CELLULAR AND MOLECULAR REGULATION OF EMBRYONIC SKELETAL DEVELOPMENT AND MORPHOGENESIS

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Abstract

Embryonic skeletal development is the result of programmed differentiation and morphogenesis of the embryonic mesoderm. This review summarizes recent cellular and molecular findings on the mechanisms underlying development of the cranial, axial, appendicular skeletal components. Specifically, the identities and activities of patterning and morphogenetic genes are presented, as well as the regulation and functional consequences of cell-cell and cell-matrix interactions important for cell differentiation and organogenesis. Finally, this review also surveys a number of *in vitro* and *in vivo* experimental systems currently in use for the study of skeletal development.

Key Words: Bone, cell-cell interaction, cell differentiation, cell-matrix interaction, embryonic mesoderm, *in vivo*, *in vitro*, morphogenesis, skeletal development.

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Introduction

Embryonic skeletal development represents the sequential events of patterning, cell differentiation and maturation, and morphogenesis, which together give rise to the full compendium of cartilage and bone. That skeletal structures function as the scaffolding of the vertebrate animal underscores the importance of stringent regulation of skeletogenesis at multiple levels. This review describes the major pathways of ossification, the cellular origin of cartilage and bone, the molecular mechanisms of skeletal patterning of the embryonic skeleton, and various model systems currently being used to study the mechanisms of skeletal cell differentiation.

Ossification Pathways

There are two principal pathways of skeletal development: endochondral and intramembranous (Hall, 1988). In endochondral ossification, such as that seen in the long bones and vertebrae, mesenchymal cells condense, undergo chondrogenesis and form cartilage, which subsequently matures and undergoes hypertrophy, and is eventually replaced by bone (Caplan and Boyan, 1994). In this manner, the cartilage anlage also serves to spatially define the future marrow environment where the hematopoietic compartment is housed and where blood cell differentiation proceeds. On the other hand, intramembranous ossification involves direct differentiation of mesenchymal cells into osteoblasts, and is seen most predominantly in various craniofacial bones (Langille, 1994). The ossification centers of intramembranous bones, such as the cranial calvarial bone, then fuse to form plates of woven bone. No cartilage intermediate is found in the intramembranous bones. Thus, two different pathways operate to give rise to histologically similar tissue structure. The underlying mechanisms, which govern the specific choice of ossification pathways, are clearly important for proper skeletal development.

Interestingly, during embryonic skeletogenesis, different cell lineages contribute to the primary endochondral bone than to the primary intramembranous

bone. For example, the craniofacial bones (intramembranous) are primarily derived from the cranial neural crest cells (Couly *et al.*, 1993; Langille, 1994; Noden, 1988), whereas the limb mesenchyme and the prevertebral sclerotome (both endochondral precursors) are of lateral plate mesoderm (Newman, 1986) and paraxial mesoderm origin (Tam and Trainor, 1994), respectively. However, neural crest cells will differentiate into cartilage, provided that they interact with the appropriate epithelium during their migration to the rostral part of the developing embryo (Hall, 1991). For example, during migration, the differentiation of cranial neural crest cells with anchorin CII receptors for collagen type II may be influenced by their interaction with collagen type II (Thorogood *et al.*, 1986). Also, premigratory neural crest cells from the mesencephalic region cultured *in vitro* with cranial ectoderm or retinal epithelium undergo chondrogenic differentiation, whereas the same cells combined with maxillary epithelia produce both cartilage and bone (Bee and Thorogood, 1980). Thus, at least for early neural crest cells, regulation of their differentiation fate may depend on the influence of the epithelia. In a similar manner, the formation of intramembranous bone by the mesenchyme of the palate (Tyler and McCobb, 1981), maxilla (Tyler, 1978), and mandible (Tyler and Hall, 1977), all require direct contact with the epithelia. In an analogous manner, chondrogenic differentiation of the lateral plate mesoderm derived mesenchyme of the limb bud is also regulated by interaction with the limb epithelium (Solursh *et al.*, 1981), most likely mediated via the action of fibroblast growth factor members (Crossley and Martin, 1995; Fallon *et al.*, 1994; Niswander and Martin, 1992, 1993).

Interestingly, under certain conditions, the calvarium, a normally intramembranous bone, may exhibit a cartilage-like phenotype. For example, in chick embryos which are rendered severely calcium-deficient as a result of long-term culture *ex ovo* (without the eggshell), the calvarium expresses collagen type II, a cartilage matrix associated component, and cells with chondrocyte-like phenotype are found (Jacenko and Tuan, 1986; McDonald and Tuan, 1989; Tuan and Lynch, 1983). Another situation is callus formation in a fractured calvarial bone, which also involves chondrogenesis (Girgis and Pritchard, 1958; Hall and Jacobson, 1975). Thus, the two apparently different pathways of ossification, intramembranous and endochondral, may actually share some common underlying mechanisms, particularly with respect to the differentiation potential of the initial mesenchymal cell population.

It is reasonable to speculate that, since the mesenchymal cells contributing to the final intramembranous bone have the potential to undergo chondrogenesis, a specific set of regulatory signals must operate during normal intramembranous ossification to either enhance osteogenesis or suppress chondrogenesis. Conversely, the environment and cell-cell/cell-matrix interactions operating during endochondral ossification must be of such a nature as to promote chondrogenesis of the condensing mesenchyme. Understanding the nature of these influences is important for elucidating the basic mechanisms of embryonic skeletal development, as well as designing approaches to treating or enhancing the healing of defective or damaged skeletal components.

Cellular Origin and Morphogenesis of the Axial, Appendicular, and Craniofacial Skeleton

Although the skeleton is derived wholly from the embryonic mesoderm, different lineages contribute to the axial, appendicular, and craniofacial components. This section briefly summarizes the ontogeny of the vertebra, the long bones of the limb, and the cranial calvarial bone.

