Genetics and developmental biology of closed dysraphic conditions

Victoria J. Jones, Nicholas D. E. Greene and Andrew J. Copp

Newlife Birth Defects Research Unit, Institute of Child Health, University College London, WC1N 1EH, UK

Key words: developmental biology, genetics, neural tube, neurulation, mouse models, malformations, spinal cord, notochord, vertebrae

Abstract

Closed spinal dysraphic conditions are typically considered malformations of caudal development, and have prompted intense speculation on possible pathogenic mechanisms. Ultimately, an understanding of developmental processes, both normal and abnormal, requires an experimental evidence base. This chapter surveys the experimental literature for clues to the genetics and developmental biology of human spinal dysraphism, based largely on studies in mouse models. Current trends in human disease gene identification, and the development of mouse genetic disease models, are reviewed, as well as several key areas of developmental biology progress that relate to development of the caudal body axis. Open neural tube defects (e.g. myelomeningocele) are relatively well understood owing to the many mouse models of faulty neural tube closure. Closed lesions in which the spinal cord is tethered and associated with spinal lipoma are much less well represented in mouse models; only preliminary clues to their likely developmental origins can currently be discerned. Some closed sacro-caudal conditions have a more defined genetic and developmental biology basis: for example, dorsal and ventral vertebral anomalies, caudal regression syndrome and Currarino triad. A future concerted research effort is needed to bring together clinical observations with research in developmental biology in this important area of pediatric clinical management.

Introduction

Closed spinal dysraphism comprises a number of diverse pathologies with varying morphology and severity, which can be isolated or associated with anomalies in other body systems. The term 'spinal dysraphism' is generally understood to mean "congenital abnormalities of the vertebrae and spinal cord or nerve roots". On the other hand, the basis of the word dysraphism - 'raphe' - is strictly "a groove, ridge or seam in an organ or tissue, typically marking the line where two halves fused in the embryo". Hence, spinal dysraphism actually refers to midline fusion defects. Appending the word 'closed' effectively rules out almost all neural tube fusion defects which generally involve open not skin-covered lesions (e.g. myelomeningocele); hence spinal dysraphism in its current usage is actually a misnomer. In this chapter, we consider the genetics and developmental biology of a range of skin-covered malformations of the low spine and/or nerve roots, and their associated vertebral anomalies.

Traditionally, the rare and often complex spinal dysraphic pathologies have prompted intense speculation on possible pathogenic mechanisms. The starting point for such speculation is usually the anatomical appearance of affected fetuses, or more often postnatal individuals, at the time of diagnosis, surgery or even death. A process of 'backwards extrapolation' is then carried out, to deduce the embryonic and fetal events that are surmised to underlie the pathology. While such an approach is a useful hypothesis-generating exercise, it has rarely been followed by experimental testing. In consequence, pediatric neurosurgery and related fields suffer from a plethora of untested and largely unsubstantiated hypotheses regarding the pathogenic mechanisms underlying low spinal malformations.

As an alternative approach, in this chapter we summarise the genetics and developmental biology of low spine formation, based on research in animal models, and ask to what extent these principles of normal development may apply in interpreting human pathology. We draw attention to the extreme paucity of definitive information on the genetic basis of closed spinal dysraphism. In a few cases, animal models have proven useful and may suggest possible causative factors (mainly genetic) and pathogenic mechanisms that may underlie the origin of closed spinal dysraphism in humans.

Approaches to the genetic analysis of congenital disease, including spinal dysraphism

The genomics revolution of the last 25 years has enormously impacted research into congenital disorders [1]. First, it has provided tools to perform an unbiased search through the genetic material of affected individuals, for example by whole genome or exome sequencing, and then to evaluate candidate gene variants for disease association and possible causation. A second innovation has been the development and analysis of animal models of human spinal disorders where the genetic causation, and sometimes the pathogenic sequence of events, can be experimentally identified. In the

following sections, we review the main features of these patient-oriented and animal model-based approaches, and then consider the extent to which they have impacted our understanding of the causation and pathogenesis of closed spinal dysraphism.

Patient-oriented genomic approaches

High-throughput DNA sequencing is an increasingly popular, unbiased approach to identify genetic variants as candidates for disease causation. The strategy requires only small numbers of cases, which makes it particularly attractive for conditions like closed spinal dysraphism, where relatively few patients are usually available. Ideally, the analysis is performed in a family of affected and unaffected individuals, but it can also be undertaken in a group of unrelated (sporadic) cases, provided the phenotype (and hence, presumed causation) is relatively homogeneous. Either the coding regions of all genes are sequenced, to generate the 'exome', or the entire coding and non-coding genome is sequenced. Thousands of variations from the 'standard reference' human exome or genome sequence are identified and these need to be filtered sequentially to remove likely non-significant variants: e.g. harmless polymorphisms. The aim is to generate a manageable list of potential candidate genes that harbour a potentially damaging 'mutation' in cases but not in unaffected controls. Candidate genes are often ranked in priority at this stage, based on their known involvement in cellular functions that might suggest a role in disease causation.

Genome-wide association study (GWAS) is an alternative unbiased approach to disease gene identification, which seeks 'associations' between candidate loci and disease. Single nucleotide polymorphisms (SNPs) are highly abundant genomic variants that can serve as 'tags' for particular genes. The method assesses whether disease occurrence is in 'linkage disequilibrium' with candidate gene SNPs: i.e. non-random associations suggest genetic linkage. In GWAS, the method evaluates association with SNPs mapping across the genome. However, large numbers of affected individuals are needed for this approach and GWAS studies are difficult to apply to relatively rare conditions such as closed spinal dysraphism.

Establishing causation for a gene variant is often very challenging, especially when a variant exists only in a single family or in a few sporadic cases. Software (e.g. SIFT or PolyPhen-2) can analyse sequence data and predict which variants are 'damaging' or 'benign', but this is not an infallible approach: mutations with known functional effects are sometimes labelled 'benign' by such software, and vice versa. Expressing the mutant and wild type proteins in cultured cells, to study effects on *in vitro* function, is a useful test of effect [2] but can only give clues to disease causality. In a few cases, direct comparison of mutant and wild-type protein function has been possible: e.g. where the gene product is an enzyme [3]. Probably the most powerful approach is to introduce the putative mutation into an animal model to determine whether a similar phenotype is produced as in human. The advent

of CRISPR/Cas9 technology for rapid gene editing [4], should make such functional studies in both cells and animal models more feasible in future.

Insights from mouse genetic models

Animal models offer an opportunity to conduct experimental studies to reveal pathogenic mechanisms of congenital disorders. Such studies are not possible in humans where only descriptive analysis can be undertaken. Hence, the analysis of animal models is usually an essential part of the journey towards an in-depth understanding of a disease process. However, a number of factors may limit the value of animal models for understanding human pathogenesis. Many models are in lower vertebrates (e.g. birds, amphibia or fish) or even invertebrates (e.g. the fruit fly *Drosophila*, or the nematode *Caenorhabditis*) and, although such models offer valuable insights into general mechanisms, our ability to extrapolate directly to humans is limited. In contrast, the mouse as a mammal has much greater extrapolation potential and, importantly, has excellent genetic tools that enable experimental analysis of disease mechanisms.

The International Mouse Phenotyping Consortium (IMPC; www.mousephenotype.org/) aims to "produce and phenotype knockout mouse lines for 20,000 genes": i.e. for most of the genes in the genome. Several thousands of genes have already been inactivated in mice, through gene targeting technology [5], and a wealth of developmental and other phenotypes has been identified. For example, more than 200 different genes yield open neural tube defects (NTDs) when individually inactivated in mice [6;7], attesting to the genetic complexity of neural tube closure. A range of phenotypes is observed, with the majority of mutants exhibiting exencephaly (the developmental forerunner of anencephaly) and a smaller number yielding open spina bifida (i.e. myelomeningocele), often with tail flexion defects. While such single-gene, loss-of-function models are invaluable, it should be noted that human developmental disorders can arise from increased gene expression, which is not routinely modelled in mice, and that gene-gene or gene-environment interactions are also likely to be of critical importance. Such interactions can be modelled in mice, but this requires a knowledge of likely candidate genes for the condition.

