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Review

Divide, accumulate, differentiate: cell condensation in skeletal development revisited

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Introduction

This paper updates a review on the importance of cell condensations in skeletal development that we published in 1992. The following conclusions emerged from that analysis:

Condensations are the aggregations of cells from which cartilages and bones form during embryonic development and from which chondrogenesis and osteogenesis are initiated during repair and/or regeneration. Condensations are so important for whether skeletal elements will form and/or whether they will form normally, that Grüneberg (1963) termed the condensation phase "the membranous skeleton". Many mutant genes that affect skeletal development act at the condensation stage; *Talpid*^{3}, *Brachypod* (*bp*^{H}), *Phocomelia* (*Pa*) and *Congenital Hydrocephalus* (*ch*) were discussed by Hall and Miyake (1992) as perhaps the best studied examples. Condensations are initiated by alteration of the mitotic activity, aggregation of cells towards a center, or by failure of cells to move away from a center. Condensations are cellular products of the epithelial-mesenchymal tissue interactions that initiate cell differentiation and morphogenesis in virtually every organ in the vertebrate body. In craniofacial skeletogenesis, the connection between tissue interactions and condensation is a very close one. It is less close in the developing limb skeleton. Molecular characteristics of prechondrogenic condensations discussed in 1992 included hyaluronan, hyaladherins, heparan sulphate proteoglycan, chondroitin sulphate proteoglycan, versican, tenascin, syndecan, N-CAM, retinoic acid and homeobox-containing genes.

Condensations mark the onset of selective gene activity. In prechondrogenic condensations there is a 7-fold increase in mRNA for type II collagen, a 100-fold increase in the number of copies of mRNA for type II collagen, expression of mRNA for the core protein of cartilage proteoglycan, and a 10-fold increase in mRNA for type IX collagen over non-condensed mesenchyme; see Hall and Miyake (1992) for details. The concurrent expression of the three chondrogenesis-specific products, collagens type II and IX and core protein for proteoglycan, illustrates the importance of condensation for the initiation of cell differentiation. In the sense that the onset of differentiation may be identified with synthesis of gene products specific to the particular cell type, condensations mark the onset of cell differentiation.

Understanding condensation formation and function is critical for understanding skeletal development, deviations from normal development and to provide the proximate mechanisms for evolutionary change in skeletal morphology. Atchley and Hall (1991) developed a model for skeletal development and evolution in which condensations and distinct cell lineages played a pivotal role. Cellular parameters identified as fundamental developmental units in that model were:

(i) number of cells,
(ii) time of condensation initiation,
(iii) the mitotically active fraction,
(iv) rate of cell division, and
(v) rate of cell death.

The ability of such developmental units to vary individually or in concert generates an intrinsic variability of developmental processes that is expressed in morphology, initially through condensations. In a very real sense, cell condensations are the raw material of morphology (Hall, 1992a).

We now update research on condensations, especially prechondrogenic condensations, performed during the past three years. We provide a summary of molecules characteristic of the condensation phase and a model for how condensations are initiated and for transit from condensation to overt differentiation.

Condensation formation

Condensation refers both to an aggregation of similar cells and to the process of forming that aggregation. Individual condensations form by one or more of altered mitotic activity, absence of movement of cells away from a center, and/or aggregation of cells toward a center (see Ede, 1983; Hall and Miyake, 1992; Langille, 1994a for literature). Activin – a member of the TGFß superfamily – enhances expression of N-CAM, condensation size and stimulates a 5-fold increase in chondrogenesis in precartilaginous limb mesenchyme (Jiang *et al.*, 1993). That cell number is not altered by such treatment, either implicates recruitment of cells to the condensation as the mechanism of action, or indicates that activin does not act on condensation initiation. That condensation size was increased after activin treatment favors the former mechanism. Activin may increase condensation size by direct enhancement of cell aggregation or indirectly by up-regulating expression of N-CAM, which, in turn, enhances condensation (see below). Recruitment of cells into the chondrogenic differentiation pathway is blocked by culture of prechondrogenic cells in carboxymethyl cellulose, a treatment that reduces cell aggregation and therefore cell to cell interactions (Tacchetti *et al.*, 1992). This study both provides a method to study aggregation and reinforces the importance of condensation for the initiation of overt differentiation.

Abbreviations used in this paper: Barx-1, mammalian homeodomain gene in *Drosophila* Bar H-1 complex; ß-D-gal-Nac-D-gal, beta D-galactose-N-acetyl-D-galactosamine; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; *bp*^{H}, *brachypod* mouse mutation; *Cart*-1, cartilage specific gene; *ch*, *congenital hydrocephalus* mutation; *Cdxa* (*CHox*-4), chicken homeobox gene; *Ck-erg*, chicken erg gene; Cx42, 43, 45, connexin 42, 43, 45; *Dlx*-3, mammalian *Distal-less* homeobox gene; EGF, epidermal growth factor; *egr*-1, transcription factor; *ets*, transcription factor; GDF, growth and differentiation factor; *Hox*, *Hoxa*, *Hoxd*, mammalian homeodomain genes in *Drosophila Antennapedia* complex; *Hoxd*-4 (*CHoxa*), *Hoxa*4 (*CHox*-1), chicken homeobox gene; LacZ, *E. coli* ß-galactosidase reporter gene; *MHox*, mouse homeobox gene; *Msx*, muscle specific homeobox gene; MyoD, myogenesis determining gene; N-cadherin, neural cadherin; N-CAM, neural cell adhesion molecule; NCD-2, neural cadherin monoclonal antibody; *Pa*, *Phocomelia* mouse mutation; *Pax*-1, mammalian paired box homeobox gene; PNA, peanut agglutinin; *ta*, *Talpid* chick mutation; TGFß, transforming growth factor beta.

Visualization

As discussed by Hall and Miyake (1992), condensations are most readily visualized using peanut agglutinin (PNA), a galactose-specific lectin that recognizes ß-D-gal-Nac-D-gal, a terminal carbohydrate moiety of cell surface glycoproteins or proteoglycans (Davies *et al.*, 1990; Oakley *et al.*, 1994). PNA binds preferentially to the surfaces of cells in condensations (references in Hall and Miyake, 1992; Götz *et al.* , 1991; Milaire, 1991).