Axial skeleton

The prevertebra is one of the earliest skeletal structures, and is of paraxial mesodermal origin (Bellairs, 1979; Tam and Beddington, 1987; Tam and Trainor, 1994). The paraxial mesoderm is formed from the early mesoderm which streams into the paraxial regions on either side of the regressing primitive streak of the gastrulating embryo. At the completion of the head process stage during early embryonic development, the paraxial mesoderm gives rise to the unsegmented mesoderm, located at the caudal end of the embryo and lying on either side of the developing neural tube and notochord. The next important stage of paraxial mesoderm development in the gastrulating embryo is somitogenesis (Fig. 1): the mesenchymal cells located at the rostral aspect of the paraxial mesoderm begin to condense, forming structures known as somitomeres; these structures subsequently emerge as pairs of well segmented somites, one on each side, separated by distinct somitic furrows. The nascent somites then undergo mesenchymal-epithelial transformation, such that a polarized epithelial cell layer engulfs a central core of cells contained in a somitocoel cavity. Upon further maturation towards the rostral end of the embryo, the somites differentiate into a dorsal dermamyotome and a ventral mesenchyme-like sclerotome (Bellairs, 1979; Ordahl, 1993). Further maturation involves the bifurcation of the sclerotome into rostral and caudal halves, a process known as re-segmentation, which allows the caudal half of an anterior somite to fuse with the rostral half of the immediately posterior somite upon their migration medioventrally towards the notochord to give rise to a perichordal sheath to form the prevertebra (Keynes and Stern, 1988). In this manner, the prevertebra is located one half segment out-of-register with the original somite. This re-segmentation process is of great importance for the musculature and neuronal orientation, with respect to the
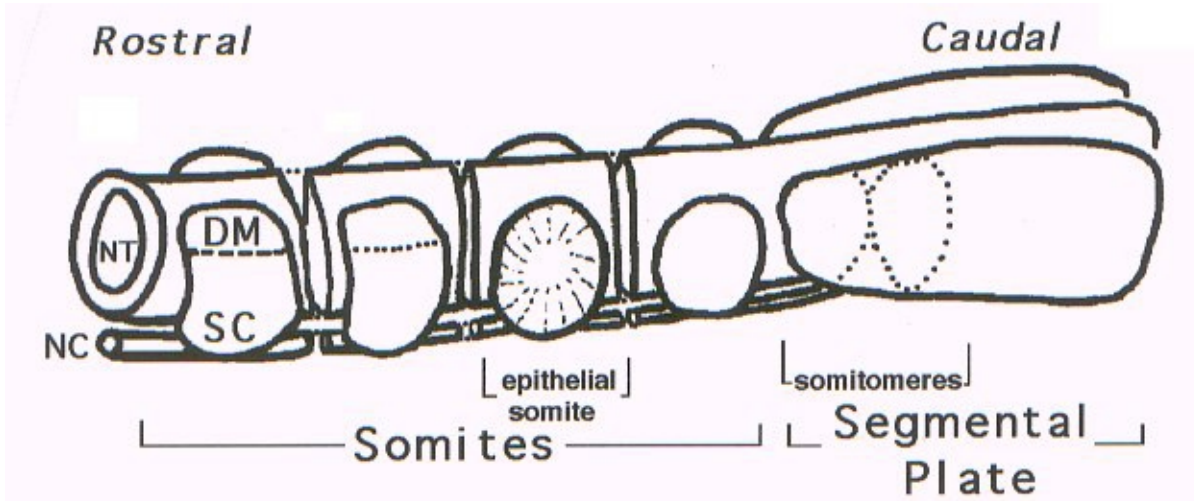
Figure 1. Schematic of somite development in the early chick embryo. Somites are formed as aggregates of paraxial mesodermal cells derived from the unsegmented mesoderm, segmental plate, localized at the caudal end of the gastrulating embryo. At the rostral end of the segmental plate, mesodermal cells condense to form somitomeres, and later develop into mesenchymal aggregates of early somites. A mesenchymal-to-epithelial transformation converts the somites into a epithelium-circumscribed structure and a somitocoel cavity containing central cells. Further differentiation of the somite gives rise to the dermamyotome (DM) dorsally and the sclerotome (SC) ventrally. The sclerotomal mesenchymal cells migrate ventrally to eventually give rise to the prevertebral component. NT: neural tube; NC: notochord.

vertebra, to allow anchoring of the newly developing muscle to adjacent vertebrae. The sclerotomal mesenchymal cells at the developing prevertebrae then condense and subsequently form cartilage. Upon maturation, hypertrophy, and mineralization, the cartilage is replaced by bone to yield the final vertebral structure.

Limb bud

The lateral plate mesoderm contributes the mesenchymal cells which migrate and accumulate under specific sites of the epidermal tissue; from this limb field, the limb bud emerges. These mesodermal cells eventually give rise to the chondrocytes of the cartilage anlage by first undergoing cellular condensation, i.e., the cells begin to aggregate and come into close contact with one another (Thorogood and Hinchliffe, 1975). This process is accompanied by the disappearance and alteration of the extracellular matrix, and the appearance of various cell adhesion molecules (see **Limb chondrogenesis**). Subsequently, the mesenchymal cells undergo overt chondrogenic differentiation, and begin copious production of cartilage matrix molecules, such as collagen type II, aggrecan, etc. The chondrocytes continue their developmental process, consisting of proliferation, maturation, hypertrophy and mineralization; in the growth plate, bone is formed subsequent to vascularization. It is generally believed that the osteoprogenitor cells are recruited from the local mesenchyme, as a result of cartilage maturation

and hypertrophy, and the establishment of the vasculature (Caplan and Boyan, 1994).

Calvaria

In contrast to the endochondral bones, the cranial calvarial bone develops in an intramembranous manner, i.e., the mesenchymal cells undergo limited condensation and aggregation and differentiate directly into osteoblasts (Hall, 1988; Hall and Miyake, 1992). As stated earlier, most of the craniofacial bones which ossify via the endochondral pathway are derived from cranial neural crest cells (Couly et al., 1993; Langille, 1994), with contribution also from the cranial somites (Huang et al., 1997). It should be noted that although intramembranous bones lack a cartilage model, cartilage can form on these bones, but only secondarily, i.e., arising only after osteogenesis is already established (Hall, 1991; Hall and Miyake, 1992; Vinkka-Puhakka and Thesleff, 1993). The best example is the condylar secondary cartilage of the condylar process in the mammalian mandible, a cartilage that contributes to the vertical growth of the jaws and neighboring regions of the head. Typically, these secondary cartilages are mechanically induced and require the movement-related mechanical stimulation to maintain their phenotype.

Molecular Mechanisms of Skeletal Patterning

Axial patterning

Somitogenesis is a crucial event in vertebrate em-

bryogenesis, as the somitic mesoderm is the first embryonic tissue to become "segmented," i.e., to become organized into repeated blocks of cells. The derivatives of each somite always arise from a particular position in the original series of somites and always in register with the other tissues derived from the same individual somite, thus generating the functionally important segmented pattern of the vertebrate trunk (Bellairs, 1979; Keynes and Stern, 1988; Ordahl, 1993). Additionally, the repeating pattern of somites is in close register with the other aspects of the body pattern, such as the limbs, nervous system, and internal organs. The paraxial mesoderm is therefore the tissue site where proper formation of the original metameric pattern of the vertebrate embryo is regulated.

