VERTEBROGENESIS: WHAT THE DISCOVERIES OF THE 21ST CENTURY ADDED INTO THE CLASSICAL UNDERSTANDING OF THE EMBRYOGENESIS OF THE SPINE IN GENERAL AND OF THE CRANIOVERTEBRAL ZONE IN PARTICULAR. SCIENTIFIC REVIEW

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Classical concepts of embryogenesis of the spine, supplemented by modern data on the role of extracellular matrix factors, specific cell adhesion molecules, signaling molecules, and Hox and Pax genes are presented. They allow us to get closer to understanding the molecular genetic cascades possibly regulating the development of the axial skeleton. Particular attention is paid to the data on the influence of these factors on the morphogenesis of the craniovertebral zone and its defects, primarily associated with segmentation disorders.

Key Words: vertebrogenesis, embryogenesis of the spine, craniovertebral zone.

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Understanding the mechanisms of spinal embryogenesis is required to explain not only the diverse anatomical variations of its development, but also possible abnormal processes associated with it [1–4]. Among all the parts of the human skeleton, the craniovertebral junction (CVJ) has the most complicated range of functions that provide head movements, protection of medulla oblongata and cranial cervical spinal cord. Such unique functionality requires specific development of the structures that form the CVJ – the occipital bone, the C1 and C2 vertebrae and ligaments related to them.

The objective is to provide a present-day idea of embryonic development of the spine and possible ways of its improvement in experimental vertebrology, and possibly – in practical one.

The Present-day Idea of Embryogenesis of the Spine

NB! Preparation of this review didn't include the task of describing all the stages of embryogenesis; therefore, we will focus on stages that are of crucial significance for vertebrogenesis.

Normal embryonic development of the spine includes 6 sequential, however, overlapping phases.

1. *Phase of gastrulation and formation of somites and notochord.* During the first two weeks after fertilization, a human embryo goes through several stages of cell division and restructuring and forms a one-layer embryoblast that divides into epi- and hypoblast, and then transforms into a three-layer embryo. Knowing the early phases of vertebrogenesis includes a fundamental fact that the embryonic cells acquire spatial ventral-dorsal and craniocaudal polarity; its markers are the occurrence of the primitive streak, Hensen's node and the elongating notochord surrounded on both sides by the somitic mesoderm that is the base for the subsequent development of axial skeleton [1–3, 5–9].

2. *Condensation of somitic mesoderm, formation of somites.* The first somites (condensates of mesodermal cells) develop at the beginning of the third week of embryogenesis in the future cervical area. There are up to five cranial somites by the start of the closure of the developing neural tube; the subsequent ones are growing in the rostral-caudal direction and are accompanied by a simultaneous wave of neurulation. It is thought that the axial differentiation of vertebrae is determined by the various expression of different homeotic genes (Hox genes, homeobox genes) at the appropriate level (the so-called "Hox profile") and by the transcription factors controlled by them (please see below). As a result, in the week 4 of pregnancy, the paraxial mesoderm adjacent to the neural tube and segments of the notochord develops in 42 somites: 4 occipital, 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 8-10 coccygeal [3, 10–15].

3. *Development of sclerotome and dermomyotome.* Differentiation of somite cells regulated by the notochord and the ventral plate of neural tube floor results

in the formation of its segments – medial (sclerotome that gives rise to the axial skeleton), external (dermatome that forms the dermis and subcutaneous tissues of the back), and internal (myotome that determines the development of myocytes of the dorsal muscles of the trunk). These segments express different molecular markers: sclerotome – Pax-1 and Pax-9, dermomyotome – Pax-3 and Pax-7, as well as Myo-D and others.

The events of embryogenesis phases 1–3 are provided in Fig. 1.

The subsequent development of somites is usually divided into 3 subsequent phases [3, 12, 16].

