tube and body formation literature is an important tool that organizes and synthesizes ~ 100 years of observations from precious human samples.

Weaknesses:

(1) The non-pathologic presence of multiple polarizations in human tails compared to the rodent pathogenic counterpart is interesting given that rodents obviously maintain this appendage that is lost in humans. A clear mechanism for how the secondary tube becomes continuous with the primary tube and how this relates to the presence of multiple polarizations in humans remains elusive.

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Reviewer #2 (Public review):

This study utilizes an extensive series of neurulation human embryos to address several open questions about the similarities and differences between human primary and secondary

neurulation in the tail. Results are compared to other model systems, such as the chicken and rodent. Histology, in situ hybridization, and apoptosis analysis provide molecular data about how the tail regresses in the human embryo. The number of embryos utilized for the analysis and the quality of the histological analysis provide robustness to the findings.

Comments on revised version:

The authors have meticulously addressed all the concerns raised by the reviewers, using new data and modifications to the text to further strengthen the quality of the manuscript.

This is a fabulous manuscript. I have nothing more scientifically to critique.

https://doi.org/10.7554/eLife.88584.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review)***
>
> *(1) The identification of the proximal to distal degeneration of the tailgut within the human tail is difficult to distinguish with the current images present in Figure 3. A picture within a picture of the area containing the tail gut could be provided to prominently demonstrate the cellular architecture. Additionally, quantification of the localization of apoptosis would strongly support this observation, as well as provide a visualization of the tail's regression overall. For example, a graph plotting the number of apoptotic cells versus the rostral to caudal locations of the transverse sections while accounting for the CS stage of each analyzed embryo could be created; this could even be further broken down by region of tail, for example, tailgut, ventral ectodermal ridge, somite, etc.*

To provide more information on apoptosis, we prepared serial sections from an additional 6 human tails, 5 of which were processed for fluorescence anti-caspase 3 immunohistochemistry with DAPI staining (Fig 4) and H&E (Fig 6). This confirmed our previous finding of apoptosis especially in the tailgut and ventral mesoderm. We have not quantified the apoptosis, given the difficulty of deciding whether anti-caspase signals represent single or multiple dying cells. Instead, we performed a tissue area analysis from caudal to rostral along the tail (new section on p 9). This shows a progressive enlargement of the neural tube, no change in the notochord and a striking reduction in tailgut area (Fig 4C,D). The smaller tailgut has fewer nuclei in cross section rostrally compared with more caudally (Fig 4E). Given that apoptosis is present in the tailgut at all rostro-caudal levels, this is consistent with a rostralto-caudal loss of the tailgut, as is also found in mouse and rat embryos.

> *(2) The identification of the mode of formation of the secondary neural tube is probably the most interesting question to be addressed, however, Figure 7's evidence is not completely satisfying in its current form. While I agree that it is unlikely that multiple polarization foci form within the most caudal part of the tail and coalesce more rostrally, I am equally unsure that a single polarization would form rostrally and then split and re-coalesce as it moves caudally, as is currently depicted by 7B. Multiple groups have recently shown the influence of geometric confinement on neuroectoderm and its ability to polarize and form a singular central lumen (Karzbrun 2021, Knight 2018), or the inverse situation of a lack of confinement resulting in the presence of multiple lumens. The tapering of the diameter of the tail and its shared perimeter and curvature with the polarization bears a striking resemblance to this controlled confinement. An interesting quantification to depict would include the number of lumens versus the transverse*

> *section diameter and CS stage to see if there is any correlation between embryo size and the number of multiple polarizations. Anecdotally, the fusion of multiple polarizations/lumens tends to occur often in these human organoid-type platforms, while splitting to multiple lumens as the tissues mature does not. Other supplements to Figure 7 could include 3D renderings of lumens of interest as depicted in Catala 2021, especially if it demonstrates the recoalescence as seen in 7B. The non-pathologic presence of multiple polarizations in human tails compared to the rodent pathogenic counterpart is interesting given that rodents obviously maintain this appendage while it is lost in humans.*

The additional 6 sectioned human embryo tails (as described above) provide further information in support of the original findings of the paper: (i) that the secondary neural tube formation initially involves a single lumen, and (ii) that neural tube duplication occurs in many tails at more rostral levels. Neural tube duplication was observed in 15/25 of our sectioned tails: hence, overall 60% of human tails exhibited neural tube duplication in this study. We have replaced all the cross sectional images in the original Fig 7 (now Fig 6) to better illustrate the findings of neural tube duplication at relatively rostral levels of the human tail. Additionally, the axial position of sections containing duplicated neural tube are indicated by arrows in the graph of neural tube areas (Fig 4C). From this analysis it appears that neural tube duplication is not contingent on an increasing tail diameter, as raised by the reviewer, because some tails show a transition to neural tube duplication, and then return to an single lumen morphology more rostrally. While the 3D renderings of lumens would be interesting, we consider it beyond the scope of the present study.