While the morphogenetic events of somite formation are well described, the mechanisms by which these events occur at the cellular and molecular level are less understood. Cell adhesion and extracellular matrix molecules are clearly implicated in the complex pathways of cellular organization and migration; several such molecules have indeed been specifically shown to be functionally involved in these cellular events. For example, the cells of the segmental plate and somitic cells exhibit Ca^{2+} dependent and independent cellular adhesion systems of cellular aggregation (Cheyney and Lash, 1984; Duband et al., 1987; Hatta et al., 1987). These systems can be inhibited respectively by the addition of antibodies to N-cadherin or N-CAM, suggesting that these cell adhesion molecules play a role in pre-somitic aggregation and somite segmentation (Duband et al., 1987). N-cadherin has also been implicated in the epithelial conversion of the outer somitic cells as well as in the migration and subsequent differentiation of sclerotomal cells (Duband et al., 1987; Radice et al., 1997). Among matrix components, fibronectin has been shown to be involved in pre-somitic aggregation in the paraxial mesoderm (George et al., 1993), and interruption of fibronectin function and the addition of anti-fibronectin antibodies alter somite numbers (Lash et al., 1984, 1987). Similarly, the inhibition of collagen synthesis has been shown to affect somite number, size, and shape (Bellairs and Veini, 1980). Although a significant body of information has suggested the involvement of these specific cell adhesion and extracellular matrix molecules in somite development, the exact molecular mechanisms regulating somitogenesis and somite segmentation remain to be elucidated.

Paraxis A novel family of basic helix-loop-helix (bHLH) proteins has recently been identified, one member of which, *Paraxis* (Burgess et al., 1995), displays a unique localization pattern in the paraxial mesoderm and newly formed somites. Structurally identical cDNAs to mouse *Paraxis* have also been cloned in human, bHLH-EC2 (Quertermous et al., 1994), hamster, Meso1 (Blanar et al., 1995), and most recently in chicken (Barnes et al., 1997;

Sosic et al., 1997). Whole-mount *in situ* hybridization showed that the pattern of *Paraxis* expression in the early gastrulating chick embryo is specifically associated with the mesoderm. Initially, *Paraxis* expression is found around Hensen's node, the organizer of the early embryo, followed by two symmetric crescents of expression just rostral and lateral to Hensen's node and the primitive streak, strongly indicating a functional involvement in the development of the somitogenic paraxial mesoderm. It is of particular interest to note that this early expression profile of *Paraxis* shows a striking resemblance to that of *Wnt-11* (Eisenberg et al., 1997) and *Ch-Tbx6L* (Knezevic et al., 1997). *Wnt-11*, a member of the *Wnt* family of secreted signaling proteins, has been proposed to play a role in promoting embryonic patterning by regulating cell-cell associations. On the other hand, *Ch-Tbx6L*, a T-box gene related to mouse *Brachyury*, is speculated to play some role in regulating somitogenesis (Chapman et al., 1996; Papaioannou, 1997). How the activities of these genes and others, such as *Notch I* (Conlon et al., 1995), may relate to that of *Paraxis* during early paraxial mesoderm development remains to be elucidated.

To consider the function of *Paraxis* in mesoderm development, it is instructional to revisit the morphogenetic events of somitogenesis (Tam and Trainor, 1994). The first recruitment stage refers to prospective mesodermal cells from the primitive streak being recruited to form the paraxial mesoderm. It has been shown by tissue transplantations that, at this stage, the cells which are destined to form the somites are still multipotent or undetermined with respect to their differentiation fate. This stage is followed by a stage of "organization," during which the cells of the paraxial mesoderm are arranged into 6 to 7 pre-somitic units referred to as somitomeres (Meier, 1979, 1984; Jacobson, 1988; Tam and Trainor, 1994). The organization stage describes the cells of the paraxial mesoderm extending from the tail end of the segmental plate up to a distance of approximately 1-2 somite lengths from the caudal, most nascent, clearly delineated somite. The next stage of somitogenesis is "segmentation," which covers the rostral 1-2 somite lengths of the segmental plate. This is the region of the paraxial mesoderm in which cell compaction increases, presumably via upregulation of cell adhesion molecules, such as N-cadherin and N-CAM, or changes in cellular interaction with extracellular matrix molecules, such as fibronectin and collagen. Once segmented, paraxial mesoderm development continues with the mesenchymal to epithelial conversion of the outer cells of the emerging somite. The cells of the epithelial somite are still "plastic" with respect to differentiation (Aoyama, 1993), and the potential of distinct regions is realized through the influences of the surrounding cells and tissues, which modulate the expression of genes involved in the differentiation pathways of the individual

somitic compartments (e.g., *Pax-3* and *Pax-7* for the dermamytomal compartment and *Pax-1* and *Pax-9* for the sclerotomal compartment) (Christ and Ordahl, 1995). This has been demonstrated by surgical rotation of somites and by isolation of the segmental mesoderm, which is able to continue to form somites but lacks further dermamyotomal and sclerotomal differentiation, processes that require extrinsic influences form the notochord, lateral mesoderm and other surrounding tissues (Aoyama and Asamoto, 1988; Brand-Saberi *et al.*, 1993; Hall, 1977; Kenney-Mobbs and Thorogood, 1987; Packard and Jacobson, 1976; Pourqie *et al.*, 1993). Thus, the epithelial somite is the site of early dorso-ventral and medio-lateral patterning of the somite.

Results of our spatiotemporal analysis of *Paraxis* expression in the somitic stage chick embryo strongly indicate the importance of *Paraxis* in somite development. By Northern blot analysis, *Paraxis* expression begins early in embryonic development and is clearly detectable by 36 hours of incubation when the embryo has formed the first 8 to 10 pairs of somites. In addition, it has been previously reported that both *Paraxis* and Meso1, a homolog of *Paraxis*, are detectable in the paraxial mesoderm just prior to the formation of the first somite (Blanar *et al.*, 1995; Burgess *et al.*, 1995). In fact, in both reports, low levels of *Paraxis* expression are first detectable in mouse embryos at Day 7.5 *post coitus* (p.c.), approximately 12 hours prior to the formation of the first somite. During chick embryonic somite formation, the level of *Paraxis* expression remains relatively constant, subsequently diminishing by Day 4 of incubation and becoming undetectable by Day 5, correlating with the cessation of somite segmentation from the paraxial mesoderm. This finding is in agreement with the recent mouse study of Burgess *et al.* (1995), in which *Paraxis* expression is diminished to undetectable levels by Day 13.5 p.c. Thus, the temporal profile of *Paraxis* expression in both the chick and murine embryo correlates with somite formation.