4. *Development of membranous somite and resegmentation.* At week 5 of pregnancy, all somites, with the exception of several cranial ones, undergo a reorganization of their rostral-caudal borders, i.e. resegmentation. Upon that, each sclerotome divides into cranial and caudal halves expressing different cellular and molecular markers. Dense caudal part of the sclerotome combines with the less dense cranial part of the underlying one; in this manner a vertebral body is formed that does not correspond to the initial segmentation (this process is called a "metameric shift"). A cleft between new vertebrae filled with mesenchyme (von Ebner's fissure) is transformed into an intervertebral disc, with a single formation, nucleus pulposus, that originates entirely from the notochord in its center. After the metameric shift, spinal nerves that initially emerged from the neural tube are located between the precartilaginous vertebral bodies; they get through the center of sclerotome and innervate segmental myotomes. Segmental arteries are developed near the center of each precartilaginous vertebral body.

The CVJ develops from the fourth occipital sclerotome and the first and second cervical sclerotomes. The fourth occipital sclerotome, or so-called transitional sclerotome of the proatlas, originates from the fusion of the caudal half of the fourth somite and the rostral half of the fifth somite (in Russian literature, "proatlas" usually refers to the bone between the occipital bone and the C1 vertebra). A break line is developed at the segmentation border that is the final cell-level separation of the skull and spine. Thus, the condyles and condylar processes, the foramen magnum (FM) ring, part of the C2 dens, as well as the apical, cruciate, and alar ligaments raise from the fourth occipital sclerotome (somites 4 and 5). The first cervical sclerotome (somites 5 and 6) gives rise to all components of the atlas and most part of the C2 dens; the second cervical sclerotome (somites 6 and 7) gives rise to the other parts of the second cervical vertebra. As a result, seven cervical vertebrae develop from eight cervical somites (Fig. 2) [3, 12, 16–19].

During resegmentation, somites 4–7 give raise to the proatlas (PA), the first (C1) and second (C2) cervical sclerotomes, and the intervertebral rim zones (IRZ_{1-3}) that, in turn, form the following anatomical structures:

– PA – clivus of the occipital bone (C), its anterior tubercle (AT), basion (B), occipital condyles (OC), edges of the FM