A limitation of mouse models of congenital disease is the marked difference in gestational length from human. The mouse is born at a relatively immature stage and while it may efficiently model early-arising human defects, those that develop at later embryonic and fetal stages (which may be the case with some forms of closed spinal dysraphism) are likely to be less well reproduced. There is also a paucity of mouse models for certain disease entities, including closed spinal dysraphism This contrasts with open NTDs where there are many models, and probably reflects the relatively 'subtle' external appearance of skin-covered spinal developmental lesions compared with open NTDs. Even if

a closed spinal defect is present in a newly created knockout line, it may go unnoticed during the initial characterisation of the mutant line.

Developmental biology events relevant to low spinal development - an overview

In this section, we review some of the main events of embryonic development, considering which are likely to have direct relevance to the pathogenesis of conditions classified as 'closed spinal dysraphism'.

Gastrulation. This process converts the bilaminar embryo into a trilaminar structure, generating the three definitive germ layers and specifying rostro-caudal and left-right body axes. Mesoderm and endoderm are generated when cells of the pre-gastrulation epiblast layer pass through the primitive streak and node, respectively, while ectoderm arises from epiblast that remains on the dorsal surface. At first sight, gastrulation might be considered a prime candidate for disruption leading to congenital spinal defects, as cells with ectodermal, mesodermal and endodermal characteristics often co-exist in a disorganised fashion in dysraphic states. However, in recent years it was demonstrated that gastrulation generates tissues only at cranial and cervical levels of the body axis, as far caudally as the sixth somite [8]. More caudal levels arise from progenitor cells in the 'tail-bud' region (also called the caudal eminence or end-bud). Hence, the gastrulation process itself is unlikely to be implicated in the origin of closed spinal dysraphism.

Neuro-mesodermal progenitors (NMPs). The existence of self-renewing progenitor cells in the caudal embryonic region (Fig 1) was originally inferred from clonal analysis in mice: cells of both neurectodermal and mesodermal types were found to arise as the mitotic descendants of single tail-bud cells [9]. As endoderm was rarely observed among these descendants, the parent cells were named 'neuro-mesodermal progenitors', but they might have broader potential. Such cells are thought to be defined by co-expression of the transcription factors Brachyury and Sox2, which play master regulatory roles in the subsequent development of mesoderm and neurectoderm respectively [10]. Moreover, it has proven possible to generate cells resembling NMPs from embryonic stem (ES) cells, of both mouse and human origin, by treatment in vitro with specific 'differentiation protocols'. For example, fibroblast growth factor (FGF) and Wnt exposure are essential to generate cells with NMP potential. Mesoderm differentiation can then be induced by continued exposure to Wnt, while neural differentiation is induced by exposure to retinoic acid plus stimulation of sonic hedgehog (Shh) signalling [11]. Hence, bi- or multi-potential cells exist within the caudal embryonic region and these are strategically placed to participate in the generation of low spinal dysraphic conditions.

Convergent extension (CE). This embryonic shaping process recently came to prominence as a vital event for initiation of neural tube closure. During and following gastrulation, cells in both

neurectoderm and underlying mesoderm alter their positions, causing a net lateral-to-medial displacement of cells in the plane of the tissues. Cells intercalate in the midline causing the body axis to narrow and simultaneously elongate rostro-caudally. CE cell movements require Planar Cell Polarity (PCP) signalling, a non-canonical Wnt/frizzled/dishevelled pathway that regulates cytoskeletal function and hence cell shape and motility [12]. If CE is compromised through mutation of PCP genes, embryos develop an abnormally short, broad neural plate that fails to initiate neural tube closure, yielding the severe open NTD craniorachischisis [13]. There is also increasing evidence that CE plays a role in shaping the developing neural tube at later developmental stages, and hence could be involved in the pathogenesis of dysraphic conditions in the low spine.

Primary and secondary neurulation. The embryonic neural tube is the development precursor of the entire brain and spinal cord, and so its development is expected to be pivotal in an understanding of spinal malformations. In terms of embryonic morphogenesis, neurulation begins with folding and fusion of the neural plate, so-called 'primary' neurulation, which initiates at the hindbrain-spinal cord boundary and then spreads throughout the brain and gradually along the spinal region. Progressively lower levels of the spinal cord are formed as closure, which resembles the travel of a zip fastener, gradually passes down the body [14]. At low spinal levels (upper sacral region), primary neurulation is completed and neural tube formation transitions to a different process, 'secondary' neurulation, in which the emerging neural tube is internal, covered by future epidermis, throughout its development (Fig 2). The secondary neural tube forms through a process of dorsal midline aggregation of mesenchymal tail-bud cells, followed by 'canalisation' in which the cells adopt an epithelial morphology, arranging themselves around a lumen to form the secondary neural tube [15]. Hence, defects of primary neurulation yield open NTDs, as confirmed by studies of mouse embryos, whereas secondary neurulation disorders are associated with skin-covered defects, as exemplified by closed spinal dysraphism.

Neural crest (NC). In the spinal region, this population of cells separates from the dorsal part of the recently closed neural tube and migrates along specific routes to contribute to neural and non-neural tissues [16]. Sensory and sympathetic ganglia are formed by NC, as are all the melanocytes of the skin. Hence, differentiation of NC cells has the potential to generate a variety of tissue types and, if the cells became misrouted and/or altered in differentiation, they could be involved in spinal dysraphic conditions. However, it is not yet clear to what extent NC cells actually arise from the secondary neural tube in the low spinal region. Sensory ganglia are not formed at this level, suggesting a reduced differentiation repertoire of 'secondary' NC. The chick has a similar lack of sensory ganglia adjacent to the caudal-most levels of the secondary neural tube; NC cells arise, but their developmental potential is restricted to melanocytes and glia [17]. There is no information on NC origins and differentiation potential in the secondary neural tube region of mammalian embryos.

Vertebral development. Formation of the vertebral column proceeds in parallel with neural tube formation in the spinal region. Vertebrae arise from the paraxial mesoderm which is formed in the low spine by differentiation of NMPs in the tail bud region (see above). Paraxial mesoderm becomes segmented to form the epithelial somites, which are specified by a molecular oscillator that involves cyclical expression of a series of 'clock' genes in the paraxial mesoderm. Each cycle specifies a single somite on each side of the body and ensures the somites are regularly spaced [18]. During subsequent development, the somites partly lose their epithelial structure, forming loose sclerotomal cells ventrally that migrate around the notochord and neural tube, later undergoing cartilaginous and then bony differentiation (Fig 2). Cells from the medial part of the sclerotome give rise to vertebral bodies and intervertebral discs, while the lateral regions of the sclerotome form the vertebral arches and ribs [19]. The caudal half of one sclerotome, and the rostral half of the next, together form a vertebral segment. Failure of the molecular oscillator to properly specify somites produces segmentation defects that alter number or size of vertebrae, whereas faulty sclerotomal migration around the neural tube and notochord is responsible for various malformations of the vertebrae.

Notochord. During gastrulation, the rostral end of the primitive streak (the 'node') migrates caudally, leaving in its wake a midline population of cells called the 'head process', which intercalates into the hypoblast (primitive endoderm) layer, forming the definitive gut endoderm [20]. Cells in the midline of the head process 'pinch off' dorsally to form the notochord, a narrow rod-like structure situated between the neural tube and the gut. In humans, the notochord is considered to comprise rostral and caudal parts, separated by the 'primitive pit' that forms the 'neurenteric canal' through which the amniotic cavity communicates with the yolk sac cavity [21]. A neurenteric canal is not generally recognised in mouse development, where the notochord is a continuous, solid structure throughout its length, even though it has several distinct developmental origins [22]. Whether this is a human-mouse species difference is yet to be established. The notochord fulfills important roles, both as a signalling center through its secretion of Shh which induces and patterns the surrounding neural and mesodermal tissues, and as a 'nucleation' center for sclerotomal cells to form the vertebral bodies. Ultimately the notochord forms the nucleus pulposus of the intervertebral discs [23].