Evidence for the utilization of PNA as a marker for condensations continues to accumulate. PNA has been used to visualize condensations of perinotochordal and other mesenchyme in chick embryos that act as barriers to axon advance (Tosney and Oakley, 1990; Oakley and Tosney, 1991; Oakley *et al.*, 1994), condensations for developing teeth where PNA is visualized after tenascin expression (Jowett *et al.*, 1992), and prechondrogenic condensations in mice with the 'apparent' exception of the nasal septal and Meckel's cartilages (Sasano *et al.*, 1992). We say 'apparent' exception because PNA-binding can be masked by terminal sialic acid residues in tissues not pre-treated with neuraminidase. Figure 1 shows the effect of pre-treatment with neuraminidase on visualization of PNA-binding in the first (mandibular) arch of a 12 day-old mouse embryo. Neuraminidase treatment also reveals a gradient of sialic acid residues along the condensation for Meckel's cartilage in the mouse (Miyake *et al.*, 1995a).

Condensation duration

Condensation is a transient stage on the way to overt cell differentiation. The normal duration of a condensation in skeletogenesis is some 12 h (Hall and Miyake, 1992; Dunlop and Hall, 1995; Miyake *et al.*, 1995a,b). The condensation for the elastic cartilage of the rat external ear, however, is initiated at 15 days of gestation and persists until one week after birth, a duration of 14 days (Bradamante *et al.*, 1991).

In the embryonic chick the osteogenic condensations for mandibular and maxillary membrane bones have similar durations and are initiated at the same stage, Hamilton and Hamburger stage 26, or approximately 5 days of incubation (Dunlop and Hall, 1995). This developmental coupling is because the condensations in the upper and lower jaws begin as a single condensation of cells that migrated from the cranial neural crest. These cells accumulate at the base of the branchial arches before migrating into the primordia for the upper and lower jaws (see Fig. 4 in Dunlop and Hall, 1995). It now appears that preosteogenic cells of the upper and lower jaws are induced by a common epithelial-mesenchymal interaction acting on the cells as they accumulate outside the branchial arch primordia.

In the craniofacial skeleton, epithelial-mesenchymal interactions immediately precede condensation. The association is less direct in skeletogenesis within limb buds where several types of epithelial-mesenchymal interactions occur. These include an early interaction before the limb bud is evident and that is required for chondrogenesis to be initiated, interactions to initiate and maintain the apical ectodermal ridge, interactions to maintain distal mesenchyme as a proliferative, undifferentiated

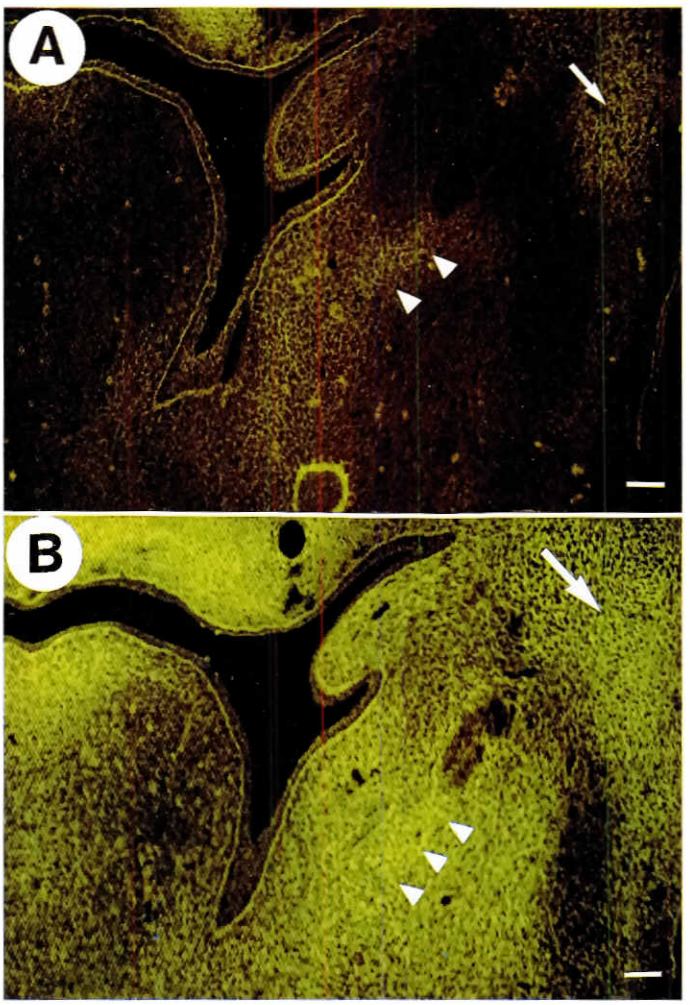

Fig. 1. Visualization of PNA-binding in the first (mandibular) arch of a 12-day-old (sub-stage 20.31) mouse embryo without (A) and with (B) pre-treatment with neuraminidase. *Arrows indicate the caudal component of the single first arch chondrogenic condensation. Arrowheads indicate the core component of the condensation from which the major portion of Meckel's cartilage develops. See Miyake et al. (1995a) for neuraminidase and PNA-binding methodologies and Miyake et al. (1995b) for detailed criteria for staging mouse embryos.*

population of cells, and a later inhibition of chondrogenesis by limb bud epithelium; see Hall (1983) and Solursh (1989) for reviews.

Within a condensation cells may exist in regions that remain at the condensation stage for different periods of time. This is especially seen when a single condensation subdivides or produces more than one element. Miyake *et al.* (1995a) demonstrated that the single first branchial arch prechondrogenic condensation in C57BL/6 inbred mice has three components, each with different fates and each with a different duration. This condensation consists of:

(a) a rostral component from which the symphyseal cartilage will form, and which is present for 28 h;

(b) a core component for the bulk of Meckel's cartilage, which is present for 12 h, and

(c) a caudal component from which the caudo-lateral portion of Meckel's cartilage and the incus and malleus of the middle ear arise, and which is present for 30 h.

Both timing of onset and duration of condensations vary between inbred strains of mice (Miyake and Hall, unpublished observations), indicating that both components can be modified by selective inbreeding. The presumption is that both can be modified during evolutionary change in morphology (Atchley and Hall, 1991).

Onset of a tissue or organ is often used in the study of heterochrony as a mechanism of evolutionary change (Hall, 1984a, 1990, 1992a; Hall and Miyake, 1995). Indeed, there is a constancy of species-specific allometric growth throughout amphibian metamorphosis. Species can be separated even at very early ontogenetic stages, solely on the basis of their allometric growth trajectory. Adult morphological differences are established early in embryonic development through the size of condensations and the rate of division of their cells (Strauss and Altig, 1992). Indeed, Hall and Miyake (1995) concluded that embryos measure time in relation to cell cycles and causal sequences of critical developmental events using cell autonomous oscillators. Condensation is one of the first critical developmental events in skeletogenesis.