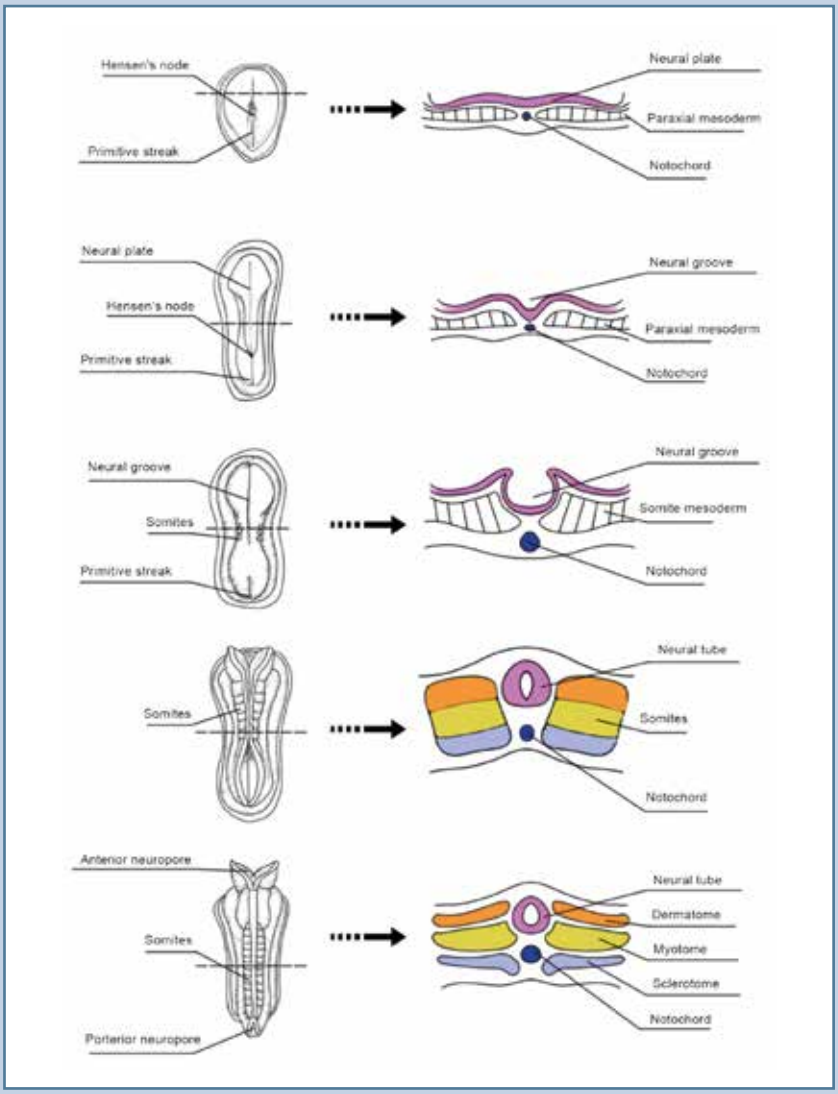

Fig. 1
Events of phases 1–3 of embryogenesis: explanations in the text (drawing by Ya.E. Sazonov based on the authors' sketch)

(EFM), opisthion (O), and the apex of the C2 odontoid process (AOP);

– C1 – basal segment of the C2 odontoid process (BSOP), as well as the anterior arch of the C1 atlas (AAA), its lateral masses (LMs), superior and inferior articular surfaces (SAS and IAS), and the posterior arch (PA).

– C2 – the epistropheus body (AB), its superior and inferior surfaces (SS and IS);

– IRZ_1 – the superior synchondrosis of the odontoid process (SSOP);

– IRZ_2 – the inferior synchondrosis of the odontoid process (ISOP);

– IRZ_3 – the first intervertebral disc (ID).

5. *Chondrification phase.* The development of chondrification centers starts in the week 6 of embryogenesis in the cervicothoracic region and spreads cranially and caudally; it is induced by the substances secreted by the notochord and neural tube. As a rule, three paired chondrification centers develop in each vertebra: ventral (vertebral body) ones surround the notochord ventral to the neural tube; dorsal ones form the posterior arches and spinous processes; the third (intermediate) ones develop between the dorsal and ventral pairs giving raise to the transverse processes and costal arches. Ventral centers develop earlier than the dorsal ones, and the intervertebral disc ring is originated from cells that gather around the notochord, while its physaliferous cells develop in the centrally located gelatinous nucleus. The anterior and posterior longitudinal ligaments (ALL and PLL) are developed at the same stage from the mesenchymal cells surrounding the cartilaginous vertebrae [3, 16].

6. *Ossification phase.* Vertebral ossification starts in the week 8-9 of embryogenesis and continues after birth. Ossification of the vertebral body centers occurs slightly earlier than the ossification of the dorsal arch; it starts in the thoracolumbar region (T10–L1), and quickly spreads cranially to T2 and caudally to L4; more cranial and caudal vertebrae are involved later. Ossification of the dorsal arches from C1 to L1, in turn, starts simultaneously and continues in the craniocaudal direction. All ossification centers are visible by 14 weeks of pregnancy [3, 16, 20].

Cartilaginous endplates are differentiated cranially and caudally to the ventral ossification centers; on their periphery, between the intervertebral disc and the growing ossification center of the vertebra, there is an ellipsoid ring apophysis with relatively stronger lateral and ventral parts. Secondary ossification centers in apophyses and apexes of the spinous and transverse processes develop only by 11–14 life years; they merge by 15–16 years. Complete fusion of the primary and secondary ossification centers occurs in late adolescence.

In summary, the fundamental periods of vertebrogenesis can be represented as follows (Table 1).

Contribution of the 21st Century Discoveries to the Understanding of Congenital and Hereditary Vertebral Abnormalities

Progress in embryology and molecular genetics has helped to come closer to understanding the mechanisms of encoding genetic programs and their dependence on microenvironmental factors. Information on biochemical gradients, morphogens, mechanical forces, cell migration, and cell adhesion is the base for a general concept of the embryogenesis of congenital spine abnormalities including the craniovertebral junction zone [12, 19].