Development of closed spinal dysraphism - clues from animal models and human genetics

The causes and pathogenesis of skin-covered spinal anomalies are poorly understood, and in need of a concerted research effort. A prenatal origin is suggested by the frequent occurrence of these conditions in fetuses and young children, and most authorities consider the defects to be 'malformations', resulting from disturbed low spinal and vertebral development. However, an acquired pathogenesis for example involving vascular insults later in gestation could apply in some cases, and should not be discounted as a possible mechanism. Here, we first review what is known

about the developmental genetics of open NTDs, where there is a significant evidence base, and then consider our less complete knowledge of the embryonic development of closed spinal dysraphism.

Open NTDs and the genetic basis of neural tube closure.

The commonest types of human and mouse NTDs are anencephaly and myelomeningocele (open spina bifida) which arise from failure of primary neurulation in the cranial and spinal regions, respectively. Craniorachischisis, a rarer NTD variant in which most of the brain and the entire spine remains open, arises from failure of closure initiation (called Closure 1 in mice). The fact that the forebrain is often closed in fetuses with craniorachischisis points to an independent closure initiation site at the extreme rostral end of the neural plate, and this has been confirmed in both human [24] and mouse [25;26] embryos. However, some other neural tube closure sites that were predicted for human embryos, based on examination of NTD patterns in late-stage fetuses [27], have not been confirmed in neurulation-stage embryos [24]. This reinforces the hazardous nature of 'backward extrapolation' to determine embryonic mechanisms.

Owing to the > 200 genetic models of open NTDs in mice, we have a good understanding of the link between faulty neural tube closure and NTD development. Moreover, this genetic mouse resource has provided clues to the genes and pathways that may be critical for open NTD development in humans. For example, the close association of craniorachischisis with PCP gene mutations in mice has demonstrated the vital role of convergent extension in establishing a narrow, elongated neural plate to allow closure initiation. Genomic studies of PCP genes in humans with NTDs have also yielded positive findings: human fetuses with craniorachischisis were found to harbour mutations in the *CELSR1* and *SCRIB* genes of the PCP pathway [2].

Interestingly, individuals with later-arising NTDs have also been found to carry putative mutations in core PCP genes including *CELSR1*, *DVL1, DVL2, FZD6, PTK7, PRICKLE1, VANGL1* and *VANGL2*, and in PCP-related genes including *DACT1*, *FUZZY*, *LRP6, SEC24* and *SCRIB* [28]. This suggests that PCP-dependent convergent extension plays a critical role throughout neural tube closure, not just at the start. Importantly, the patients are almost invariably heterozygous for a PCP gene variant, which is often transmitted from an unaffected parent. Mice heterozygous for PCP mutations are mostly unaffected (only homozygotes develop open NTDs), so it seems unlikely that a heterozygous PCP 'mutation' in humans can be solely responsible for an open NTD. This suggests that the PCP variants may interact with other, as yet unidentified, gene mutations to cause defects.

Other genes that are implicated in the causation of open NTDs in both mice and humans include those encoding enzymes of folate one-carbon metabolism which function in mitochondria. Around 70% of the cell's one-carbon units are generated in the mitochondria, and then exported as formate to the cytoplasm, entering the folate cycle to produce pyrimidines and purines for DNA synthesis. One-

carbon units also enter the methylation cycle which transfers methyl groups to nucleic acids, proteins and lipids, for example in the regulation of gene expression. Genes encoding enzymes of the glycine cleavage system, aminomethyltransferase (AMT) and glycine decarboxylase (GLDC), harbour a number of missense (i.e. amino acid-changing) genomic alterations in patients with open NTDs, but not in unaffected controls [3;29]. In the case of *GLDC*, these variants diminish enzyme activity indicating a functional effect on folate metabolism. Both *Amt* and *Gldc* mouse mutants display NTDs and, strikingly, supply of exogenous formate to pregnant females prevents NTDs in *Gldc* mutant embryos [30]. Hence, disturbed mitochondrial folate metabolism is implicated in causation of open NTDs in mammals.

Spinal cord tethering: incomplete separation of the secondary neural tube

As reviewed above, most of the spinal region (all post-cervical levels) develop by differentiation of neural and mesodermal lineages (and perhaps also endodermal) from a self-renewing population of neuro-mesodermal progenitors (NMPs) in the remnant of the primitive streak, called the 'tail-bud' [31]. Spinal neural derivatives of the NMPs sequentially form the primary and then secondary neural tube. Hence, the very different morphology of open NTDs and closed dysraphic conditions must reflect the differing modes of neural tube morphogenesis, not the origin of the neuroepithelial cells which is closely similar in both.

'Tethering' of the spinal cord refers to the failure of the cord to 'ascend' normally during fetal and infant development [32]. Differential growth between spinal cord and vertebral column causes the conus to ascend from its original sacral level to the level of the L2 vertebrae by adulthood. Moreover, the normal cord shows mobility with respiration, whereas this is lost in tethering; the result is that the terminal spinal cord and nerve roots become stretched, and neurophysiology is abnormal. In open spina bifida, failure of the primary neural tube to separate ('disjoin') from non-neural ectoderm (i.e. the future epidermis) results in tethering at the neural placode. This has been found to cause attenuation of spinal cord diameter immediately above the open lesion in mouse fetuses [33]. However, tethering is also frequently observed in closed dysraphism, where it seems likely that incomplete separation of the secondary neural tube from the surrounding tail-bud tissue is responsible. A mesenchyme-to-epithelium transition (MET) occurs as the secondary neural tube forms [34], with genes becoming expressed that characterize the epithelial state: e.g. E-cadherin, while genes that typify mesenchyme are down-regulated: e.g. fibronectin. It seems likely that failure to complete MET may underlie some cases of cord tethering in closed lesions. Additionally, other factors are known to contributing to cord tethering including the presence of abnormal lipoma tissue (see below), which can anchor the spinal cord to subcutaneous fat, and prevent spinal cord ascent.

Junctional neurulation disorders: transition from primary to secondary neurulation

In recent years, 'junctional neurulation defects' have been described in several unrelated patients where structurally and functionally normal primary and secondary neural tube derivatives are separated by a non-neural inert band [35;36]. This striking phenotype has been related to the previous finding of a 'junctional neurulation zone' in the chick embryonic neural tube. Here, dorsal cells which express the neural marker Sox2 contribute to the caudal end of the primary neural tube while, at the same axial level, ventral Sox2-negative cells undergo epithelial-mesenchymal transition, migrate caudally and contribute to the secondary neural tube [37]. This finding corresponds to the dorso-ventral demarcation at the primary-to-secondary 'transition zone' which was previously described for chick embryos, where the caudal end of the primary neural tube overlaps dorsally with the rostral end of the secondary neural tube ventrally [38]. However, no such transition zone occurs in the mouse embryo [15], raising a question about the relevance of this chick neurulation feature to humans. The gene *Prickle1* has been suggested to be essential for this junctional neurulation process, in view of its expression at this axial level and the neural tube defects that result from inhibition of *Prickle1* expression in the caudal region of chick embryos [37]. In mice, there are four *Prickle* genes, providing scope for functional redundancy. *Prickle1* has been shown to regulate body growth generally, with a partial loss-of-function phenotype resembling human Robinow syndrome [39], but with no low spinal dysraphic findings. To date, therefore, the mechanisms underlying these rare junctional neurulation defects in humans remain unexplained developmentally.