Condensation size

Duration of the condensation phase affects condensation size. Grüneberg (1963) demonstrated that many mutations exert their primary action on condensations. This is in large part because of the need for a minimal cell number before prechondrogenic or preosteogenic cells can differentiate and because many mutations affect condensation size. Too small a condensation prevents a skeletal element from forming, while too large a condensation can lead to abnormally large elements that are initiated early. Too small or too large a condensation can also affect the number of skeletal elements that form; see Grüneberg (1963) and Hall and Miyake (1992) for examples. Ontogenetic fusions are often associated with such early initiations of ossification. Phylogenetic fusions may have a similar developmental basis. Reduction of condensations below a critical size is a developmental mechanism for evolutionary loss of skeletal elements (Hall, 1984a,b, 1990, 1992a; Smith and Hall, 1990, 1993; Atchley and Hall, 1991; Thomson, 1991).

The importance of the condensation phase is reflected in its retention even when the skeletal element or organ that would normally develop from the condensation does not form in the species. This is seen in the hind limbs in whales, limbs in snakes, or teeth in monotremes. The occasional reappearance of these structures as atavisms attests to the developmental potential retained within such condensations (Hall, 1984b, 1995; Thomson, 1991). Mechanisms for retaining condensations of a particular size may include intrinsic properties of the condensation, lateral inhibition and/or inhibition from neighboring tissues such as epithelia; see Hall and Miyake (1992).

Molecular characteristics of condensations

Prechondrogenic condensations have been best studied in the developing limb buds of birds and mammals (Milaire, 1965; 1974, 1978; Hall, 1978; Hinchliffe and Johnson, 1980; Ede, 1983; Thorogood, 1983; Johnson, 1986). These condensations segment and bifurcate to produce the diversity of limb patterns found among the vertebrates (Hinchliffe, 1994).

Condensation is associated with changes in cellular ultrastructure and formation of gap junctions. Sire and Huysseune (1993), on the basis of shape, organization and localization, separated two condensation types in the cichlid fish, *Hemichromis bimaculatus*, one for scales and lepidotrichia, the other for bones and scutes. Recently, Minkoff *et al.* (1994) demonstrated expression of the gap junction protein connexin Cx43, coincident with condensation of preosteogenic condensations. Cx45 was associated with later stages of osteogenic differentiation – osteoblast differentiation and matrix synthesis. Cx42 was not detected at any stage.

Extracellular matrix and cell surface molecules

Extracellular matrix and cell surface molecules are important in condensation because cell shape is maintained by cytoskeletal-cell surface-extracellular matrix interactions (Daniels and Solursh, 1991; Ettinger and Doljanski, 1992; Tsukahara and Hall, 1994). Hyaluronan and hyaladherins, two species of heparan sulphate proteoglycan, a large chondroitin sulphate proteoglycan, tenascin, syndecan, versican, N-CAM and N-cadherin are all characteristic of chondrogenic condensations (Edelman, 1986; Gallagher, 1989; Mark *et al.*, 1989; Thesleff *et al.*, 1990a,b; Salmivirta *et al.*, 1991; Shames *et al.*, 1991; Hall and Miyake, 1992). We are aware of at least thirty recent papers dealing directly with the effect on condensations of such extracellular matrix and cell surface molecules as chondroitin-4-sulphate, versican, syndecan and tenascin.

The formation of a condensation is associated with reduction in hyaluronan (hyaluronate, hyaluronic acid) and increase in chondroitin sulphate. Hyaluronan blocks chondrogenesis; removal of hyaluronan permits chondrogenesis; see Hall and Miyake (1992) for a review. An additional study (Fernandez-Teran *et al.*, 1993) has confirmed that chondroitin-4-sulphate is found in prechondrogenic condensations.

Versican, a hyaluronic acid-binding chondroitin sulphate proteoglycan is found in prechondrogenic condensations. Versican is co-localized with tenascin but follows onset of PNA-binding (Gallagher, 1989; Shinomura *et al.*, 1990; Bignami *et al.*, 1993; Fernandez-Teran *et al.*, 1993; Perides *et al.*, 1993; Yamagata *et al.*, 1993).

Knowledge of the role played by syndecan, a cell surface proteoglycan receptor, has been expanded; see Bernfield *et al.* (1993) for a review. Syndecan binds to tenascin with an avidity that is tissue specific, binding very tightly in teeth, but weakly in mammary glands (Salmivirta *et al.*, 1991 and see below).

New members of the syndecan family are emerging. Syndecan-3 is present in large amounts in prechondrogenic condensations (Gould *et al.*, 1992). The promoter for syndecan-1 has recognition sites for such regulatory genes as *Hox* and *MyoD*. Syndecan-2 (fibroglycan) is found in both prechondrogenic and preosteogenic condensations and at epithelial-mesenchymal boundaries, implying a role in epithelial-mesenchymal interactions (David *et al.*, 1993). Indeed, syndecan appears to be one of the molecules modulating transit of cells from condensation (proliferation) to initial differentiation (Goldring and Goldring, 1991; Hofbauer and Denhardt, 1991; Baserga and Rubin, 1993).