Molecular basis of morphogenesis. Histo- and organogenesis is based on decreased cell pluripotency and differentiation of cell lines; these processes provide positional information and biochemical specificity of cells starting from their first divisions [21]. Upon that, morphogenesis is determined by a limited set of division processes, as well as changes in shape, composition, migration, and growth of cells. The extracellular matrix (ECM) is a highly organized structure that includes collagen, proteoglycans and other macromolecules; it is considered to have an effect on these processes.

According to the idea of the migration system, at the differentiation stage, interaction with it results in the cells acquiring morphofunctional polarity, ability to diffuse into the corresponding areas of the embryo and to form specific associations with the help of cell adhesion molecules [22–25].

In addition to recognizing ECM signals, migrating cells also communicate via long-distance signaling molecules (morphogens) that regulate differential gene expression and control cell specification in tissues due a concentration gradient. Morphogen molecules are released from a local but active source, aggregate together and/or with other molecules, and move through the extracellular medium due to limited diffusion. Gradient type is determined by the release rate, the diffusibility of the morphogen, and the kinetics of its clearance in target tissues. By interacting with heparan sulfate proteoglycans (HSPG) and other extracellular proteins, morphogens bind to the cell surface having an effect on their transport and reception. Linear transfer of morphogen can have an effect on intracellular signaling pathways resulting in stepwise activation of the transcription (reading) effect; the latter is involved in the target gene response to the concentration and duration of morphogen exposure. Thus, target genes are regulated not only by the morphogen signaling pathway, but also by preexisting factors and cross-talk. Feedback mechanisms equilibrate fluctuations in morphogen generation, have an effect on the interpretation of signals, and scale up the development of the pattern mediated by them [26–28].

Key vertebrogenesis-regulating genes. It is considered that genes related to the homeobox (Hox) and paired box (Pax) have a critical role in controlling the development of the craniocervical junction [29].

Hox genes are involved in the development of a molecular coordinate system that determines the basic plan of embryo's structure. Works on the functions of proteins encoded by Hox genes revealed that they were involved in cell differentiation and organ development, as well as participated in hematopoiesis, development of neural circuits, and tissue regeneration even in adulthood