> *(3) Of potential interest is the process of junctional neurulation describing the mechanistic joining of the primary and secondary neural tube, which has recently been explored in chick embryos and demonstrated to have relevance to human disease (Dady 2014, Eibach 2017, Kim 2021). While it is clear this paper's goal does not center on the relationship between primary and secondary neurulation, such a mechanism may be relevant to the authors' interpretation of their observations of lumen coalescence. I wonder if the embryos studied provide any evidence to support junctional neurulation.*

We agree this is an important point to address in the paper, and a new section has been inserted in the Discussion: ‘Transition from primary to secondary neurulation’ (pp 13-14). In brief, we find no evidence for a specific mode of ‘junctional neurulation’ in the human embryos. In any event, its existence is hypothetical in humans, suggested largely as an ‘embryological explanation’ for the finding of rare interrupted spinal cord defects in neurosurgical patients (Eibach, 2017). In chick neurulation there is longitudinal dorso-ventral overlap between the primary and secondary neural tubes (Dryden, 1980), with the junctional zone derived from ingressing cells at the node-streak border (Dady, 2014), a known source of neuromesodermal progenitors (NMPs). However, this is a very different developmental situation from the human so-called ‘junctional neurulation’ defect (Eibach, 2017), in which the spinal cord is physically and functionally interrupted, with only a rudimentary filament connecting the rostral and caudal parts.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) Figures 3, 4, and 7, would be easier to digest quickly with inclusions of labels that mark the rostral and caudal transverse sections. For example, "caudal" over 3G and "rostral" over 3F.*

Figures 3 and 4 have been combined to form revised Figure 3, and the rostral/caudal sections are no longer included, as these are superseded by the new Figure 4. Similarly Figure 7 has been replaced by new images in the revised Figure 6, with clear labelling of axial levels.

> *(2) The manuscript does a nice job of comparing and contrasting the human findings to mouse, however, there are several instances where it would be nice to continue this trend within the text, such as including the rate of somite formation for rodents in the sections that you state the quantified human and published organoid findings, as well as the total number of somite rodents' exhibit. Additionally, the last sentence of the "Morphology of human PNP closure" section correctly states that human PNP's seem to close via Mode 2 neurulation that is seen in the mouse. However, my read of the literature (published by Dr. Copp) demonstrates that the PNP in mice actually closes via Mode 3 at the most caudal portion. If this is the case, it would be pointed to explicitly state that regionally dependent morphogenetic difference between the two species.*

We agree these are important points to include. The additional somite data (for mouse) has been inserted in the Results section on 'Somite formation' (p 8), and the apparent absence of Mode 3 during human spinal neural tube closure is now included in the new Discussion section, 'Transition from primary to secondary neurulation' (pp 13-14).

> *(3) The introduction to secondary neural tube formation with the hypothesis diagrams in Figure 7 is slightly jarring. At the beginning of the Figure, a schematic depicting the morphogenetic differences between primary and secondary would be helpful in introducing the readership to these complex embryologic events. An example of this could be similar to Figure 1 in Dr. Copp's paper:*

Nikolopoulou, E., et al. Neural tube closure: cellular, molecular and biomechanical mechanisms. Development 144, 552-566 (2017).

We feel that a summary diagram of primary and secondary neurulation would simply reproduce diagrams that are already widespread in the literature. As noted by the Reviewer, our article in Development (Nikolopoulou, 2017) contains just such a summary diagram as Figure 1. Therefore, we prefer to explicitly cite this article/figure in our Introduction (see modified first sentence, third paragraph, p 3), so that readers can consult the freely accessible Nikolopoulou review for more detail. The diagram in Figure 7 (now revised Figure 6) has been completely redrawn to make much clearer the hypotheses being examined in the study of human secondary neural tube formation, and neural tube duplication.

> *(4) Finally, a matter of semantics, the second paragraph of the introduction describes myelomeningocele as a neurodegenerative defect, while it is true amniotic fluid further degrades exposed neural tissue while exposed, to me, the term neurodegenerative defect suggests a lifelong degeneration, which is not the case for human patients. Perhaps shortening to neurological defect is a compromise. Thank you for the important and interesting work.*

We agree that 'neurodegenerative' can mean different things to different people. Literally, it refers to degeneration of neural tissue, which of course includes neuroepithelial loss due to amniotic fluid action in the uterus. Nevertheless, to avoid confusion, the word has been removed and the sentence expanded to include a reference to the adverse effects of amniotic fluid on the exposed neuroepithelium (see Introduction, second paragraph, p 3).

> ***Reviewer #2 (Public Review)***
>
> *It is not clear how the gestational age of the specimens was determined or how that can be known with certainty. There is no information given in the methods on this. With this in mind, bunching the samples at 2-day intervals in Figure 1J will lead to inaccuracies in assessing the rate of somite formation. This is pointed out as a major difference between*

> *specimens and organoids in the abstract but a similar result in the results section. The data supporting either of these statements is not convincing.*

Human embryos were assigned to Carnegie stages based on standard morphological criteria. This was stated, with references, in the first Results paragraph, and we have now also included this information in the Methods (first paragraph, p 19). We assigned the embryos to 2-day intervals based on the standard literature timing of these Carnegies stages, as described in O'Rahilly and Muller (1987). We have clarified both Carnegie staging and assignment of embryos to 2-day intervals in a new sentence within the Methods, first paragraph, p 19. "Embryos were assigned to Carnegie Stages (CS) using morphological criteria (O'Rahilly and Muller 1987; Bullen and Wilson 1997) and to 2-day post-conception intervals for regression analysis based on timings in Table 0-1 of O'Rahilly and Muller (1987)." This has also been inserted in the legend to Figure 1J.