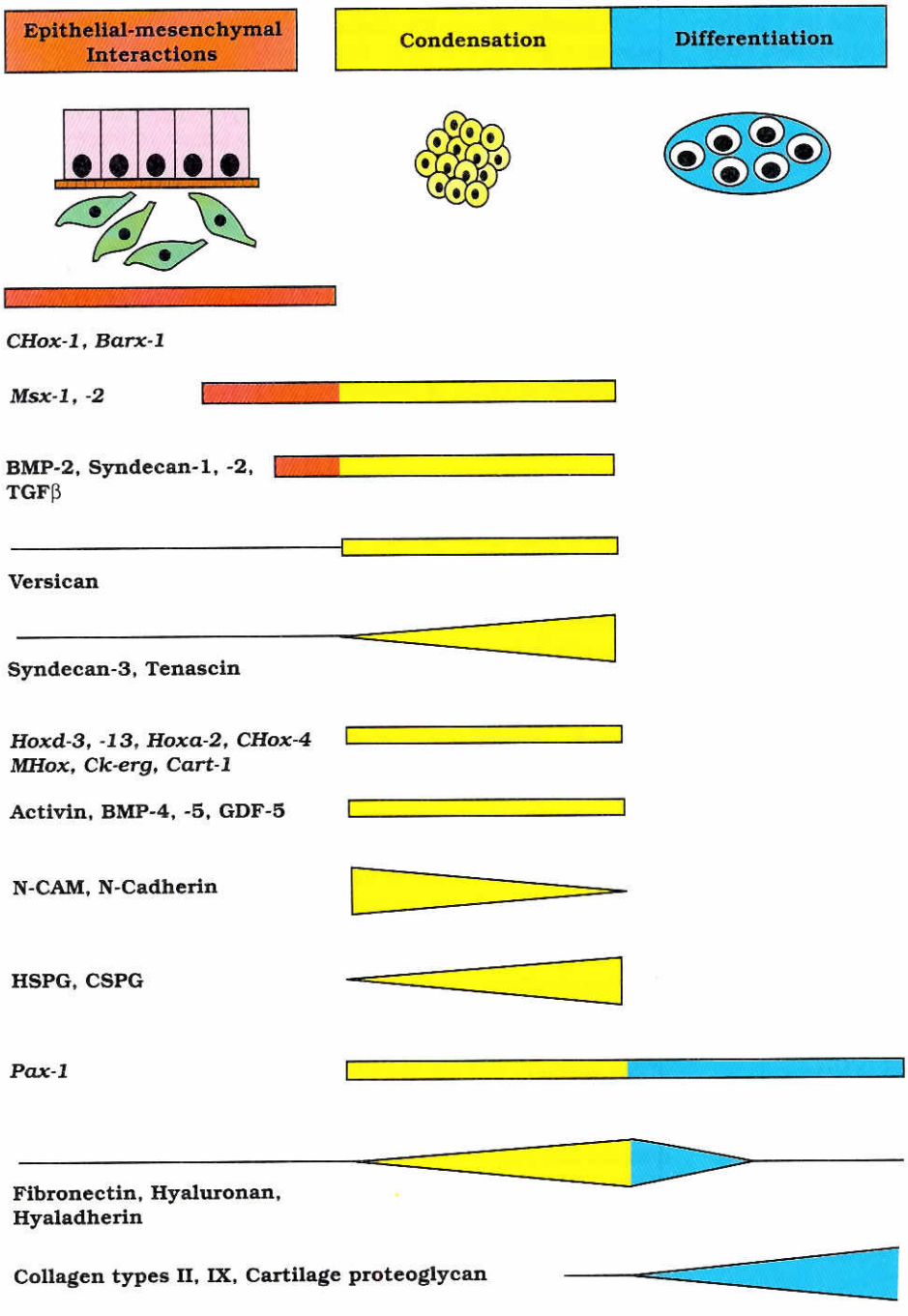

Fig. 2. A summary of the molecules known to be associated with the three major phases of chondrogenesis in the craniofacial skeleton. *The three phases are: pre-condensation, characterized by epithelial-mesenchymal interactions (brown), condensation (yellow) and differentiation (blue). The pre-condensation phase is characterized by expression of Hox genes (CHox-1 [Hoxa4], Barx-1), Msx-1, -2, the growth factors BMP-2 and TGF-ß, and syndecan-1. Versican, syndecan-3 and tenascin, which are present in low concentrations pre-condensation, are up-regulated at condensation. Other Hox genes and transcription factors (Hoxd-3,-13, Hoxa-2, Cdxa [Chox-4], Mhox, Ck-erg and Cart-1) and other growth factors (activin, BMP-4, -5 and GDF-5) are expressed at condensation. The cell adhesion molecules N-CAM and N-cadherin, also appear with condensation but are down-regulated during condensation. Heparan sulphate and chondroitin sulphate proteoglycans appear at condensation and are up-regulated during condensation. The transcriptional factor Pax-1 is present during and following condensation. Extracellular matrix molecules such as fibronectin, hyaluronan and hyaladherin increase during condensation (yellow) but are down-regulated thereafter (blue). Collagen types II and IX and cartilage proteoglycan appear post-condensation, although mRNAs for the collagens and for the core protein of the proteoglycan are up-regulated during condensation. See text for details.*

Tenascin, the large glycoprotein that binds to chondroitin sulphate proteoglycan continues to be much studied. Tenascin promotes chondrogenesis in part by counteracting inhibitory effects of fibronectin. Syndecan also interferes with fibronectin-integrin interactions (Chiquet-Ehrismann *et al.*, 1988). Fibronectin promotes condensation; its removal promotes differentiation (Newman, 1988; Leonard *et al.*, 1991, and see Hall and Miyake, 1992 for a discussion). That tenascin contains repeat domains analogous to EGF makes tenascin an important modulator of cell growth and development (Jiang and Chuong, 1992; Riou *et al.*, 1992). Dunlop and Hall (1995) demonstrated that tenascin was present in the mandibular arch of embryonic chicks, but only in the mandibular epithelium, not in preosteogenic or prechondrogenic mesenchyme. Timing of epithelial expression of tenascin is consistent with a role in epithelial-mesenchymal interactions.

Paradoxically, mice deprived of tenascin by replacing the tenascin gene with the *Lacz* gene develop normally (Saga *et al.*, 1992). Fibronectin, laminin, collagen and proteoglycan appeared normal as did gross and histological morphology. Given all the evidence for multiple developmental roles played by tenascin, another molecule(s) may (must?) compensate for the tenascin missing in these mice. There are different forms of tenascin,

each of which is differentially sensitive to induction by TGFß-1 and bFGF (Tucker, 1993; Tucker *et al.*, 1993), but just how mice manage to survive and survive so normally without tenascin is a mystery.

Tenascin enhances the mitogenic activity of EGF and is itself mitogenic for several cell types, including condensing mesenchyme (End *et al.*, 1992). Systems in which EGF or structural molecules with EGF-repeats provide the initial mitogenic signal in a two-step signaling process – first division, then initiation of differentiation – are ideal models in which to explore mechanisms controlling both initiation of condensation and transit of cells from condensation to differentiation (Coffin-Collins and Hall, 1989; Hall and Ekanayake, 1991; Hall, 1992b, 1994; Stein and Lian, 1993a,b; Dunlop and Hall, 1995).

In osteoblast-enriched cell cultures, tenascin is only found in cell aggregates expressing alkaline phosphatase (Mackie and Tucker, 1992). Alkaline phosphatase is a marker for osteoblast differentiation (see Hall, 1978; Milaire, 1974; Doty and Schofield, 1990 and Dunlop and Hall, 1995 for a few of many discussions of this point). Consequently, Mackie and Tucker's results would be taken to mean that tenascin is expressed in osteogenic cells *after* condensation. However, Dunlop and Hall (1995) found that alkaline phosphatase was expressed *before* condensation in chick preosteogenic mandibular arch mesenchyme, where condensation was visualized with PNA. A similar situation appears to be obtained in murine mandibular arch mesenchyme (Miyake, unpublished observations). Another difference between osteo- and chondrogenic condensations is the retention of blood vessels in the former but their exclusion from the latter (see Hall and Miyake, 1992 for a discussion).