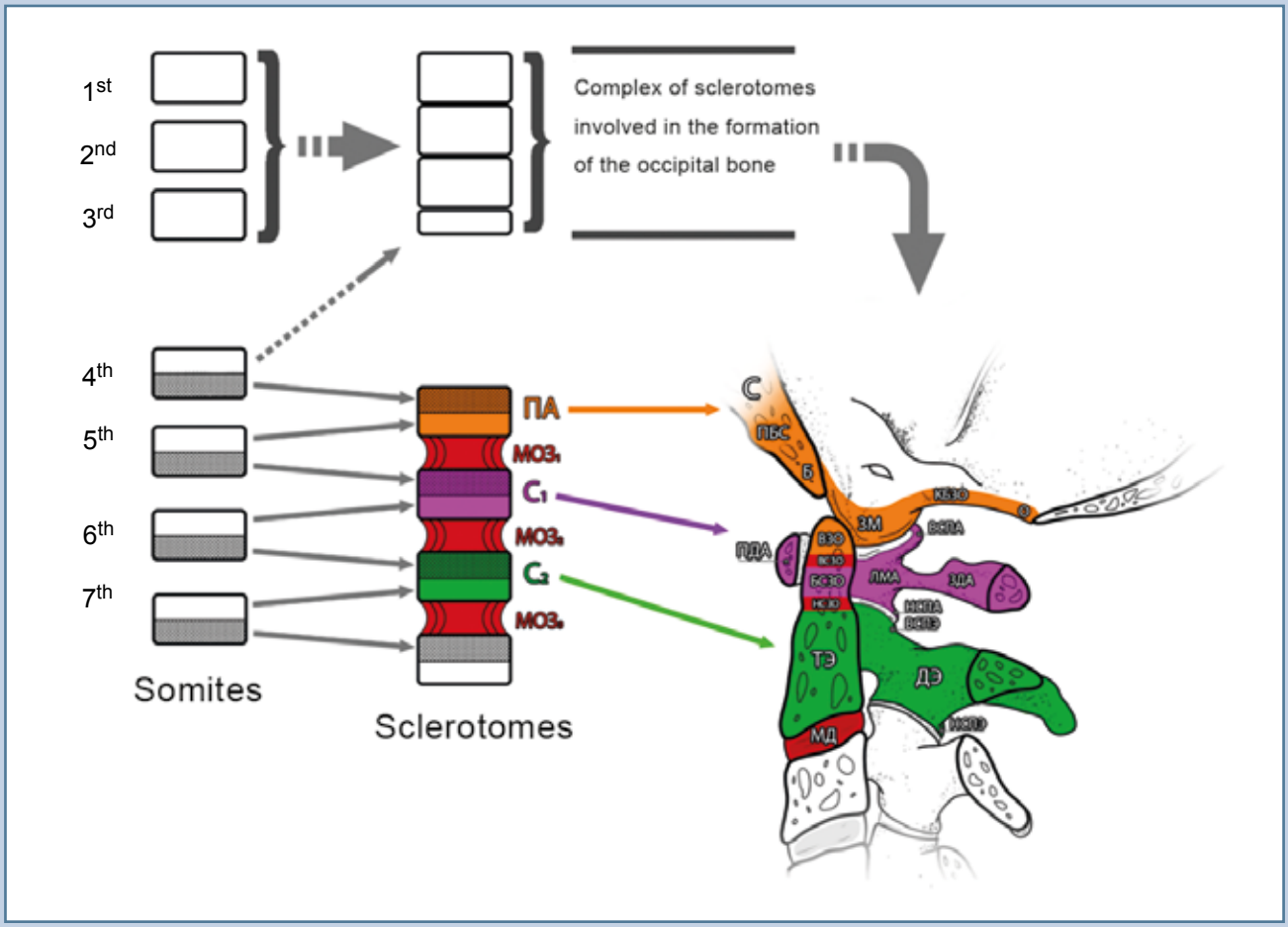

Fig. 2
Scheme of somite distribution as a result of resegmentation (drawing by Ya.E. Sazonov based on the authors' sketch)

Table 1
Deterministic periods of prenatal and postnatal vertebrogenesis

Prenatal phases of vertebrogenesis	
Somitization of paraxial mesoderm	Week 4
Formation of the membranous somite and resegmentation	Week 5
The occurrence of chondrification centers	Week 6
The beginning of vertebrae ossification (the occurrence of ossification nuclei)	Weeks 8–9
Visualization of all spinal ossification centers	Week 14
Postnatal phases of vertebrogenesis	
The occurrence of the ossification nucleus of C2 dens	3 years
Fusion of the odontoid process and the body of the C2 vertebra, complete ossification of the dens	4-6 years
Fusion of the ossification nucleus of the dens and the odontoid process of the C2 vertebra	6 years
The occurrence of ossification foci of the vertebral endplates	11–14 years
Fusion of ossification foci of the vertebral endplates	15 years
Fusion of primary and secondary ossification centers of the spine	15–16 years
Completion of postnatal formation of the bone components of the cervical spine, synostosis of the apophyses of the vertebral bodies	14–17 years

[30–34]. Hox proteins are associated with a wide range of abnormalities that develop in connection with mutations or improper regulation [35, 36], including vertebral transformations: for example, excessive expression of Hox-7 in mice results in posterior translocation of the cervical vertebrae followed by the development of an aberrant bone, the proatlas, from the last occipital somites, instead of the occipital bone. The true atlas is instead developed with a complete vertebral body [3].