The spatial localization of *Paraxis* expression in the somitic stage embryo also suggests a role in the segmentation of somites from the paraxial mesoderm (Fig. 2). *Paraxis* expression is clearly seen in the rostral portion of the paraxial mesoderm of the segmental plate, approximately 2 somite lengths from the newest clearly delineated somite, similar to that previously reported in the mouse embryo (Burgess *et al.*, 1995). This localization places *Paraxis* expression in the region of the segmental plate where the segmentation stage of somitogenesis is occurring, according to the scheme of somitogenesis described by Tam and Trainor (1994). Furthermore, we observe that the expression of *Paraxis* in the most rostral portion of the segmental plate pre-configures the next somite to be formed. In serial transverse sections of embryos post-*in situ* hybridization, the strongest staining for *Paraxis* expression appears just

caudal to the last clearly delineated somite in the region of the next somite to form. It is noteworthy that the highest level of *Paraxis* expression in the somitic stage chick embryo occurs in the portion of the segmental plate preparing to undergo the mesenchymal to epithelial transition and subsequent segmentation into a new somite. This expression pattern further implicates *Paraxis* as having a role in somite formation.

Paraxis may also play a role in the subsequent maturation and differentiation of the embryonic somite. After the segmentation of a newly formed epithelial somite, *Paraxis* expression is initially homogeneous, before becoming medially localized as the somite matures. In more differentiated somites, expression appears in both the sclerotome and dermamyotome, as previously reported (Blanar *et al.*, 1995; Burgess *et al.*, 1995; Quertermous *et al.*, 1994), eventually becoming preferentially expressed in the dermamyotome, before becoming restricted to the dermatome of the most rostral somites which express *Paraxis*. This later restricted expression in the dermatome suggests that *Paraxis* may play multiple roles in somite development and differentiation, perhaps distinct from its early role in epithelial somite formation.

Additional evidence supporting the hypothesized role of *Paraxis* in the segmentation of somites from the paraxial mesoderm comes from our observation on the effect of perturbing *Paraxis* expression through the use of a *Paraxis* specific antisense oligonucleotide, administered to the paraxial mesoderm by two routes (Barnes *et al.*, 1997). Treatment of the segmental plate with *Paraxis* antisense oligonucleotide, by either topical application or direct injection, results in segmentation anomalies in a significant number of embryos. Neither treatment with control oligonucleotides nor mock injections produced similar results. Specifically, topical treatment with *Paraxis* specific antisense oligonucleotide results in anomalies in 67% of treated embryos. Most of these embryos (85%) display complete absence of somites in various regions, whose location suggest a temporal correlation between the presence of the perturbing antisense oligonucleotide at the time of somite formation and the disruption of somitogenesis. These regions of observable somitic anomalies also exhibit diminished *Paraxis* expression as determined by whole-mount *in situ* hybridization, which also reveals that these regions of segmentation deficiency correlate with diminished *Pax-1* expression, a marker of epithelial somites and sclerotome formation (Barnes *et al.*, 1996a; Ebensperger *et al.*, 1995; Peters *et al.*, 1995) (also see below), further demonstrating the lack of proper somite formation and maturation. (Note: whether down-regulation of *Pax-1* expression is a direct consequence of inhibited *Paraxis* expression or an indirect consequence of somite dysmorphogenesis, is an intriguing possibility that remains
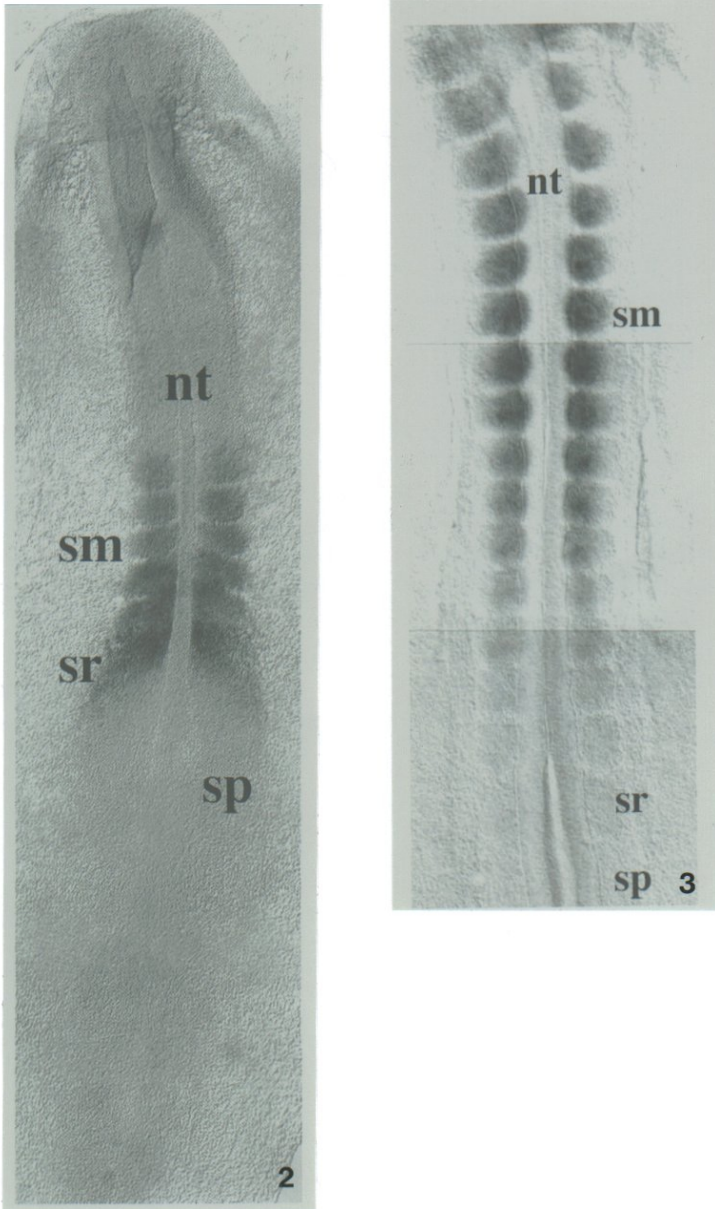
Figure 2. Expression of *Paraxis* in a 6-somite stage chick embryo localized by whole-mount *in situ* hybridization. *Paraxis* expression, detected by an alkaline phosphatase catalyzed color reaction, is seen in the rostral aspect of the segmental plate (sp), where somitomeres (sr) are formed. Newly formed somites (sm) express *Paraxis*, but as they mature, gene expression level decreases. This pattern of expression suggests that *Paraxis* is likely to be involved in the segmentation of the paraxial mesoderm and the initial formation of the somites. nt: neural tube.