The implications of such findings for how we consider the relationship between condensation and initial differentiation in osteogenic and chondrogenic mesenchyme were explored by Dunlop and Hall (1995).

The sequence of events in chondrogenesis is:

(a) an epithelial-mesenchymal interaction(s) preceding condensation of prechondrogenic mesenchyme;

(b) condensed cells transform into prechondroblasts;

(c) prechondroblasts differentiate into chondroblasts;

(d) deposition of extracellular matrix, and

(e) terminal differentiation ± mineralization depending on the cartilage type.

The sequence of events in osteogenesis is:

(a) an epithelial-mesenchymal interaction(s) initiates transit from osteogenic mesenchyme to preosteoblast;

(b) preosteoblast condensation increases the number of committed preosteoblasts;

(c) transformation of preosteoblasts to osteoblasts;

(d) deposition of extracellular matrix, and

(e) terminal differentiation and mineralization.

We can code the major developmental events in skeletogenesis as follows:

1, epithelial-mesenchymal interaction;

2, condensation;

3, differentiation of preosteo- or prechondroblasts;

4, differentiation of osteo- or chondroblasts;

5, deposition of extracellular matrix, and

6, terminal differentiation ± calcification.

In chondrogenesis the sequence is 1, 2, 3, 4, 5, 6. In osteogenesis it is 1, 3, 2, 4, 5, 6. Therefore in osteogenesis, differentiation of preosteoblasts precedes condensation which then amplifies the number of committed osteogenic cells. In chondrogenesis, condensation precedes the appearance of prechondrogenic cells, i.e. precedes commitment; see also the two threshold (proliferation/differentiation) model of Stein and Lian (1993a,b) and Dunlop and Hall (1995) for a discussion of a third possibility, that condensation and differentiation are synchronous. Further elaboration of this issue and understanding of these phenomena will require a more rigorous understanding of cell commitment, when differentiation is initiated, how commitment and differentiation are measured and the relationship of cell division to onset of differentiation. These are all issues that lie at the core of modern day cellular developmental biology.

Neural-cell adhesion molecule (N-CAM)

Earlier studies demonstrated N-CAM in the dermal condensations of feather primordia, mesenchyme, and pre- and post-migratory (but not in migratory) neural crest cells (Edelman, 1986 for review). Such localization and the known role of N-CAM in mediating cell adhesion, suggested that N-CAM may play a similar role in the initiation of condensation.

More recently, N-CAM has been investigated in chondrogenesis in avian limb buds, both *in vivo* and *in vitro*, confirming localization in prechondrogenic condensations (Oberlender and Tuan, 1994; Tavella *et al.*, 1994). N-CAM is co-expressed with N-cadherin. Expression of N-CAM and type II collagen are mutually exclusive; i.e. as cells 'exit' the condensation and differentiate, N-CAM is down-regulated. Calcium-dependent aggregation of limb mesenchymal cells is inhibited by NCD-2, a monoclonal antibody to N-cadherin, in both a dose- and a time-dependent manner (Oberlender and Tuan, 1994). This suggests that N-cadherin initiates and N-CAM maintains cells in condensations. Injection of NCD-2 into limb buds *in ovo* perturbed and deformed development of the limb cartilages.

Exposure of prechondrogenic mesenchyme to Fab fragments of an antibody against N-CAM inhibits cell aggregation, while over-expression of N-CAM enhances chondrogenesis by enhancing condensation (Widelitz *et al.*, 1993). These authors also showed that N-CAM was up-regulated by activin (a member of the TGFß superfamily of growth factors) and by fibronectin; see also the discussion of the work by Jiang and colleagues under Formation.

Talpid, a mutation in the chick, affects segregation of prechondrogenic mesenchyme into condensations during limb development so that cartilages fuse or are duplicated to produce polydactylous limbs. Cells within *Talpid* condensations are more adhesive than cells from wild type embryos (Ede, 1983) and *Talpid* embryos have abnormally large prechondrogenic condensations (see Johnson, 1986 and Hall and Miyake, 1992 for reviews). *Talpid* also has elevated N-CAM expression in its over large condensations. That the size of these condensations can be reduced by exposure to anti N-CAM antisera (Chuong *et al.*, 1993) establishes a link between over-expression of N-CAM and production of abnormally large condensations. *Talpid* therefore illustrates the profound effect that a change in a cell adhesion at the condensation stage can have on skeletal morphogenesis.