All Pax genes in vertebrates include a highly conserved DNA sequence called the "paired box". Encoded Pax proteins, often referred to as master regulators, are tissue-specific transcription factors that control the development, structuring, and function of organ tissues during cellular differentiation by maintaining cell identity. Despite the well-known function of Pax proteins in the development of abnormalities, the role of their isoforms in the embryogenesis of craniovertebral deformities is further studied [37–39].

The key to understanding the anatomy of vertebral malformations may be in the regulation of segmentation mechanisms: while Hox genes determine the rostral-caudal identity of cells within somites after primary segmentation, Pax-1 expression is of the first importance in resegmentation because of its effect on cell differentiation into tissues [40]. Out of the nine known Pax genes, all, except Pax-1 and Pax-9, are involved in the development of the central nervous system. The exceptions, especially Pax-1, control boundary formation between tissues (cell populations): it is thought to encode a transcription factor due to regulating molecules expressed by different populations of surface cells [41, 42] that are required for the resegmentation of cell adhesion molecules (such as NCAM (cytotactin)) and intercellular communication (such as connexins) [41, 43–46]. At the same time, the effect of Pax-1 on the intervertebral boundary zone (IBZ), with the mesenchyme of the future intervertebral disc, helps in the zonation (segregation) of sclerotomes.

Pax-1 expression is detected in predifferentiated somites very early: SHH (Sonic Hedgehog) protein-mediated signals from the chord and ventral plate of the neural tube floor induce somite dividing into sclerotome and dermomyotome; this coincides with intense Pax-1 expression of ventral part of sclerotome, suggesting that Pax-1 also plays a mediating role in the dorsoventral specification of somites [47, 48]. After somitic differentiation, Pax-1 expression is found within both lateral and axial sclerotomes where its fluctuations coincide with crucial resegmentation events. For example, during condensation of the sclerotome into the loose and dense halves, Pax-1 expression is weak within the loosely cellular prevertebrae but intense within the IBZ [41, 48]. Later, during chondrification and development of a homogeneous vertebral body, Pax-1 is repressed, however, persists in high levels at the interbody zones, where the centers are separated until intervertebral disc development is underway. Pax-1 level is also enhanced during condensation of the lateral sclerotome to form the neural arch [48–51].

On the contrary, normal fusion of certain adjacent sclerotomes takes place only when Pax-1 expression is turned off. At the CVJ of chick embryos, Pax-1 repression is timed exactly when the occipital sclerotomes fuse to form the basioccipital. The fusion of the two C2 odontoid process primordia with its body also coincides with the down-regulation of the Pax-1 gene, while ectopic Pax-1 expression disrupts normal assemblage of the dens axis and base of the occipital bone [48, 51]. Pax-1 is also highly expressed within the junctional zone between the proatlas and the first cervical sclerotome. It may thus also play a role in the separation of the head from the trunk. Murine Pax-1 mutants are associated with multiple fusion of vertebral bodies and fusion of the odontoid process with the anterior atlantal arch, reminiscent of the human Klippel – Feil syndrome. [51, 52].

Hyper- and hyposegmentation defects in humans are likely to be explained by over- and under-expression of Pax-1 during certain periods of vertebral development. Under the control of the Pax and Hox genes, the proatlas hypocenter forms the anterior tubercle of the clivus, while its center gives raise to the dens apex and apical ligament. The neural arch is divided into a ventral-rostral segment that forms the occipital condyles, the anterior margin of the FM, alar and cruciate ligaments, and a dorsal-caudal segment that forms the posterior arch and lateral masses of the atlas [12, 39]. In early development, a dense band of connective tissue forms an arch that is ventral to each vertebral segment. The proatlas-related hypochordal arch usually regresses, and the one related with the first cervical somite contributes to the development of the C1 anterior arch [12, 53, 54].