Spinal lipoma: possible aberrant differentiation of neuro-mesodermal progenitors

The ability of the self-renewing NMP cell population in the embryonic tail-bud to differentiate into a variety of neural and mesodermal derivatives makes it a prime candidate for the origin of lumbosacral lipomas. Moreover, the recent development of methods to study NMP differentiation in culture offers an opportunity to define the differentiation signals that might divert such cells towards adipose development. An understanding of the in vivo origins of spinal lipoma, however, require the development of an animal model, and only one has so far been described, as follows.

Ectopic expression of the gene *Gcm1* was achieved in the mouse tailbud by linking the *Gcm1* coding sequence to a *Hoxa7* enhancer, and creating transgenic lines [40]. Expression of beta-galactosidase (encoded by the *LacZ* gene) confirmed that only low spinal expression of *Gcm1* occurred in the transgenic embryos (Fig 3A-C). At late embryonic and fetal stages, several of the transgenic lines exhibited different dysraphic conditions including longitudinally duplicated spinal cord, resembling diastematomyelia, and caudally located open spina bifida. Importantly, formation of a lipoma was described at the tip of the spinal cord (Fig 3D-H), therefore providing a striking parallel to the situation in human closed spinal dysraphism.

The mammalian *Gcm1* gene is orthologous to the *glial cells missing* (*gcm*) gene of *Drosophila*, which functions as a master regulator of glial cell fate specification. Loss of *gcm* function in flies leads to a paucity of glia whereas increased *gcm* expression induces ectopic glia. Strikingly, the mammalian *Gcm1* gene can substitute for the *Drosophila* version in fly development, arguing for a high level of conservation of function. *Gcm1* is a transcription factor that activates *Hes* genes [41] which mediate the downstream effects of Notch signalling, in regulating neural stem cell fate. In the *Gcm1* transgenic mice, *Notch1* and *Tbx6* were down-regulated, which was interpreted as allowing more cells than normal to assume a neuroepithelial fate. However, the observation of ectopic lipoma formation in this model, in conjunction with structural defects of the low spinal neural tube (both primary and secondary), argues strongly for a more extensive maldifferentiation of NMPs, and is consistent with a fundamental defect of progenitor cell differentiation leading to spinal lipoma in humans.

Other explanations have been suggested for the origin of lumbosacral lipomas. For example, the theory of 'premature dysjunction' suggests that neural and non-neural ectoderm separate before closure of the neural tube, thus allowing paraxial mesoderm to migrate into the open neural tube preventing closure and differentiating into fat cells [42]. However, an experimental test of this idea in the chick embryo, involving surgical incision of a unilateral neural fold, gave variable developmental anomalies but no histologically identified lipoma formation [43].

Sacrococcygeal teratoma: multi-lineage differentiation from a caudal progenitor cell

As the most frequent congenital teratoma, and with a caudal location, sacrococcygeal teratomas have long been suggested to arise from the embryonic tail-bud. Even before the demonstration of a caudally-located NMP progenitor cell population, experiments both in vivo and in vitro demonstrated that the chick and mouse tail-buds are capable of differentiating into tissues characteristic of all three germ layers [34;44]. Moreover, there is a clinical association between sacrococcygeal teratomas and lumbosacral lipomas [45], consistent with a developmentally linked origin. It remains to be determined, however, under what developmental circumstances a multi-germ layer, teratomatous differentiation pattern would occur, as opposed to a highly specific maldifferentiation in which adipocyte formation is the primary pathogenic outcome.

A parallel to sacrococcygeal teratoma comes from recent studies of chordoma, a low-grade but highly recurrent tumour that typically occurs around 50-60 years of age. Chordoma originates in cells remaining from the embryonic notochord, and cell-fate-tracking experiments in mice have identified notochordal cell remnants [46] whose distribution match the sites where chordomas typically arise (skull, mobile spine and sacrum). Genetic studies show that the gene *Brachyury*, which is essential for mesoderm formation generally and for notochord development specifically, is duplicated in familial chordoma [47], consistent with the finding that sporadic chordomas typically over-express *Brachyury*

[48]. Hence, the origin in adulthood of a tumour arising from embryonic cell remnants is the result of over-expression of a gene necessary for formation and maintenance of the same tissue in embryogenesis. Analogous over-activation of one or more embryonic pathways might prove to be implicated in spinal dysraphic conditions, and sacro-coccygeal teratoma is perhaps a prime candidate.

Sacro-caudal agenesis: premature arrest of axial elongation

The spinal axis grows caudally throughout the time that the primary and then secondary neural tube are forming. However, this caudally-directed growth can become arrested at almost any stage, generating body axial truncation. In humans, this is most often encountered as sacral agenesis (also called 'caudal regression syndrome'), which can be isolated, associated with maternal diabetes mellitus, or as part of a recognised syndrome including OEIS complex, VACTERL association and Curriano triad. In each case, it seems likely that the axial arrest has its origin in a defect of the developing tail-bud. This contrasts with the origin of another caudal malformation, sirenomelia, in which the caudal axis is grossly abnormal with failure of lower limb separation. This can be strongly associated with an aberrant abdominal umbilical artery that arises from the aorta, suggesting a 'vascular steal' condition, involving redirection of blood flow from the lower extremities during development [49].

The genetic and cellular requirements for axis elongation during spinal development have been well established through analysis of mouse, chick, amphibian and fish models. In mice, continued proliferation of the NMP cell population is vital, as this is the source of cells for newly formed axial levels. NMP proliferation and multipotency depend on *Wnt3a* and *Fgf8* gene function. Wnt3a is a ligand for canonical Wnt signalling, necessary for cell proliferation and mesodermal differentiation, in part by regulation of Fgf8-mediated signalling via its receptor Fgfr1. Null mutations in either *Wnt3a* or *Fgf8* result in body axis truncation, as does excessive retinoid (vitamin A derivative) exposure, which inhibits Wnt3a expression and leads to precocious cell differentiation within the tail-bud. A similar outcome is seen when the retinoid metabolising enzyme Cyp26a1 is mutated [50], reflecting the need for the tail-bud to maintain low endogenous retinoid levels for continued NMP 'stem cell' behaviour. Embryos developing in a diabetic maternal environment are predisposed to this retinoid-mediated axial truncation [51].

It remains to be determined whether axial truncation phenotypes in humans similarly reflect defects in WNT/FGF/retinoid pathways as in mice. One issue to be borne in mind is that the mouse is a tailed mammal, so that axial truncation with absent tail is extremely obvious. In humans, while a caudal appendage is formed in the embryo, this is entirely reabsorbed into the body during subsequent development. Hence, some caudal defects of axial elongation may be less obvious in humans than in mice.

Split cord malformations

Split cord anomalies are regularly observed in humans and occur in a number of mouse genetic mutants [52]. Strikingly, when mouse tail-bud development is arrested, the default pathway of NMP differentiation is neural, with formation of multiple neural tubes. These can exist in a chaotic arrangement, for example after retinoid-induced arrest of axial elongation [53], or as a precisely organised structure, as in mice lacking the *Tbx6* gene where bilateral ectopic neural tubes form instead of somites either side of the midline neural tube [54]. Other genes whose mutants exhibit duplicated neural tube [52] include *Wnt3a* (the vestigial tail mutant), *Axin1* (the Fused and Kinky mutants) and *Brachyury* (the T mutant). All of these genes are required for mesodermal differentiation of NMPs, providing strong evidence to implicate faulty NMP development in the origin of split cord. Additionally, convergent extension may also play a role, as when faulty this can produce a broad, sometimes bifurcated notochord in mice with PCP gene mutations [55]. In humans, it has been suggested that split cord malformations result from adhesions between ectoderm and endoderm, that lead to an accessory neurenteric canal around which an endomesenchymal tract condenses, bisecting the developing notochord. This is seen as causing formation of two hemineural plates [56] sometimes with pre- and post-vertebral enteric cysts, posterior enteric sinus and posterior enteric remnants [57]. These hypotheses remain to be tested experimentally.