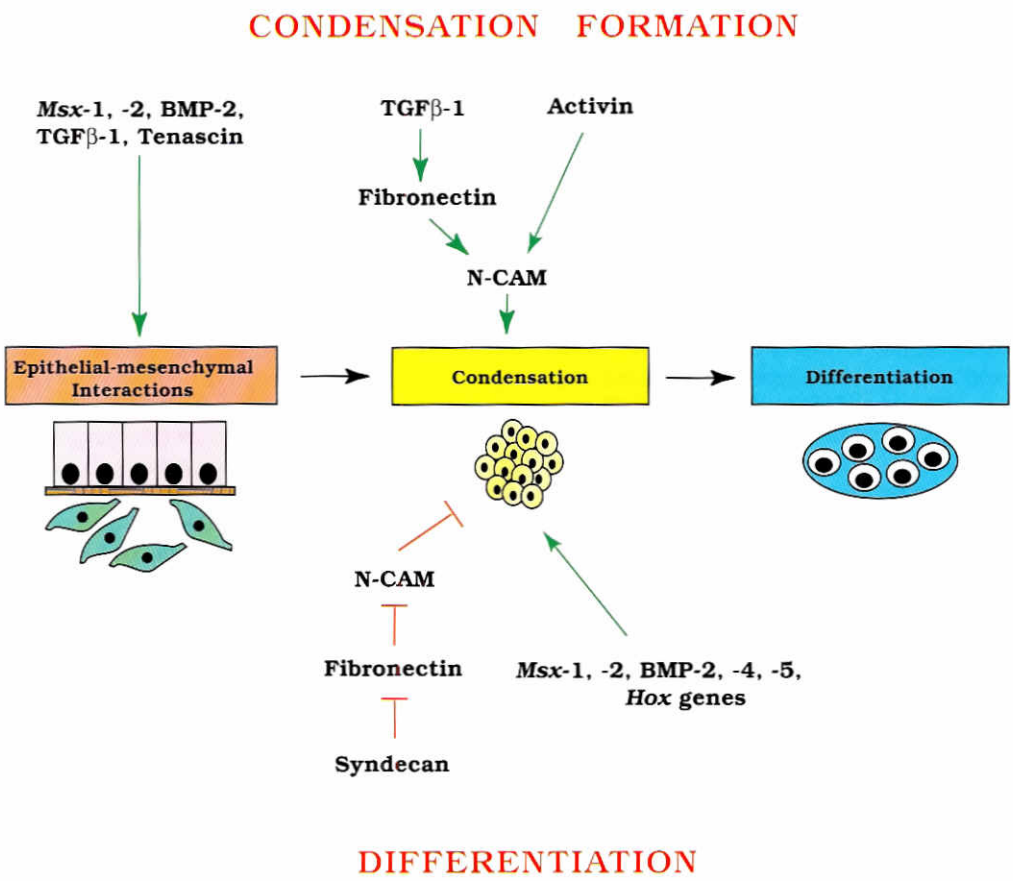

Fig. 3. A summary of the molecular pathways leading to condensation formation and to differentiation of prechondrogenic cells in the three major phases of chondrogenesis shown in Figure 2. *Condensation is initiated by Msx-1, -2, growth factors and tenascin regulating epithelial-mesenchymal interactions that in turn control condensation. TGFß-1 by up-regulating fibronectin and activin by direct action stimulate accumulation of N-CAM and so promote condensation. Transition from condensation to overt cell differentiation is mediated negatively by suppression of further condensation and positively by direct enhancement of differentiation. Syndecan by inhibiting fibronectin, breaks the link to N-CAM and so terminates condensation formation Cessation of activin synthesis has the same effect. A number of Hox and Msx genes and BMP-2, -4, and -5 enhance differentiation directly by acting on condensed cells. See text for details.*

Tavella and colleagues (1994) described how N-CAM expression was lost with differentiation of chondroblasts to hypertrophic chondrocytes, but was re-expressed as hypertrophic chondrocytes transformed into osteoblasts, as they do in some instances of endochondral ossification.

Up-regulation of N-CAM concomitant with osteogenesis and down-regulation concomitant with chondrogenesis has also been shown by Fang and Hall (1995) for secondary chondrogenesis and intramembranous ossification from common periosteal cells on the quadratojugal of embryonic chicks. At joints and sites of ligament or muscle insertion where local mechanical factors are acting, bipotential periosteal cells switch from osteogenesis to secondary chondrogenesis. Elsewhere along the bone, periosteal cells differentiate as osteoblasts. Chondroblasts fail to differentiate and cartilage to form in embryos paralyzed with neuromuscular blocking agents. N-CAM is expressed in osteoprogenitor cells, osteoblasts, prechondrogenic cells (as in condensations) but not in chondroblasts. Down-regulation in periosteal progenitor cells coincides with the switch of progenitor cells to chondrogenesis. This switch does not occur in paralyzed embryos in which progenitor cells remain as N-CAM-positive osteogenic progenitors. N-CAM, being responsive to embryonic movement, is a likely signaling molecule transducing biomechanical signals into progenitor cell switching and initiation of chondrogenesis (Fang and Hall, 1995). According to Lee and Chuong (1992) N-CAM expression coincides with expression of collagen type I and alkaline phosphatase but occurred after expression of fibronectin in osteogenic condensations in both intramembranous and in endochondral ossification, further implicating N-CAM in the transition of preosteogenic cells to osteoblasts during bone formation.

The TGFß superfamily of growth factors

Links have now been established between N-CAM, condensation and members of the TGFß superfamily of growth factors, especially BMP-4 and activin. TGFß stimulates the production of fibronectin and fibronectin receptor, which promotes condensation through its action as a cell adhesion molecule; mRNA for fibronectin increases 5 fold at condensation initiation (Newman, 1988; Kulyk *et al.*, 1989a; Ohya and Watanabe, 1994). By stimulating fibronectin production, TGFß facilitates initiation of prechondrogenic condensations, followed by selective expression of cartilage specific gene products. Two hour exposure of limb bud mesenchyme to TGFß is sufficient to initiate a 3-6 fold increase in synthesis of mRNA for both type II collagen and proteoglycan core protein (Kulyk *et al.*, 1989b).

TGFß and BMP-2 act on chondrogenesis, but pre- and post-condensation respectively. Roark and Greer (1994) showed that prechondrogenic limb mesenchymal cells from the embryonic chick express mRNA for TGFß-1, -2, and -3 during chondrogenesis in micromass culture. Application of exogenous TGFß-3 or BMP-2 promoted chondrogenesis as assessed by accumulation of extracellular matrix. TGFß was more effective on pre-condensation cells, BMP-2 more effective on post-condensation cells or

on cells undergoing differentiation (Asahina *et al.*, 1993; Carrington, 1994; Reddi, 1994). TGFß-1 regulates differentiation of otic capsule cartilage by regulating epithelial-mesenchymal interactions at condensation. TGFß can substitute for the epithelium that normally induces chondrogenesis in the otic capsule (Frenz and Van de Water, 1991; Frenz *et al.*, 1992; see Hall, 1991 for a discussion of the specificity of this chondrogenic induction).

A morphogenetic specificity underlies condensation cell lineages and the shapes of the skeletal elements formed from them. Fore- and hind-limb bud prechondrogenic condensations respond differently to TGFß and retinoic acid and, furthermore, display different morphological patterns *in vitro*, hind limb bud cells forming nodules and wing cells sheets of cartilage (Downie and Newman, 1994). For further analysis of the morphogenetic patterning of specific populations of chondrogenic or osteogenic cells see Hall (1981, 1985, 1989), Langille (1994b) and Richman (1994).

Brachypod is an autosomal recessive mutation in the mouse with small digits because of an effect on prechondrogenic condensations (Milaire, 1965; Grüneberg and Lee, 1973). Grüneberg and Lee (1973) concluded that mesenchyme was not properly allocated between distal condensations. *Brachypod* condensations have altered cell surface properties, reflected in altered cell adhesion, delayed cartilage formation and reduction in the number of cartilages; see Hall and Miyake (1992) for literature. It was suggested that the alterations in the cell surface were mediated by alteration in surface galactosyltransferases (Elmer *et al.*, 1988). Alteration is now known to involve a member of the TGFß superfamily. Storm *et al.* (1994) identified new members of the TGFß superfamily, the growth-differentiation factors 5-7 (GDF5-7). GDF-5, a BMP homolog, is expressed in prechondrogenic limb mesenchyme at condensation. A frameshift mutation occurs in *brachypod*, affecting chondrogenesis at the condensation stage. GDF-5 is therefore responsible for the condensation-based limb deficiencies in *brachypodism.*

In transgenic mice, BMP-4 is expressed ectopically and, in the most severely affected embryos, is associated with severe craniofacial malformations, principally bony fusions and cleft palate (Blessing *et al.*, 1993). The *Short Ear* mutation in the mouse, in which some bones are lost, is the first reported mutation of a BMP gene (Kingsley *et al.*, 1992; also see Kingsley, 1994 for a review). BMP-4 and BMP-5 both act at the condensation stage. Indeed, both may act as inducers of condensation (*ibid.*; King *et al.*, 1994). *Short Ear* and *Brachypodism* are therefore both defects in members of the TGFß superfamily, BMPs and GDFs respectively. Both genes act at the condensation stage, *Short Ear* in axial, rib and ear cartilages, *Brachypod* in limb cartilages. That two members of the BMP superfamily acting at the same stage – condensation – act on totally different parts of the developing skeleton, further alerts us to the subtlety of developmental regulation of the skeleton at the condensation stage and reinforces the importance of identifying and understanding the behavior of sub-populations of skeletal cells (Atchley and Hall, 1991; Tickle, 1994).