> *The regression analysis of somite number against days post-conception (Figure 1J) allowed a conclusion to be drawn on the rate of somite formation in early human embryos. We have added 95% confidence intervals to our finding of a new somite formed every 7.1 h in humans. We consider this to be important for comparison with non-human species and organoid systems. On p 8, second paragraph, we simply state our finding of a 7.1 h somite periodicity in human embryos, compared with 5 h in the organoid system (and 2 h in mouse and rat – as suggested by Reviewer 1). We are careful not to say it is a 'major difference' or 'similar result' in different parts of the paper, as the Reviewer has drawn attention to.*
>
> *Whenever possible, give the numbers of specimens that had the described findings. For example, in Figure 2C - how many embryos were examined with the massive rounded end at CS13? Apoptosis in Figures 3 and 4?*

Numbers of embryos analysed in Figures 2 and 3 (the latter now a combined version of the original Figures 3 and 4) are shown in Table 2. We have also created a new Supplementary Figure 1 to show additional examples of human embryonic tails, which illustrate the consistency of morphology through the stages from CS13 to CS18. Numbers of samples that contributed to Figures 4-6 are detailed in the legends.

> *For Figure 2I-K, it would be informative to superimpose the individual data points on the box plots distinguishing males from females, as in Figure 1I.*

This was attempted but the data points overlie the box plots and look confusing. Instead, we have created Supplementary Table 2 which gives the raw data on which Figure 2I-K are

based. We have also drawn attention to the fact that not all embryos yielded all types of measurement, especially tail lengths.

> *Is it possible to quantitate apoptosis and proliferation data?*

We have not quantified apoptosis, given the difficulty of deciding whether anti-caspase signals represent single or multiple dying cells. Instead, we performed a new tissue area analysis along the body axis, which has shed light on the possible direction (rostral to caudal) of tailgut loss in the human caudal region (see response to Reviewer 1 above). Since the cell proliferation data were limited in extent, and not a major focus of the paper, we have removed that analysis completely from the revised version.

> *The Tunel staining in Figure 3 is difficult to make out.*

We have extended our analysis of anti-caspase 3 immunohistochemisty and removed the TUNEL images.

> ***Reviewer #2 (Recommendations For The Authors)***
>
> *The anatomy of the sections in Figures 3, 4, and 7 is difficult to discern. Is it possible to insert adjacent panels tracing and labeling the structures in each panel? Also, drawings showing the axial level of each section would be helpful.*

To clarify the axial levels of sections, we have inserted images of mouse and human embryos as parts A and B of the revised Figure 3. We have tried to clarify the morphology of sections by labelling all relevant structures in the sections themselves.

> *High-magnification views of the tailbud in Figure 5 would be more informative. Staining is difficult to see after CS13. The low-magnification views can be shown in an insert. Figures 5 and 6 can be combined.*

At the reviewer's suggestion, we have merged Figures 5 and 6 into a revised Figure 5. Now, the sections provide higher magnification images of the areas of expression as shown in the lower magnification whole mount images. We feel this makes the gene expression findings much clearer than before.

> *Some of the writing in the abstract, introduction, and results is very descriptive, with a lack of summary and integration of information. For instance, the abstract could be rewritten to include an overall conclusion at the end and a better description of the longstanding questions addressed. Moreover, the abstract suggests multiple lumens are not found in human specimens. Another example is the second paragraph of the introduction lists various NTDs but doesn't provide an integrative conclusion of the information. The discussion is much better but lacks a conclusion at the end.*

We agree that more concluding sentences should be used, as the Reviewer suggests. To this end, we have rewritten the Abstract (p 2) to emphasise the long-standing questions that our study addresses, and concluding sentences are now included in other places (e.g. somite results, p 8). A new 'Conclusions' section has been added at the end of the Discussion (pp 17-18).

ADDITIONAL CHANGES MADE TO REVISED MANUSCRIPT

Title. This has been amended to: "Spinal neural tube formation and tail development in human embryos" to reflect the greater focus on developmental events, and less on tail regression.

Additional studies have been added to Supplementary Table 1, to include the main transcriptomic studies of human embryos in the primary/secondary neurulation stage range. This takes the number of previous studies to 28 and the total number of embryos to 925. See p 4, top and p 12, first paragraph for corresponding changes to the text.

We added a sentence to the Discussion (p 13, first paragraph) to counter the claim that humans have undergone 'tail-loss', as included in Xia et al, 2024, "On the genetic basis of tail-loss evolution in humans and apes". Nature 626:1042-8. Clearly, the human embryo is tailed, which undermines these authors' statement.

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