Currarino syndrome: an emerging gene regulatory network

A phenotype related to caudal regression syndrome occurs in the Currarino triad which comprises a sickle-shaped sacrum, presacral mass (either teratoma or anterior meningocele) and anorectal anomaly [55]. In practice, only 1 in 5 cases exhibit all three of these features, while associated malformations include Hirschsprung's disease, renal and gynaecological anomalies [58]. The occurrence of familial cases exhibiting autosomal dominant inheritance has led to the identification of mutations in the *MNX1* gene (also called *HLBX9*) which encodes a homeodomain-containing transcription factor [59]. As regards animal models, a knockout mouse lacking *Mnx1* was developed, and while this exhibited pancreatic defects, revealing a role for the gene in foregut development [60], no caudal developmental disorders were observed. Other genes of interest are *Pcsk5* and *Gdf11*, whose loss of function phenotypes include VACTERL/caudal regression/Currarino-like malformations [61]. Pcsk5 is a proprotein convertase that cleaves and activates Gdf11 protein, regulating downstream genes including *Mnx1* and caudally expressed *Hoxa, Hoxc* and *Hoxd* genes; these members of the *Hox* gene family specify levels along the body axis [62]. *Pcsk5* and *Gdf11* expression was inhibited in the hindgut region of mouse embryos after retinoid treatment that induced a caudal regression phenotype [63]. Non-synonymous mutations in *PCSK5* were found in patients with VACTERL syndrome [62]. Hence, a gene regulatory network is emerging that is necessary for normal caudal development, although the precise developmental events that these genes regulate remain unclear.

Vertebral defects: faulty specification and migration of sclerotomal cells

Incomplete neural arches of 1-2 successive vertebrae (most commonly L5 and S1), called spina bifida occulta, affects 10-15% of the population and is generally asymptomatic. However, of greater severity is a range of variable vertebral disorders that are often detected in patients with closed dysraphism, including more extensive dorsal spina bifida, butterfly vertebrae, hemivertebrae and vertebral fusions. These are often found as part of multi-system malformation syndromes, for example with butterfly vertebrae in Alagille syndrome, caused by mutations in JAG1 or NOTCH2 genes, and hemivertebrae with vertebral fusions in spondylocostal dysostosis (also called Jarco Levin syndrome).

While anomalies of neural tube formation typically have secondary effects on subsequent vertebral development, a number of genes have roles specifically in vertebral development, independently of the neural tube. For example, isolated dorsal spina bifida of the entire spinal column occurs in the presence of normal spinal neural tube formation in the *Patch* mutant mouse [64] that lacks platelet-derived growth factor receptor alpha (*Pdgfra*). The posterior neural arch elements develop initially, but fail to undergo appropriate condensation. Similarly, the transcription factor gene *Zic1* is required for formation of the posterior vertebral elements, with a particularly strong dorsal spina bifida phenotype (with no neural tube involvement) in double mutants with *Gli3*, a downstream gene in the *Shh* pathway [65].

A second group of genes is required for ventral vertebral development and pedicle formation. 'Anterior' (i.e. ventral) vertebral spina bifida occurs in cases where the vertebral bodies are malformed or absent (butterfly vertebrae) and several genes produce this phenotype in mice. Loss of function of *Pax1*, a member of the Pax (Paired box) transcription factor family, causes abnormal or absent vertebral bodies and intervertebral discs. The proximal parts of ribs are also defective, whereas the neural arches are essentially normal [66]. A second mouse model is provided by the *Bapx (Nkx3.2)* mouse mutant which also lacks vertebral bodies [67]. Regulation of *Bapx* is under control of both *Pax1* and *Shh* [68], providing evidence for a key regulatory pathways in vertebral body and intervertebral disc development. A third genetic function is required for development of the pedicles and transverse processes. *Uncx4.1* gene mutants die perinatally and exhibit severe malformations of the axial skeleton in which the pedicles, transverse processes and proximal ribs are lacking along the entire length of the vertebral column [69].

Hence, development of each part of the sclerotome that gives rise to the different parts of the vertebrae is under distinct genetic control, and mutations in these key signalling pathways are strong candidates for vertebral malformations in humans.

Conclusions

This review has attempted to apply the principles that are emerging in vertebrate developmental biology and genetics to the diverse anomalies that comprise human closed spinal dysraphism. Our understanding is currently fragmentary, with a few areas having a strong experimental evidence base, and many others remaining hypothetical. A future concerted research effort is needed to bring together clinical observations with research in developmental biology. This should significantly advance knowledge in this important area in the coming years.

Acknowledgements

The authors thank Ms Lenka Filipkova for the embryo image in Figure 1. The authors' research on spinal dysraphism is supported by grants from Smiles with Grace, Great Ormond Street Hospital Children's Charity (GOSHCC) and a Child Health Research PhD studentship. AC and NG are GOSHCC Professors.

Figure legends

Fig 1

Neuro-mesodermal progenitors (NMPs) in mouse embryonic development. Transgenic mouse embryo of genotype *Nkx1.2*^{ERT2Cre}*; ROSA26*^{YFP}, 24 h after maternal intraperitoneal injection with tamoxifen (left). *Nkx1.2* is expressed in NMPs, and so tamoxifen induces Cre-mediated recombination of the floxed YFP sequence, generating green fluorescence expression in NMPs and their mitotic descendants. The strongest YFP expression is in the tail-bud (dashed circle) where the self-renewing NMPs are located. Both open neural plate (NP) and closed neural tube (NT) are also strongly YFP-positive, reflecting neural contribution of NMP descendants. Paraxial mesoderm (PM) is YFP-positive, albeit less intensely, consistent with the multi-potent nature of the NMPs. Note that YFP expression has a rostral boundary in the upper spinal region (arrow) indicating the transition level from gastrulation-derived to NMP-derived body axial tissues. Diagrammatic representation of the caudal spinal region (right) indicates the dual function of the NMPs: to mediate axial elongation while also supplying cells to both neural and mesodermal lineages. S: somite. Scale bar: 0.5 mm.

Fig 2

Diagrammatic representation of key events in low spinal development. Schematic transverse sections at three low spinal levels, as indicated by the lines on the embryo diagram. Developmental events are depicted from early to late stage in each top-to-bottom sequence. (**A**) Sections through the embryonic tail bud show future epidermis (non-neural ectoderm) overlying tail bud mesenchyme. Mesenchyme sequentially undergoes aggregation (condensation), canalisation and mesenchyme-epithelial transition to form the secondary neural tube. (**B**) At posterior neuropore level, the neural plate folds and fuses dorsally, creating the primary neural tube. Presomitic mesoderm flanking the closing neural tube undergoes mesenchyme-epithelial transition to form somites soon after closure is complete. (**C**) At the level of closed primary neural tube, somites subsequently differentiate, partially losing their epithelial structure, to generate loose sclerotomal cells ventro-medially. Sclerotomal cells migrate to surround the neural tube dorsally and notochord ventrally and undergo cartilaginous then bony differentiation, forming the vertebrae.

Fig 3

Ectopic expression of the *Gcm1* gene, under control of a *Hoxa7* enhancer sequence, causes mice to develop open and closed neural tube defects, with caudal lipoma. (**A**-**C**) Expression of β-

galactosidase, encoded by the transgene, at embryonic days (E) 9.5 (A,B) and 10.5 (C). Staining is observed in the caudal region with an anterior boundary at the level of somites 18-20, in transgenic (Tg) embryos (B,C) but not in a non-transgenic control (Wt). Arrows indicate the closing region of spinal neural tube (the posterior neuropore) at E9.5 (A,B), whereas the spinal neural tube is closed at E10.5 (C). (**D-H**) Developmental anomalies in mice ectopically expressing the *Gcm1* transgene. Transverse sections through the lumbo-sacral level at E12.5 show open spina bifida (D) and split cord (E; asterisks). Dorsal root ganglia are indicated by the arrows. Views of the filum terminale (ft) in 1-month old wild-type (F) and transgenic (G) spinal cords shows the presence of a lipoma (lp) in the transgenic spinal cord (G). Transverse section of the adult transgenic spinal cord (H) shows the attached lipoma and the split cord, with central canals (cc) marked. D,E,H: hematoxylin and eosin staining. Bars: 0.1 mm. Figure modified with permission from [40].