Figure 3. Expression of *Pax-1* in a 16-somite stage chick embryo localized by whole-mount *in situ* hybridization. *Pax-1* expression is not seen in the somitomeres (sr) nor the segmental plate (sp), and is first detected in the caudal, second to third most nascent somites (sm). As somites mature, *Pax-1* expression becomes more medioventrally localized and eventually becomes concentrated in the caudal aspect of the sclerotome (see Barnes *et al.*, 1996a). nt: neural tube.

to be elucidated.) In addition, direct injection of *Paraxis* specific antisense oligonucleotide into one side of the segmental plate produces anomalies consistent with those observed in topically treated embryos, though markedly more discrete. Specifically, such treatment consistently results in a discrete fusion between adjacent somites. The site of somite fusion is always located on the injected side of the embryo, and correlates with somites which have formed from cells located in the vicinity of the injection site at the time of injection. Thus, both types of *Paraxis* antisense treatment lead to observable somitic anomalies that correlate spatially and/or temporally with the treatment, suggesting that the specific perturbation of *Paraxis* expression is, at least in part, involved in the emergence of

these anomalies.

The results of our antisense experiments are consistent with the phenotype of the recently reported *Paraxis* null mouse (Burgess *et al.*, 1996), in which epithelial somite formation is not detectable, suggesting a disruption of normal somite formation. However, a segmental pattern within the loosely arranged mesenchymal cells of the somitic mesoderm is seen, reminiscent of that in wild-type embryos; these embryos are also born with a segmented skeletal structure including vertebrae and rib, albeit characterized by a number of patterning defects.

That *Paraxis* expression is crucial for normal somitogenesis naturally begs the experimental question of what regulates *Paraxis* expression. This is an area of current

interest in a number of laboratories. Sosic *et al.* (1997) have recently reported that ectoderm- and neural tube-derived signals are involved in the regulation of *Paraxis* expression. These investigators described two phases of *Paraxis* expression in the paraxial mesoderm: (**1**) an early phase which is dependent on signals from the ectoderm and independent of the neural tube; and (**2**) a later phase that is supported by redundant signals from the ectoderm and neural tube. Interestingly, Garcia-Martinez *et al.* (1997) recently reported a high degree of plasticity in cells of the late gastrula/early neural stage chick embryo. For example, grafts from the prospective neural plate ectoderm could readily substitute for regions of prospective mesoderm, when transplanted to the epiblast or primitive streak, undergoing an epithelial-mesenchymal transition and, where appropriate, expressing *Paraxis*. Thus, at this stage of development, germ-layer specific properties are not irrevocably fixed for prospective ectodermal and mesodermal regions, and inductive signals must still be present and available. Characterizing and identifying such signals are clearly future goals for a molecular understanding of somitogenesis.

Pax-1 Another recently identified gene which has been implicated in axial somitic and skeletal patterning is *Pax-1*, a member of the *Pax* gene family, which are vertebrate homologs of the *Drosophila* pair-rule genes and contain a highly conserved sequence known as the paired box (Mansouri *et al.*, 1996). Analysis of a mouse skeletal patterning mutant, *undulated*, which has a phenotype of kinky tail and fused or asymmetric vertebrae, mapped the mutation to the *Pax-1* gene allele with a single base change in the paired box sequence (Balling *et al.*, 1988; Dietrich and Gruss, 1995). Recent work from this author's laboratory has further elucidated the functional importance of *Pax-1* in somite development (Barnes *et al.*, 1996a,b; Love and Tuan, 1993; Smith and Tuan, 1995). As shown in Figure 3, in the early chick embryo, *Pax-1* gene expression is seen evenly distributed in the somites as soon as epithelialization begins. Upon differentiation of the somite, *Pax-1* expression appears to be associated primarily with the mesenchymal sclerotome, even as they migrate ventromedially towards the notochord. Specifically, expression appears to be preferentially found in the caudal aspect, the condensing half, of the sclerotome. This spatiotemporal profile of *Pax-1* gene expression strongly suggests a functional involvement in the maintenance of somitic segmentation, and/or the subsequent differentiation of the sclerotomal cells into precartilage cells of the prevertebrae. Upon disruption of gene expression using *Pax-1* antisense oligonucleotides, a number of somitic anomalies are seen, including fused somites, scrambling of aggregates of the somitic mesoderm, and shortened body axis (Barnes *et al.*, 1996b; Smith and Tuan, 1995). It should be pointed out

that the patterns of somite dysmorphogenesis produced by *Paraxis* and *Pax-1* antisense treatments are related but not identical. Specifically, *Paraxis* down-regulation primarily results in poor condensation (and perhaps epithelialization) of the paraxial mesoderm during somitogenesis, whereas *Pax-1* down-regulation is accompanied by a breakdown of the segmented structures of the somites, with subsequent compromised sclerotomal development. The phenotype of the *Pax-1* antisense-treated embryos are thus consistent with that of the *undulated* mouse embryo, i.e., asymmetric vertebrae, hemivertebrae, fused vertebrae, and kinky tail (Balling *et al.*, 1988; Dietrich and Gruss, 1995). Finally, it is important to note that *Paraxis* expression is highest in the segmental plate, the precursor of the somites, and is attenuated as the somites develop and mature (Barnes *et al.*, 1997, Burgess *et al.*, 1995). On the other hand, *Pax-1* gene expression is somite-specific, and shifts to an exclusive association with the sclerotome as the somite develops. It is thus reasonable to speculate that *Paraxis* acts as an early regulatory gene in paraxial mesoderm development, apparently involved in its segmentation process, whereas *Pax-1* is likely to be a crucial gene in the maintenance of the somite boundaries, and *Pax-1* specifies the precartilage pathway for the sclerotomal cells. Since both *Paraxis* and *Pax-1* are intracellular, candidate DNA-binding transcription regulators, the interesting question is: what are the target genes whose expression is regulated by these patterning genes? In order to establish patterns and set up boundaries, distinct cellular or matrix components must participate in an interactive manner to functionally express this spatiotemporal information. The extensive information on skeletal cell biology clearly points to cell-cell and cell-matrix interactions as prime regulatory steps in patterning skeletal architecture. It is thus tempting to speculate that, in vertebrates, these patterning genes act by regulating the expression of gene related to these activities, i.e., cell adhesion and extracellular matrix genes. This is a most exciting area that awaits further analysis.

Limb patterning

The developing limb represents another morphogenetic system where pattern formation is under stringent regulation. As stated above, the precartilage mesenchyme of the limb bud are derived from the lateral plate mesoderm. While these mesodermal cells eventually give rise to the chondrocytes of the cartilage anlage, they are also inductive such that the overlying ectoderm is stimulated to produce a structure known as the apical ectodermal ridge (AER), located at the junction of the future dorsal and ventral ectoderm (Saunders, 1948; Todt and Fallon, 1984). Elegant surgical and transplant manipulations by Saunders and co-workers (Saunders, 1948; Saunders and Gasseling, 1968) clearly showed that the AER is needed for the sustained outgrowth of the embryonic limb, and is responsible for

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specifying the proximal-distal positional information. The target cell population for the AER signalling, which has been shown to be mediated by members of the fibroblast growth factors family (FGF-2, FGF-4 and FGF-8), are those residing underneath the AER, within a region known as the progress zone (Crossley and Martin, 1995; Fallon *et al.*, 1994; Niswander and Martin, 1992, 1993). These growth factors are able to maintain limb outgrowth when the AER is surgically removed.