BMP-4 is present in condensing mesenchyme of limb buds and facial processes in 8.5 day mouse embryos (Jones *et al.*, 1991). Interestingly, BMP-2 and -4 have both recently been reported to be down-regulated in the diastemal (non-tooth forming) region of the developing mouse maxilla (Tureckova *et al.*, 1995). Such lack of persistence of these BMPs (and of *Msx*-2; see below) may explain, in part, the failure of teeth to form in the diastema.

BMP-2 is present in condensing chondrogenic and osteogenic mesenchyme in the embryonic chick (Kolodziejczyk and Hall, 1993). Considerable evidence now exists demonstrating that BMP-2 is an inductor of osteogenesis from osteoprogenitor cells of membrane bones, marrow stromal cells, established fibroblastic and osteosarcoma cell lines and can convert myogenic cells into osteoblasts (C3H10T1/2 and 3T3) (Asahina *et al.*, 1993; Knutsen *et al.*, 1993; Wang *et al.*, 1993; Carrington, 1994; Ghosh-Choudhury *et al.*, 1994; Katagiri *et al.*, 1994; Rickard *et al.*, 1994).

BMP-4 appears to mediate epithelial-mesenchymal interactions in both tooth and hair follicle development (Blessing *et al.*, 1993; Vainio *et al.*, 1993). In murine tooth development, epithelial expression of BMP-4 induces expression of BMP-4 in the adjacent dental mesenchyme, which in turn induces expression of such homeobox genes as *Egr*-1 and *Msx*-1, but not such molecules as syndecan or tenascin. As indicated above, the diastema lacks *Msx*-2. In this way expression of *Msx*-1 and *Msx*-2 both depend on epithelial-mesenchymal interactions in tooth as they are in bone development (Takahashi and Le Douarin, 1990; Takahashi *et al.*, 1991; Jowett *et al.*, 1993; Liu *et al.*, 1995; Mina *et al.*, 1995). Four *Msx* genes are expressed during development of the median and paired fins in the zebra fish and in the blastema during fin regeneration, appearing as early as one day after amputation. The appearance of *Msx* correlates with proliferation and aggregation of cells in the regeneration blastema (Akimenko *et al.*, 1995).

Msx-1 minus homozygote mice display clefting of the secondary palate, defective alveolar bone development in both mandibular and maxillary skeletons and inhibited tooth development. The upper incisors are absent as is the alveolar process normally associated with the upper molars. The action of *Msx* was traced to the condensation stage: a tooth rudiment is present but fails to progress beyond the bud stage or to produce more than a trace of condensed dental mesenchyme (Satokata and Maas, 1994). Within the mandible and maxilla, *Msx*-1 plays a specific role in only one of the cell populations identified by Atchley and Hall (1991), viz. the alveolar population. The body (ramus) of the mandible (the osteogenic population) and the three bony processes at the rear of the dentary bone are unaffected by loss of the *Msx*-1 gene. Not only do the separate cell lineages postulated by Atchley and Hall exist, but they are under differential genetic control.

Msx-2 has now been shown to be associated with craniosynostosis and ectopic calvarial bone formation in mice and with Boston-type craniosynostosis in at least one human family; see Liu *et al.* (1995). *Msx*-2 exerts its action on condensed and progenitor cells of calvarial periostea.

Homeobox genes and transcription factors

Our knowledge of the role played by homeobox genes in condensation has increased over the past several years but we are still a long way from understanding just what their role(s) is (are). Some information on *Egr*-1 and *Msx* has already been noted. Some *Hox* genes, such as *Hoxa*4 (*Chox*-1) discussed below, are expressed early in regions that exceed the limits of subsequent

condensations and are involved in skeletal patterning. Other *Hox* genes are restricted to condensations. *Pax*-1, a transcriptional regulator of the gene *undulated*, is expressed in murine sclerotomal and pectoral girdle mesenchyme, plays a role in the transition from condensation to initiation of chondrogenesis and so may be one of the elements that move cells out of the condensation stage. In mice lacking *Pax*-1, skeletal elements fuse, are malformed or lost (Timmons *et al.*, 1994; Wallin *et al.*, 1994). Strong expression of *Dlx*-3 is found in the condensing preosteogenic mesenchyme of the mouse mandibular arch, although its role in development is unclear (Robinson and Mahon, 1994). *Dlx*-3 and *Dlx*-4 are also seen in otic vesicle epithelium before induction of the otic vesicle in the zebra fish, a site and timing consistent with a possible role in otic capsule induction (Ekker *et al.*, 1992; Akimenko *et al.*, 1994).

Mice in which *Hoxd*-13 has been mutated display skeletal alterations along the entire axis because of failure of the primary condensations to divide, failure of ossification or bony fusions, indicating multiple roles for *Hoxd*-13 at various stages of limb skeletal development (Dollé *et al.*, 1993). Other homeobox genes are expressed in very early chick limb bud mesenchyme. Expression ceases with condensation but they do play a role in skeletal patterning, *Hoxa*4 (*CHox*-1) has been implicated in proximo-distal axial gradient, and *Cdxa* (*CHox*-4) in condensation branching (Yokouchi *et al.*, 1991). This patterning role is beautifully demonstrated by *Hoxd*-3 and *MHox*. As revealed by differential visualization with peanut agglutinin lectin, different parts of the same vertebra are affected differentially because of the role that these genes play in controlling division of populations of sclerotomal cells (Condie and Capecchi, 1993; Götz *et al.*, 1993; Martin *et al.*, 1995). Compound mutations of *Hoxa*-4, *b*-4 and *d*-4 or of *Hoxa*-11 and *d*-11 reveal much more pronounced deletions and/or homeotic transformations than evident with single gene mutations (Davis *et al.*, 1995; Horan *et al.*, 1995). That effects are dose-dependent indicates the degree to which *Hox* genes control skeletal patterning. *MHox*, a nuclear factor that binds to the creatine kinase enhancer, is up- regulated by the epithelial-mesenchymal interactions that initiate condensation formation (Martin *et al.*, 1995).