References

(1) Webber DM, MacLeod SL, Bamshad MJ, Shaw GM, Finnell RH, Shete SS, Witte JS, Erickson SW, Murphy LD, Hobbs C. (2015). Developments in our understanding of the genetic basis of birth defects. *Birth Defects Res A Clin Mol Teratol* **103**: 680-691.

(2) Robinson A, Escuin S, Doudney K, Vekemans M, Stevenson RE, Greene ND, Copp AJ, Stanier P. (2012). Mutations in the planar cell polarity genes CELSR1 and SCRIB are associated with the severe neural tube defect craniorachischisis. *Hum Mutat* **33**: 440-447.

(3) Narisawa A, Komatsuzaki S, Kikuchi A, Niihori T, Aoki Y, Fujiwara K, Tanemura M, Hata A, Suzuki Y, Relton CL, Grinham J, Leung KY, Partridge D, Robinson A, Stone V, Gustavsson P, Stanier P, Copp AJ, Greene ND, Tominaga T, Matsubara Y, Kure S. (2012). Mutations in genes encoding the glycine cleavage system predispose to neural tube defects in mice and humans. *Hum Mol Genet* **21**: 1496-1503.

(4) Tschaharganeh DF, Lowe SW, Garippa RJ, Livshits G. (2016). Using CRISPR/Cas to study gene function and model disease in vivo. *FEBS J* **283**: 3194-3203.

(5) Melton DW. (1994). Gene targeting in the mouse. *BioEssays* **16**: 633-638.

(6) Juriloff DM, Harris MJ. (2000). Mouse models for neural tube closure defects. *Hum Mol Genet* **9**: 993-1000.

(7) Harris MJ, Juriloff DM. (2010). An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res A Clin Mol Teratol* **88**: 653-669.

(8) Gouti M, Metzis V, Briscoe J. (2015). The route to spinal cord cell types: a tale of signals and switches. *Trends Genet* **31**: 282-289.

(9) Tzouanacou E, Wegener A, Wymeersch FJ, Wilson V, Nicolas JF. (2009). Redefining the progression of lineage segregations during mammalian embryogenesis by clonal analysis. *Dev Cell* **17**: 365-376.

(10) Henrique D, Abranches E, Verrier L, Storey KG. (2015). Neuromesodermal progenitors and the making of the spinal cord. *Development* **142**: 2864-2875.

(11) Gouti M, Tsakiridis A, Wymeersch FJ, Huang Y, Kleinjung J, Wilson V, Briscoe J. (2014). In vitro generation of neuromesodermal progenitors reveals distinct roles for wnt signalling in the specification of spinal cord and paraxial mesoderm identity. *PLoS Biol* **12**: e1001937.

(12) Tissir F, Goffinet AM. (2010). Planar cell polarity signaling in neural development. *Curr Opin Neurobiol* **20**: 572-577.

(13) Ybot-Gonzalez P, Savery D, Gerrelli D, Signore M, Mitchell CE, Faux CH, Greene NDE, Copp AJ. (2007). Convergent extension, planar-cell-polarity signalling and initiation of mouse neural tube closure. *Development* **134**: 789-799.

(14) Copp AJ, Greene NDE, Murdoch JN. (2003). The genetic basis of mammalian neurulation. *Nat Rev Genet* **4**: 784-793.

(15) Schoenwolf GC. (1984). Histological and ultrastructural studies of secondary neurulation of mouse embryos. *Am J Anat* **169**: 361-374.

(16) Graham A. (2003). The neural crest. *Curr Biol* **13**: R381-R384.

(17) Catala M, Ziller C, Lapointe F, Le Douarin NM. (2000). The developmental potentials of the caudalmost part of the neural crest are restricted to melanocytes and glia. *Mech Dev* **95**: 77-87.

(18) Pourquié O. (2003). The segmentation clock: Converting embryonic time into spatial pattern. *Science* **301**: 328-330.

(19) Christ B, Wilting J. (1992). From somites to vertebral column. *Ann Anat* **174**: 23-32.

(20) Kwon GS, Viotti M, Hadjantonakis AK. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. *Dev Cell* **15**: 509-520.

(21) Müller F, O'Rahilly R. (2004). The primitive streak, the caudal eminence and related structures in staged human embryos. *Cells Tissues Organs* **177**: 2-20.

(22) Yamanaka Y, Tamplin OJ, Beckers A, Gossler A, Rossant J. (2007). Live imaging and genetic analysis of mouse notochord formation reveals regional morphogenetic mechanisms. *Dev Cell* **13**: 884-896.

(23) Lawson LY, Harfe BD. (2017). Developmental mechanisms of intervertebral disc and vertebral column formation. *Wiley Interdiscip Rev Dev Biol* **6**.

(24) O'Rahilly R, Müller F. (2002). The two sites of fusion of the neural folds and the two neuropores in the human embryo. *Teratology* **65**: 162-170.

(25) Sakai Y. (1989). Neurulation in the mouse: manner and timing of neural tube closure. *Anat Rec* **223**: 194-203.

(26) Golden JA, Chernoff GF. (1993). Intermittent pattern of neural tube closure in two strains of mice. *Teratology* **47**: 73-80.

(27) Van Allen MI, Kalousek DK, Chernoff GF, Juriloff D, Harris M, McGillivray BC, Yong S-L, Langlois S, MacLeod PM, Chitayat D, Friedman JM, Wilson RD, McFadden D, Pantzar J, Ritchie S, Hall JG. (1993). Evidence for multi-site closure of the neural tube in humans. *Am J Med Genet* **47**: 723-743.

(28) Juriloff DM, Harris MJ. (2012). A consideration of the evidence that genetic defects in planar cell polarity contribute to the etiology of human neural tube defects. *Birth Defects Res A Clin Mol Teratol* **94**: 824-840.

(29) Shah RH, Northrup H, Hixson JE, Morrison AC, Au KS. (2016). Genetic association of the glycine cleavage system genes and myelomeningocele. *Birth Defects Res A Clin Mol Teratol* **106**: 847-853.

(30) Pai YJ, Leung KY, Savery D, Hutchin T, Prunty H, Heales S, Brosnan ME, Brosnan JT, Copp AJ, Greene ND. (2015). Glycine decarboxylase deficiency causes neural tube defects and features of non-ketotic hyperglycinemia in mice. *Nat Commun* **6**: 6388.

(31) Wilson V, Olivera-Martinez I, Storey KG. (2009). Stem cells, signals and vertebrate body axis extension. *Development* **136**: 1591-1604.

(32) Arthurs OJ, Thayyil S, Wade A, Chong WK, Sebire NJ, Taylor AM. (2013). Normal ascent of the conus medullaris: a post-mortem foetal MRI study. *J Matern Fetal Neonatal Med* **26**: 697-702.

(33) Stiefel D, Shibata T, Meuli M, Duffy P, Copp AJ. (2003). Tethering of the spinal cord in mouse fetuses and neonates with spina bifida. *J Neurosurg (Spine)* **99**: 206-213.

(34) Griffith CM, Wiley MJ, Sanders EJ. (1992). The vertebrate tail bud: Three germ layers from one tissue. *Anat Embryol* **185**: 101-113.

(35) Eibach S, Moes G, Hou YJ, Zovickian J, Pang D. (2016). Unjoined primary and secondary neural tubes: junctional neural tube defect, a new form of spinal dysraphism caused by disturbance of junctional neurulation. *Childs Nerv Syst* **33**: 1633-1647.