Another region of the limb bud which specifies positional information is the zone of polarizing activity (ZPA), a small block of mesodermal tissue near the posterior junction of the young limb bud and the body wall, which is responsible for anterior-posterior information (Saunders and Gasseling, 1968; Summerbell, 1979; Tickle *et al.*, 1975). Experimental transplantation of the ZPA to a position on the anterior side of another limb bud produces a mirror-image doubling of the digits. While retinoic acid may be involved in mediating part of the anterior-posterior signaling (Eichele, 1989), current studies have clearly implicated a secreted protein, *sonic hedgehog* (*shh*), as an important signaling molecule of the ZPA (Riddle *et al.*, 1993). *Shh* is a member of the *hedgehog* gene family, first discovered in *Drosophila* (Hammerschmidt *et al.*, 1997). The various *hedgehog* proteins consist of a signal peptide, with a highly conserved N-terminal region, and more divergent C-terminal domain. *Hedgehog* precursor proteins undergo an internal autoproteolytic cleavage to generate a 19 kDa N-terminal peptide and a C-terminal peptide of 26-28 kDa. The N-terminal peptide associates tightly with the cell surface, while the C-terminal peptide is freely diffusible. The N-terminal peptide appears to be responsible for the short- and long-range *hedgehog* signaling activities in *Drosophila* and vertebrates, and its tethering to the cell surface, recently discovered to be mediated via a covalent linkage to membrane cholesterol moieties (Porter *et al.*, 1996), allows the build-up of a high concentration of the peptide on the *hedgehog* producing cells. Whether *shh* functions to transduce signal via specific cell surface receptors, such as *patched*, remains to be established.

Finally, dorsoventral patterning of the limb, characterized by the precise positioning of the AER at the dorsoventral boundary has recently been shown to require the coordinated expression of a number of novel genes (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). *Radical fringe* (*r-fng*), a vertebrate homolog of the *Drosophila fringe* (*fng*) gene, is expressed in a restricted manner in the limb bud: *r-fng* is expressed in the dorsal ectoderm before the AER appears, and is repressed by *Engrailed-1*, which is expressed in the ventral ectoderm. Misexpression of these genes indicates that a ridge is formed wherever there is a boundary between cells expressing and not expressing *r-fng*. This pattern of regulation is thus very similar to the establishment of the margin cells at the *Drosophila* wing dorsoventral border by *fng* (Kim *et al.*, 1995). The parallel between *Drosophila* and vertebrate is further illustrated by the expression pattern of vertebrate homologs of downstream genes in the *fng* pathway; of these, *Serrate* and *Notch* are found to be exclusively expressed at the AER (Laufer *et al.*, 1997; Rodriguez-Esteban, 1997). These exciting new findings point to the developing limb bud as a fertile ground for investigating the basic regulatory mechanisms of pattern formation, as well as providing a highly accessible system for studying skeletogenesis.

It should be noted that axial and appendicular skeletal patterning are likely to share common regulatory mechanisms, particularly in view of the dysmorphogenetic phenotypes associated with gain/loss of function perturbations of the various Hox gene family members. Based on the spatiotemporal specificity of the expression of Hox genes, various mechanistic models have been advanced to account for the patterning of the axial and appendicular skeleton (see reviews by Favier and Dolle, 1997; Kuratani *et al.*, 1997).

Differentiation of Mesenchymal Cells

Limb chondrogenesis

The chondrogenic differentiation of limb bud mesenchymal cells is conveniently and faithfully reproduced in a popular *in vitro* culture system, known as the high density micromass culture (Ahrens *et al.*, 1977). This system involves the isolation and culturing of embryonic limb mesenchyme in a high density droplet to mimic the high cell density found in the condensing mesenchyme of the developing limb bud *in vivo* (San Antonio and Tuan, 1986). In this system, chondrogenesis is recapitulated, and may be qualitatively and quantitatively assessed by several methods, including the direct counting of cartilage nodules, the incorporation of radioactive sulfate into the extracellular matrix, and the expression of cartilage-specific genes (San Antonio *et al.*, 1992; San Antonio and Tuan, 1986; Tuan, 1991). In a similar manner, craniofacial mesenchyme has also been studied using the micromass culture system (Langille, 1994).

As stated earlier, a crucial step in limb mesenchymal chondrogenesis is the pre-chondrogenic event of cellular condensation. The increased cell-cell interaction as a result of condensation is presumably involved in initiating or propagating some type of signal transduction to initiate the chondrogenic differentiation pathway. A number of recent findings have provided clues as to the mechanisms responsible for cellular condensation (see review by Hickok *et al.*, 1998). Two highly specific, homotypic cell adhesion molecules, N-cadherin (Oberlender and Tuan, 1994) and N-CAM (Widelitz *et al.*, 1993), have been implicated in cellular condensation. Both molecules are expressed in

condensing mesenchyme, then disappear from the forming cartilage, and are later detected only in the perichondrial layer. A similar pattern is observed in micromass cultures of limb mesenchyme. This distribution pattern strongly suggests that these molecules are involved in mediating the cellular condensation step that gives rise to the initial precartilage core, and subsequently, in the continuing recruitment of additional chondrogenic cells into the growing cartilage. In support of this hypothesis, it is observed (Oberlender and Tuan, 1994) that treatment of limb mesenchyme micromass cultures with a neutralizing monoclonal antibody to chicken N-cadherin (NCD-2) significantly inhibits cellular condensation and chondrogenesis *in vitro*, the effect being most prominent during the first day of culture, corresponding to the initial formation of cell aggregates. Most interestingly, NCD-2 injection into the developing limb bud *in vivo* at the stage of cellular condensation also results in significant inhibition of chondrogenesis and developmental delays, gross developmental deformities, and perturbation of overall pattern formation. Thus, cellular condensation of precartilage mesenchyme is crucially dependent on N-cadherin mediated cell-cell interaction. In recent studies, using chicken limb mesenchyme (Tyndall and Tuan, 1994, 1996; Woodward and Tuan, 1999) as well as a murine mesenchymal cell line, C3H10T1/2, which undergoes chondrogenesis when cultured as high density micromass and treated with TGF-ß1 or bone morphogenetic protein (BMP)-2 (Denker *et al*., 1995a,b, 1998; Haas and Tuan, 1996, 1998), enhanced N-cadherin expression has been shown to be one of the earliest responses to the chondroinductive effect of these growth factors.