The mesoderm and ectoderm are involved in the development of the posterior cranial fossa and the growth of skull parts located anterior to the chord [55], while the parts located caudal to the dorsum sellae are of vertebral, including chordal (*chorda dorsalis*), origin [56]. The clivus has typical endochondral osteogenesis, that is, the initial development of cartilage with its subsequent reabsorption and ossification [57]. During development, the notochord emerges from the skull base onto the outer surface of the future clivus, where irregular processes are grown [56]. In the *os basisphenoidale*, the chord reaches the caudal pituitary gland where the skull base starts to develop [58]. The brain is surrounded by a thick mesenchymal mass that covers the chord and gives raise to the clivus and dorsum sellae [59]. These mechanisms fully explain the tendency for tumor growth in this area, such as chordomas and chondromas/chondrosarcomas.

The Pax-1 gene is also involved in the development of such a defect as the median (third) occipital condyle (the so-called *condylus tertius*), a residual occipital vertebra located anterior to the FM and sometimes fused with the dens or atlas; it limits movements in the CVJ [53]. In turn, atlantoaxial instability is associated with the hypoplasia of occipital condyles and *condylus tertius*; it is accompanied not only by the retaining proatlas,

but also by the odontoid process hypoplasia, stenosis of the FM and cervical spinal canal, compression of the medulla oblongata and vertebral artery, and weak transverse ligaments [54].

Groups of molecules involved in vertebrogenesis. Progress in molecular genetics over the past 20 years allowed coming closer to understanding the molecular genetic signaling cascades that are of fundamental importance in the development of the axial skeleton. Williams et al. [60] made it possible to identified groups of genes and proteins involved in the processes of embryonic vertebrogenesis, as well as typical diseases caused by the impaired activity of the corresponding molecules. Thus, in Klippel-Feil syndrome that is characterized, first of all, by the impaired segmentation of cervical vertebrae, several authors define Meox-1 (Mesenchyme Homeobox-1) as one of the triggering agents of sclerotome differentiation [61] that interacts with Pax-1 and Pax-9 inducing Bapx-1 expression [60, 62, 63]. Activation of the Bapx1-Sox-9 positive feedback mechanism allows sclerotome responding to the signal transmission by bone morphogenetic proteins (BMP) aimed at the differentiation of chondrocytes [64–66]. Thus, Bapx-1 is of critical significance for their formation during the development of vertebral bodies and intervertebral discs [67, 68]. Pang and Thompson call Pax-1 as the resegmentation gene [40]. Among the molecular markers of the abnormality, the rostral (Mesp-2 and Tbx-18) and caudal (Ripply-1 and -2, Uncx-4.1 and Pax-1/9) markers indicating the sclerotome polarity are considered critically important [69–71]. Identification of mutations in the GDF6 gene in Klippel-Feil syndrome suggests its important role in vertebral segmentation [72, 73].

Table 2
Some signaling molecules controlling the phases of embryonic vertebrogenesis

Signaling molecules	Key controlled process
Pax-1, SHH	Differentiation of the somite
Meox-1, Pax-1, Pax-9, Bapx-1, Sox-9, BMP	Differentiation of the sclerotome
TBX-18, UNCX-4.1, GDF-6, Mesp-2, Ripply-1/2, Pax-1/9	Resegmentation

Examples of several processes and their regulating agents are provided in Table 2.

Conclusion

Classical experiments on embryogenesis regulation performed at the end of the latest century were improved at the beginning of this one by the identification of types of molecular markers and genes that determine the development of certain anatomical structures and their abnormalities. The craniovertebral zone, having the most complex anatomy in comparison with all sections of the axial skeleton, can become an object for the analysis of the fundamental mechanisms of abnormal vertebrogenesis and its possible regulation.

New knowledge can not only bring us closer to the intended development of experimental models of congenital abnormalities of the axial skeleton, but also becomes the basis for targeted methods of regulating such conditions as primary abnormality of vertebrae and ribs segmentation, their secondary synostosis (including postoperative), as well as the development of a postoperative bone union. Thus, they may have unexpected applied effects that considering the rapid accumulation of scientific knowledge may become one of the future focus areas in vertebrology.

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The study was approved by the local ethics committees of the institutions. All authors contributed significantly to the research and preparation of the article, read and approved the final version before publication.

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