(36) Schmidt C, Voin V, Iwanaga J, Alonso F, Oskouian RJ, Topale N, Tubbs RS, Oakes WJ. (2017). Junctional neural tube defect in a newborn: report of a fourth case. *Childs Nerv Syst* **33**: 873-875.

(37) Dady A, Havis E, Escriou V, Catala M, Duband JL. (2014). Junctional neurulation: a unique developmental program shaping a discrete region of the spinal cord highly susceptible to neural tube defects. *J Neurosci* **34**: 13208-13221.

(38) Schoenwolf GC, De Longo J. (1980). Ultrastructure of secondary neurulation in the chick embryo. *Am J Anat* **158**: 43-63.

(39) Liu C, Lin C, Gao C, May-Simera H, Swaroop A, Li T. (2014). Null and hypomorph Prickle1 alleles in mice phenocopy human Robinow syndrome and disrupt signaling downstream of Wnt5a. *Biol Open* **3**: 861-870.

(40) Nait-Oumesmar B, Stecca B, Fatterpekar G, Naidich T, Corbin J, Lazzarini RA. (2002). Ectopic expression of Gcm1 induces congenital spinal cord abnormalities. *Development* **129**: 3957-3964.

(41) Hitoshi S, Ishino Y, Kumar A, Jasmine S, Tanaka KF, Kondo T, Kato S, Hosoya T, Hotta Y, Ikenaka K. (2011). Mammalian Gcm genes induce Hes5 expression by active DNA demethylation and induce neural stem cells. *Nat Neurosci* **14**: 957-964.

(42) Naidich TP, McLone DG, Mutluer S. (1983). A new understanding of dorsal dysraphism with lipoma (lipomyeloschisis): radiologic evaluation and surgical correction. *AJR Am J Roentgenol* **140**: 1065-1078.

(43) Li YC, Shin SH, Cho BK, Lee MS, Lee YJ, Hong SK, Wang KC. (2001). Pathogenesis of lumbosacral lipoma: A test of the 'premature dysjunction' theory. *Pediatr Neurosurg* **34**: 124-130.

(44) Tam PPL. (1984). The histogenetic capacity of tissues in the caudal end of the embryonic axis of the mouse. *J Embryol Exp Morphol* **82**: 253-266.

(45) Oliveria SF, Thompson EM, Selden NR. (2010). Lumbar lipomyelomeningocele and sacrococcygeal teratoma in siblings: support for an alternative theory of spinal teratoma formation. *J Neurosurg Pediatr* **5**: 626-629.

(46) Choi KS, Cohn MJ, Harfe BD. (2008). Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation. *Dev Dyn* **237**: 3953-3958.

(47) Yang XR, Ng D, Alcorta DA, Liebsch NJ, Sheridan E, Li S, Goldstein AM, Parry DM, Kelley MJ. (2009). T (brachyury) gene duplication confers major susceptibility to familial chordoma. *Nat Genet* **41**: 1176-1178.

(48) Vujovic S, Henderson S, Presneau N, Odell E, Jacques TS, Tirabosco R, Boshoff C, Flanagan AM. (2006). Brachyury, a crucial regulator of notochordal development, is a novel biomarker for chordomas. *J Pathol* **209**: 157-165.

(49) Stevenson RE, Jones KL, Phelan MC, Jones MC, Barr M, Jr., Clericuzio C, Harley RA, Benirschke K. (1986). Vascular steal: the pathogenetic mechanism producing sirenomelia and associated defects of the viscera and soft tissues. *Pediatrics* **78**: 451-457.

(50) Abu-Abed S, Dollé P, Metzger D, Beckett B, Chambon P, Petkovich M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev* **15**: 226-240.

(51) Lee LM, Leung MB, Kwok RC, Leung YC, Wang CC, McCaffery PJ, Copp AJ, Shum AS. (2017). Perturbation of retinoid homeostasis increases malformation risk in embryos exposed to pregestational diabetes. *Diabetes* **66**: 1041-1051.

(52) Cogliatti SB. (1986). Diplomyelia: caudal duplication of the neural tube in mice. *Teratology* **34**: 343-352.

(53) Shum ASW, Poon LLM, Tang WWT, Koide T, Chan BWH, Leung Y-CG, Shiroishi T, Copp AJ. (1999). Retinoic acid induces down-regulation of *Wnt-3a*, apoptosis and diversion of tail bud cells to a neural fate in the mouse embryo. *Mech Dev* **84**: 17-30.

(54) Chapman DL, Papaioannou VE. (1998). Three neural tubes in mouse embryos with mutations in the T-box gene *Tbx6*. *Nature* **391**: 695-697.

(55) Greene NDE, Gerrelli D, Van Straaten HWM, Copp AJ. (1998). Abnormalities of floor plate, notochord and somite differentiation in the *loop-tail* (*Lp*) mouse: a model of severe neural tube defects. *Mech Dev* **73**: 59-72.

(56) Pang D, Dias MS, Ahab-Barmada M. (1992). Split cord malformation: Part I: A unified theory of embryogenesis for double spinal cord malformations. *Neurosurgery* **31**: 451-480.

(57) Bentley J, Smith JR. (1960). Developmental posterior enteric remnants and spinal malformations: the split notochord syndrome. *Arch Dis Child* **35**: 76-86.

(58) Hofmann AD, Puri P. (2013). Association of Hirschsprung's disease and anorectal malformation: a systematic review. *Pediatr Surg Int* **29**: 913-917.

(59) Lynch SA, Wang YM, Strachan T, Burn J, Lindsay S. (2000). Autosomal dominant sacral agenesis: Currarino syndrome. *J Med Genet* **37**: 561-566.

(60) Li H, Arber S, Jessell TM, Edlund H. (1999). Selective agenesis of the dorsal pancreas in mice lacking homeobox gene *Hlxb9*. *Nature Genet* **23**: 67-70.

(61) Szumska D, Pieles G, Essalmani R, Bilski M, Mesnard D, Kaur K, Franklyn A, El OK, Jefferis J, Bentham J, Taylor JM, Schneider JE, Arnold SJ, Johnson P, Tymowska-Lalanne Z, Stammers D, Clarke K, Neubauer S, Morris A, Brown SD, Shaw-Smith C, Cama A, Capra V, Ragoussis J, Constam D, Seidah NG, Prat A, Bhattacharya S. (2008). VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5. *Genes Dev* **22**: 1465-1477.

(62) Young T, Deschamps J. (2009). Hox, Cdx, and anteroposterior patterning in the mouse embryo. *Curr Top Dev Biol* **88**: 235-255.

(63) Tsuda T, Iwai N, Deguchi E, Kimura O, Ono S, Furukawa T, Sasaki Y, Fumino S, Kubota Y. (2011). PCSK5 and GDF11 expression in the hindgut region of mouse embryos with anorectal malformations. *Eur J Pediatr Surg* **21**: 238-241.

(64) Payne J, Shibasaki F, Mercola M. (1997). Spina bifida occulta in homozygous *Patch* mouse embryos. *Dev Dyn* **209**: 105-116.

(65) Aruga J, Mizugishi K, Koseki H, Imai K, Balling R, Noda T, Mikoshiba K. (1999). *Zic1* regulates the patterning of vertebral arches in cooperation with *Gli3*. *Mech Dev* **89**: 141-150.

(66) Wallin J, Wilting J, Koseki H, Fritsch R, Christ B, Balling R. (1994). The role of *Pax-1* in axial skeleton development. *Development* **120**: 1109-1121.

(67) Lettice LA, Purdie LA, Carlson GJ, Kilanowski F, Dorin J, Hill RE. (1999). The mouse bagpipe gene controls development of axial skeleton, skull, and spleen. *Proc Natl Acad Sci USA* **96**: 9695-9700.