In addition to cell-cell interactions, cell-matrix interactions are also likely to play important functional roles in cellular condensation during mesenchymal chondrogenesis. One extracellular matrix component implicated in this pathway is fibronectin (Newman *et al*., 1987). It has been shown that fibronectin is expressed in the condensing mesenchyme of the limb bud, and may function to mediate a matrix-driven translocation of mesenchymal cells to produce pattern-specific condensation. Immunoblocking experiments suggest that the N-terminal domain of fibronectin is required for the event. Recent studies (Bennett *et al*., 1991; Gehris *et al*., 1996) have shown that alternative splicing of fibronectin mRNA takes place in the mesenchymal cell during the condensational transition to cartilage, and that the isoform containing exon IIIA (FN-A) is expressed only at condensation and disappears once chondrogenesis begins, suggesting that some type of cell-matrix interactions involving FN-A may be important for this event. This is in fact confirmed by the recent observation that treatment of limb mesenchyme *in vivo* and *in vitro* with spliced variant specific antibodies perturbs mesenchymal

chondrogenesis (Gehris *et al*., 1997). Another major cartilage matrix molecule which undergoes alternative splicing during the condensation phase of chondrogenesis is collagen type II: type IIA is found in chondroprogenitor cells, and type IIB is found in differentiated cartilage (Oganesian *et al*., 1996). Recent studies (Zhu *et al*., 1997) have strongly suggested a functional role for the collagen type IIA amino-propeptide in chondrogenesis, perhaps in the establishment of morphogen gradients via its binding to chondroinductive factors.

Recent advances in molecular genetics have greatly facilitated the identification of genes involved in either cellular condensation or cell differentiation by the analysis of the genetic basis of various skeletal diseases, as well as the characterization of skeletal phenotypes of animal models harboring exogenous copies or deleted/ mutated forms of the candidate genes. A partial list of these genes include *Hoxd-11, Hoxd-13, Pax-1, Pax-9, Pax-3, Gli3, Bmp5, Gdf5,* and *Sox9* (see review by Mundlos and Olsen, 1997).

Calvarial mesenchymal differentiation

Embryonic calvarial cells are one of the most common cell types used in studying bone cell development and biological activities (Wong and Cohn, 1974). The calvarial cells are usually derived from either rodents or avian embryos, and are able to give rise to distinct bone nodules *in vitro* that mineralize and express bone specific markers, including extracellular matrix proteins such as osteocalcin, etc. These cells are also characterized by high levels of alkaline phosphatase, and responsiveness to parathyroid hormone (Wong and Cohn, 1974). On the basis of studies using embryonic calvarial cells, it has been proposed that there are at least six stages of osteoblast differentiation, each characterized by the expression of specific molecular markers (Bruder and Caplan, 1990).

As stated earlier, although intramembranous bones, such as the calvarium, are characterized by the direct differentiation of mesenchymal cells into osteoblasts, there are conditions under which cartilage, either transient or permanent, appears. Is this cartilage a result of exogenous cells being recruited to the sites, or is it a product of differentiation of the endogenous mesenchymal cells? Recent studies from this investigator's laboratory have provided clear evidence that the embryonic calvaria contain cells with chondrogenic potential. It has been previously shown that, when chick embryos are maintained in long-term shell-less culture to produce a highly calcium deficient state, their calvarial bones begin to show sites of chondrification, and cells of distinct chondrocytic phenotype are detected (Jacenko and Tuan, 1986; McDonald and Tuan, 1989; Tuan and Lynch, 1983). When the embryonic calvarium is removed from the embryo, and explanted as a tissue graft on the chorioallantoic membrane of a calcium-deficient, shell-less embryo, it is found that
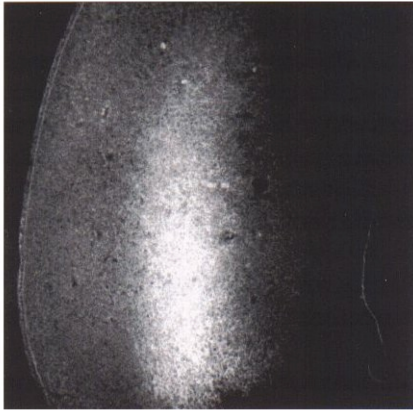
Figure 4. PNA binding to precartilage mesenchyme in the developing chick embryonic limb bud. A cryosection of Hamburger-Hamilton Stage 23/24 chick embryonic limb bud is stained with fluorescein-labeled PNA, and visualized by epifluorescence microscopy. PNA binding is clearly localized to the precartilage core of the limb bud, and appears to configure the emergence of the cartilage anlage. The distal aspect of the limb bud is located at the top of the micrograph. Photo width = 1.2 mm.

such cartilaginous regions appear in the grafted tissue, suggesting that the cells responsible for this altered phenotype are likely to be of endogenous origin (Jacenko *et al.*, 1995; Jacenko and Tuan, 1995). Further confirmation of this hypothesis is obtained from analysis of various populations of enzymatically dissociated calvarial cells fractionated by density gradient centrifugation (Wong and Tuan, 1995). Upon micromass culture *in vitro*, a relatively more dense population of calvarial cells (Fraction F, specific gravity = 1.055-1.060) consistently exhibit chondrocytic characteristics, including Alcian blue staining, increased sulfate incorporation, and expression of collagen type II and aggrecan. Interestingly, the chondrogenic activity of Fraction F cells is significantly suppressed when they are co-cultured as side-by-side micromasses with the non-chondrogenic, lower density fractions. This finding clearly indicates that the embryonic calvarium contains chondroprogenitor cells, whose ability to differentiate into chondrocytes is stringently regulated by their interaction with the more predominant osteoprogenitor cells. The presence of this chondroprogenitor cell population is further confirmed by our most recent affinity fractionation study of calvarial cells (Stringa and Tuan, 1996). The ability to bind the lectin, peanut agglutinin (PNA), has been shown as a hallmark of chondroprogenitor cells in multiple systems (Hall and Miyake, 1992), including the condensing core of the embryonic limb bud (Aulthouse and Solursh, 1987; Milaire, 1991; Zimmermann and Thies, 1984; see Fig. 4) and the caudal region of the somitic sclerotome (Bagnall and Sanders, 1989; Stern *et al.*, 1986). When dissociated calvarial cells are fractionated by PNA affinity chromatography, the PNA-binding cells exhibit the ability to undergo chondrogenesis when maintained as high density micromass cultures, with the expression of collagen type II and aggrecan. The PNA-binding cells represent a minor population (about 5%) of total calvarial cells. PNA recognizes the disaccharide [Gal(ß1,3)GalNac], and its binding to precartilage cells has been suggested to be of extracellular nature or matrix nature (Aulthouse and Solursh, 1987). The identity of the components in the precartilage recognized by PNA is not known, although some characterization of candidate molecules in the sclerotome has been reported (Davies *et al.*, 1990). Interestingly, it is reported that in keratinocytes, the PNA binding moiety is CD44, the cell surface hyaluronan receptor (Hudson *et al.*, 1995). A number of studies have clearly indicated that regulation of hyaluronan-CD44 interaction is crucial in mesenchymal cellular condensation (Knudson, 1993). Finally, it is interesting to speculate whether PNA binding mimics the action of endogenous animal lectins, a recently identified class of proteins thought to be functionally important in animal development (Zalik, 1991). Among the animal lectins, galectins, or S-type lectins, have been shown to be expressed during the development of the notochord, and bone and skin (Fowlish *et al.*, 1995). Hypothetically, the PNA-binding moieties of chondroprogenitor cells may be capable of interacting with an animal lectin type molecule, as well as other adhesion molecules, and functioning *in vivo* in the proper, normal development of the cranial vault. In addition, this interaction may be regulated under stress conditions, such as healing and repair or severe systemic calcium deficiency, in the recruitment of multipotent mesenchymal cells, and their activation and differentiation, eventually giving rise to new tissue.