Targeted deletion of *Hoxa*-2 in mice has revealed that this homeobox gene also controls skeletal patterning at the condensation stage. Second branchial arch skeletal elements are missing because second arch mesenchyme forms first arch structures in an ectopic location (Gendron-Maguire *et al.*, 1994; Rijli *et al.*, 1994). Homozygous embryos display a mirror-image duplication of the malleus, incus and tympanic bones of the middle ear as second branchial arch form more anterior structures in an ectopic location. Ectopic squamosals and Meckel's cartilages are also seen. Second arch structures such as the stapes and stylohyal cartilages are missing. We are engaged in a study of the development of early stages of skeletogenesis in these embryos to examine the action of *Hoxa*-2 at the condensation stage.

The *ets* gene superfamily consists of some 30 highly conserved transcriptional modulators which share the ETS DNA-binding domain. They have been implicated in human tumors of both mesodermal and neural crest origin. Dhordain *et al.* (1995) cloned the chicken *erg* gene (*ck-erg*) which is specifically expressed in prechondrogenic condensations of both the mesodermal and neural crest skeletons. Whether *erg* regulates skeletogenic inducers (BMP-2, BMP-4) known to be present in condensations has not yet been determined.

A new homeodomain-containing gene, *Cart*-1, is present in high levels in prechondrogenic, tendon, kidney and lung mesenchyme (Zhao *et al.*, 1994). Tissier-Seta *et al.* (1995) isolated a new homeodomain transcription factor from the mouse. *Barx*-1, the murine homolog of the *Drosophila BarH*-1 and -2 genes, was present in craniofacial mesenchyme but is down-regulated with condensation of both chondro- and osteogenic mesenchyme. *Cart*-1 may be involved in condensation initiation, *Barx*-1 in transit from non-condensed to condensed mesenchyme. *Barx*-1 was expressed in the molar but not in incisor tooth anlage, the only homeodomain protein so far identified with a tooth-type specific pattern of expression. Whether *Barx*-1 is involved in patterning molar teeth awaits further studies.

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Summary

Cell condensation is a pivotal stage in skeletal development. Although prechondrogenic condensations normally exist for some 12 h, duration can vary. Variation is seen both between condensations for different cartilages (Meckel's vs. elastic ear cartilage) and within a single condensation from which more than one skeletal element will form, as in the three components of the single first arch chondrogenic condensation. Understanding how duration of the condensation phase is established – how the condensation phase is entered and exited during cell differentiation – remains a major area for future study.

During chondrogenesis, cell-specific products such as collagen types II and IX and cartilage proteoglycan appear concomitant with condensation. Therefore, during chondrogenesis, condensation precedes commitment of cells as prechondroblasts. During osteogenesis, however, differentiation of preosteoblasts precedes condensation. Therefore, during osteogenesis, condensation amplifies the number of committed osteogenic cells. Further comparative analysis of skeletogenesis should provide us with a more rigorous understanding of cell commitment, when differentiation is initiated, how commitment and differentiation are measured and the relationship of condensation to onset of differentiation.

Current knowledge of molecules characteristic of condensations focused attention on extracellular matrix and cell surface components on the one hand, and on growth factors homeobox genes and transcription factors on the other. We have drawn together the molecular data for pre-chondrogenic condensations in diagrammatic form in Figure 2. Three major phases of chondrogenesis are identified: (a) epithelial-mesenchymal interactions that precede condensation, (b) condensation itself, and (c) cell differentiation. Although we label the third phase differentiation, it is important to recognize that phases a and b also constitute aspects of chondroblast cell differentiation (see Dunlop and Hall, 1995 for a discussion of this point. The pre-condensation phase is characterized by expression of *Hox* genes, growth fac-

tors (TGF-ß and BMP-2) and the cell surface proteoglycan receptor, syndecan-1. Expression of *Msx*-1 and *Msx*-2, growth factors and syndecan continues into the condensation phase. Other molecules, such as versican, syndecan-3 and tenascin, present in low concentrations before condensation, are up-regulated during condensation. Yet other molecules – *Hox* genes, transcription factors, growth factors (activin, BMP-4 and -5, GDF-5), cell adhesion molecules and proteoglycans – are only expressed during the condensation phase, while the transcription factor *Pax*-1, fibronectin, hyaluronan and hyaladherin are expressed both during and after condensation. During condensation mRNAs for collagen types II and IX and for the core protein of cartilage proteoglycan are up-regulated. Late in condensation and increasingly thereafter, the protein products of these genes accumulate as chondroblasts differentiate (see Fig. 2 for details).

Not all the molecules present before, during of after condensation can be placed into causal sequences. Some however can. In Figure 3 we summarize the causal sequences discussed in this paper as they relate to initiation of condensation and to transit from condensation to overt differentiation during chondrogenesis. Condensations form following activation of at least three pathways:

(1) Initiation of epithelial-mesenchymal interactions by tenascin, BMP-2, TGFß-1 and *Msx*-1 and -2.

(2) Up-regulation of N-CAM by activin.

(3) Up-regulation of fibronectin by TGF-ß, further enhancing N-CAM accumulation (Fig. 3).

It is by these three pathways that condensations are initiated and grow.

Transition from condensation to overt cell differentiation is under both positive and negative control (Fig. 3). Syndecan blocks fibronectin and so blocks N-CAM accumulation, preventing accumulation of additional cells to the condensation. By blocking condensation, syndecan enhances differentiation. BMP-2, -4 and -5, *Hox* genes and Msx-1 and -2 act directly on condensed cells to initiate differentiation.

Clearly, many steps still have to be elucidated. These include further elaboration of relationships between the molecules summarized in Figure 3 and already known to play roles in condensation or differentiation. How other molecules shown in Figure 2 (*Pax*-1, *Barx*-1, *Ck-erg*, *Cart*-1) fit into Figure 3 has to be determined. And doubtless, there are other molecules/pathways to be identified. Nevertheless, our knowledge of the importance and regulation of condensations and of the molecules that are involved when condensation formation is perturbed has advanced enormously over the last three years. *Talpid*, *Brachypod* and *Short Ear* mutations are three cases in point. Over-expression of N-CAM, a frameshift mutation in GDF-5 and a mutation in BMP-5 respectively, have been shown to perturb skeletal development by acting at the condensation phase. We look forward with eager anticipation to the next triennium.

KEY WORDS: *condensations, osteogenesis, chondrogenesis, membranous skeleton, Hox genes, growth factors*

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