(68) Rodrigo I, Hill RE, Balling R, Münsterberg A, Imai K. (2003). Pax1 and Pax9 activate *Bapx1* to induce chondrogenic differentiation in the sclerotome. *Development* **130**: 473-482.

(69) Leitges M, Neidhardt L, Haenig B, Herrmann BG, Kispert A. (2000). The paired homeobox gene *Uncx4.1* specifies pedicles, transverse processes and proximal ribs of the vertebral column. *Development* **127**: 2259-2267.

Figure 1

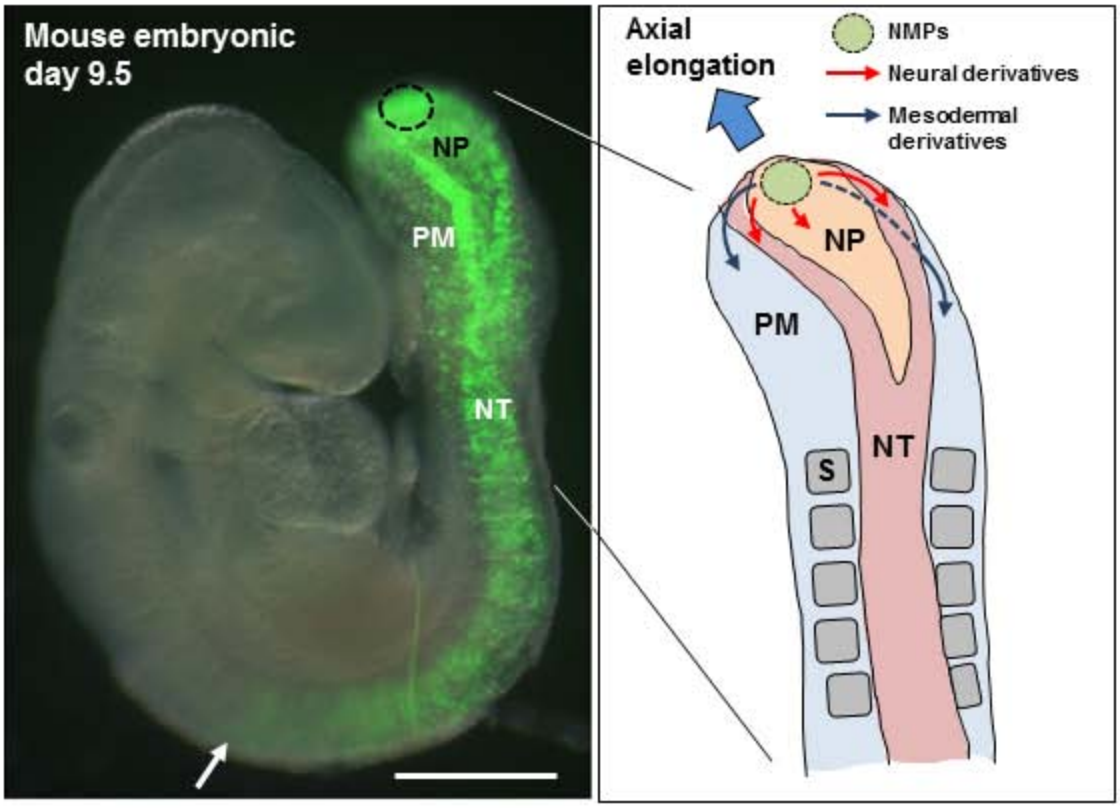
Mouse embryonic
day 9.5
NP
PM
NT
Axial
elongation
NMPs
Neural derivatives
Mesodermal
derivatives
NP
PM
NT
S

Figure 2

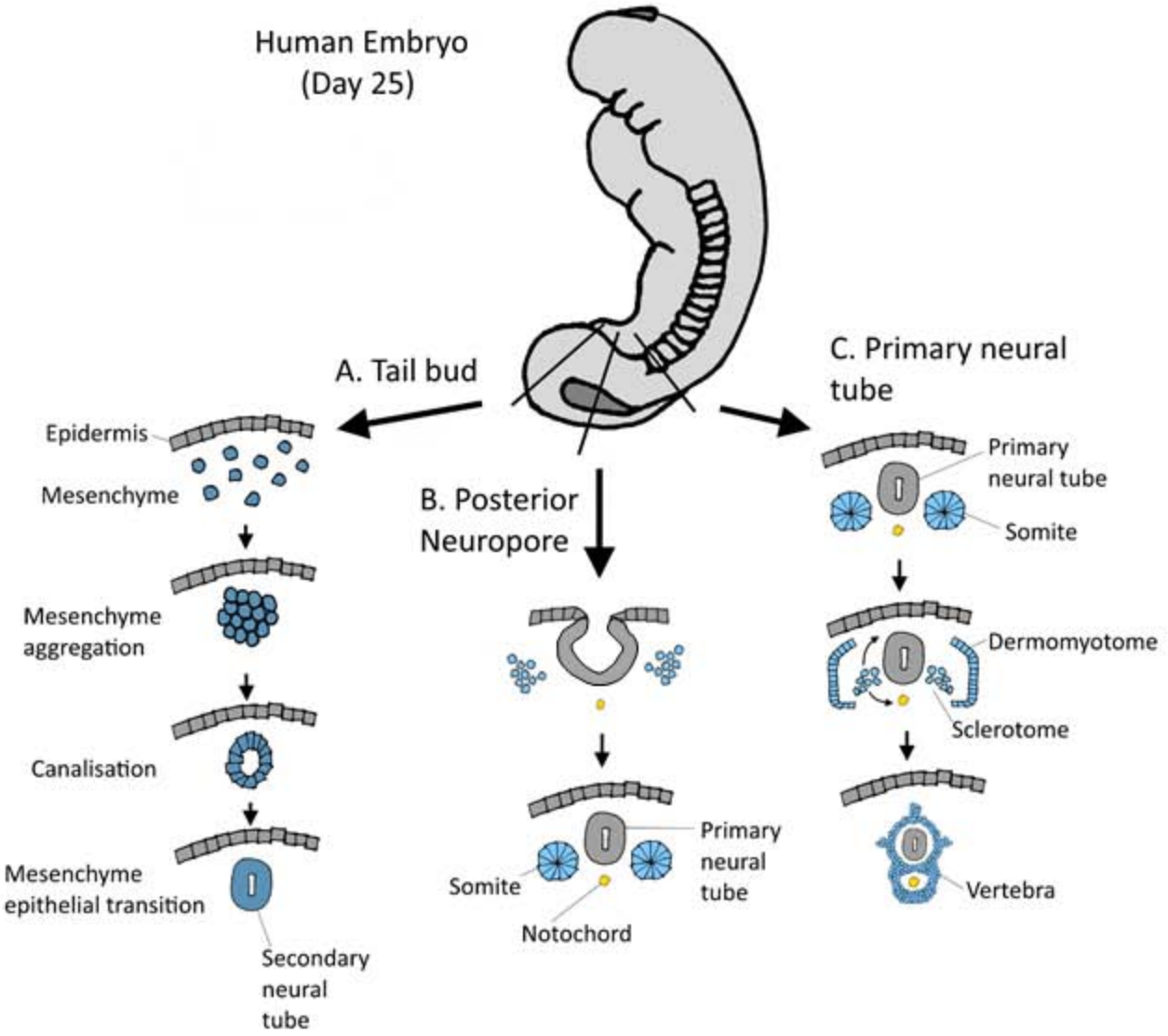
Human Embryo
(Day 25)
A. Tail bud
B. Posterior
Neuropore
C. Primary neural
tube
Epidermis
Mesenchyme
Mesenchyme
aggregation
Canalisation
Mesenchyme
epithelial transition
Secondary
neural
tube
Somite
Notochord
Primary
neural
tube
Primary
neural tube
Somite
Dermomyotome
Sclerotome
Vertebra

Figure 3