Taken together, the observations summarized above clearly indicate that the endochondral and intramembranous ossification pathways, although involving apparently different cell lineages and cellular sequences in giving rise to the bony phenotype, are nevertheless related in terms of the differentiation potential of the endogenous cells. Osteogenesis versus chondrogenesis is therefore a differentiation phenomenon that is stringently regulated as a function of cell origin, cell-cell interactions, cell-matrix interactions, and signaling by growth and differentiation factors, as well as systemic influences, all in the context of morphogenetic control by patterning genes, to develop into the three-dimensional skeletal structures and tissues.

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Discussion with Reviewers

C.-M. Chuong: As discussed in the paper, *Paraxis* seems to be a critical initial molecular event in somite segmentation. Given that *Paraxis* is a transcription factor, can you elaborate on what factors may set up *Paraxis* expression?

Author: This issue is clearly essential for any mechanistic study on patterning, i.e., how are the "regulators" regulated? At present, there is no information available on the molecular basis underlying the regulation of *Paraxis* expression. Two recent studies by Sosic *et al.* (1997) and Garcia-Martinez *et al.* (1997) have addressed the influence of ectoderm and axial structures, and the plasticity of the mesodermal cells in terms of *Paraxis* expression, respectively. These findings are discussed here, and they should serve as the basis for future work on elucidating this fundamental aspect of somite biology.

C.-M. Chuong: Is the level of *Paraxis* expression the same in different size somites of different species, or is the change in expression set at the *Pax-1* stage when borders are formed?

Author: This is a potentially interesting question, as related to the regulation of *Paraxis* expression. Currently, we do not have any information.

M.B. Goldring: Different regulatory factors and adhesion proteins are described as associated with the different forms of skeletogenesis, but it is not clear which ones are specific to a particular mechanisms or shared among the different forms.

Author: It is probably too premature to conclude that the different forms of skeletogenic pathways involve different regulatory factors and adhesion proteins. In fact, most likely similar players are involved and the "progenitor cells" share common properties. In fact, it is likely that the family of patterning genes involved are also similar, such as the Hox genes.

M.B. Goldring: While *Paraxis* is highly expressed during sclerotome formation and then decreases, a related bHLH protein, scleraxis, is also expressed in the sclerotome of the mouse embryo, as well as in the limb bud, in mesenchymal precursors that give rise to cartilage (Cserjesi *et al.*, 1995) and in cranial mesenchyme. In contrast to *Paraxis*, scleraxis expression is maintained. Is there a similar potential role for these factors in the developing chick? Could the author speculate on their potential roles in the craniofacial intramembranous ossification pathway?

Author: Compared to *Paraxis*, a "mesoderm patterning" gene, scleraxis may be best characterized as a "pre-cartilage" gene. A recent study by Huang *et al.* (1997) demonstrates that the most cranial somite of the chick embryo contributes to the sclerotomal and dermamyotomal lineages in a manner similar to that of the more caudal somites. Interestingly, scleraxis expression is found in the osteoblastic osteosarcoma ROS17/2.8 cells and is upregulated by treatment with TGF-ß (Liu *et al.*, 1996). At present, the functional relationship between the temporally sequential expression of *Paraxis* and scleraxis in the paraxial mesoderm is not known.

C.B. Knudsen: With the new information on the patterned distribution of *Paraxis* and *Pax-1*, is there any correlation with markers of the rhombomeres of the neural tube? How do the cranial somitomeres, first described by Meier (1979, 1984), and their correlative patterning with the cranial rhombomeres of the neural tube relate to differentiation pattern in the craniofacial skeleton?

Author: Both *Paraxis* and *Pax-1* are primarily associated with the more posterior somites. *Paraxis* expression is essentially downregulated once differentiation into sclerotomal and dermamyotomal regions begins. *Pax-1* is also concentrated with the condensing sclerotome in the more posterior somites. A recent study by Huang *et al.* (1997) reports on the similar differentiation potential of the first avian somite, but does not address *Pax-1* expression.

O. Jacenko: Is there any information that *Pax-1*/ *Paraxis* expression may be associated with cell cycle changes? Have any human mutations in the *Pax-1* and *Paraxis* genes been shown to be associated with altered vertebral patterning?

Author: No information is available on cell cycle association. In addition, no known human mutations are known. Our earlier study on Klippel-Feil Syndrome, a heritable cervical scoliotic disease, did not show any sequence mutations in the paired-box domain of the human *Pax-1* gene (Smith and Tuan, 1994).

O. Jacenko: Upon *Paraxis* antisense treatment, what is the long-term fate of the mesenchymal cells?

Author: We have not done any long-term studies (i.e., to incubation Day 8 and beyond). A recent study reported on the phenotype of the *Paraxis* knock-out mouse, which showed failed somite epithelialization (Burgess *et al.*, 1996).

O. Jacenko: Are cell adhesion proteins, such as N-cadherin and N-CAM, expressed in both intramembranous and endochondral skeletogenic pathways? Is N-cadherin expression associated with other chondroinductive events, such as polylysine treatment?

Author: The expression of N-cadherin and N-CAM have both been shown in skeletogenic mesenchymal condensation in the laboratories of this author and Dr. C.-M. Chuong. In our most recent unpublished study, polylysine stimulation of limb mesenchymal chondrogenesis is accompanied by an increase in N-cadherin expression and alterations in ß-catenin phosphorylation.

O. Jacenko: What is the advantage of the presence of a small percentage of chondrogenic cells in an intramembranous bone, such as the calvaria?

Author: We speculate that such cells may serve as "progenitor cells" to repair structural defects in such normally, non-cartilaginous bones, in that these cells may represent a more responsive cell type.

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