I. Introduction

Our understanding of the genetic and molecular control of development in vertebrates has dramatically increased during the last 10 years through the discovery that molecular processes that control development in invertebrates have been conserved during evolution and are also found in vertebrates. Important developmental genes were identified that are not only similar in sequence but also in their molecular function in widely diverged organisms such as C.elegans, Drosophila, zebrafish, mice and man. From Drosophila studies it is now clear that epigenetic development is regulated by cascades of gene expression. Early acting regulatory genes initiate the developmental process and induce the expression of other downstream genes.

I.1 Developmental genes in Drosophila

Phenotypic analysis of Drosophila mutants has allowed identification in the early eighties of more than 50 developmental genes that fall into three broad classes:

1. The polarity genes acting along the antero-posterior and dorso-ventral axis, their mutation resulting in defective polarity of the whole embryo (ex: bicaudal).

2. The segmentation genes involved in the formation of 14 segments (3 head segments, 3 thoracic segments and 9 abdominal segments). These zygotically active genes have been assigned to three different classes namely "gap genes", "pair rule genes" and "segment polarity genes".

3. The homeotic genes control the identity of segments and form the homeotic HOM-C complex that comprises two separate clusters Bithorax (BX-C) and Antennapedia (ANT-C)

I.2 Skeletal development in human

Skeletal development in human normally initiates at the beginning of the fourth week when somites originating from the paraxial mesoderm subdivide into three kinds of mesodermal primordium: myotomes, dermatomes and sclerotomes. These latter will give rise to the vertebral bodies and vertebral arches and contribute to the base of the skull. Appearance of the upper limb buds also occurs during the fourth week. The small bulges (upper limb buds) arise on the lateral body at the level of sclerotomes C5 to C8 while lower limb buds will appear few days later at the level of sclerotomes L3 to L5. The skeleton of the head is made of the chondrocranium (neurocranium) supporting the brain, flat bones of membranous origin which roof the skull and the viscerocranium which supports the pharyngeal arches.

II. Development of the axial skeleton

The embryonic precursors of cartilage of the vertebrae and ribs are contained within the somite. Somites are paired segmented structures that form epithelial spheres. Cells of the ventral somite delaminate from the epithelium to form the mesenchymal sclerotome. The ventral portion of the sclerotome surrounds the notochord and forms the rudiment of the vertebral body. The dorsal portion of the sclerotome surrounds the neural tube and forms the rudiment of the vertebral arches.

II.1 Signaling molecules involved in the determination of sclerotome to cartilage

Segmentation and differentiation of the somitic subunits is under the control of regulatory genes and growth factors that act through specific inductive mechanisms.
The primary signal for sclerotome induction appears to be a notochord-produced factor called Sonic hedgehog (Shh). Knock out of the Sonic hedgehog gene in mice has shown that prolonged expression of this peptide is required for sclerotome development as homozygous embryos lack a vertebral column indicating a failure of the sclerotome to form cartilaginous structures. Early sclerotome markers known to play a role in its development include the paired-box transcription factors Pax 1 and Pax 9 which are involved in mediating interactions between the notochord and the developing sclerotome.

Two basic helix-loop-helix transcription factors Twist and Scleraxis are also expressed in the sclerotome although they do not appear to be sufficient to determine sclerotome cells to the cartilage fate. In order to produce intersegmental vertebral rudiments, sclerotomes split into a cranial half and a caudal half and recombine by fusing the caudal half of each sclerotome with the cranial half of the succeeding. The resulting composite structure produces seven cervical vertebrae arising from eight cervical somites. The fibrous intervertebral discs form between the vertebral bodies.

The ribs develop from small lateral mesenchymal condensations called costal processes that form in association with the vertebral arches of all the neck and trunk vertebrae. In the thoracic region the distal tips of the costal processes lengthen to form ribs. The first seven ribs connect ventrally to the sternum via costal cartilages. The five lower ribs do not articulate directly with the sternum. All of them develop as cartilaginous precursors that later ossify through primary and secondary ossification centers.

II.2 Role of Hox genes in the regulation of vertebral segments
Appropriate differentiation of cervical, thoracic lumbar and sacral vertebrae has been demonstrated to require sequential Hox gene expression. In human the Hox gene complex, homologous to the HOM-C complex in Drosophila, comprises 39 genes organized in four different chromosomal clusters (A, B C. and D). The genes are divided in 13 paralogous families based on protein sequence similarities and genes located at the 3'end of the cluster are expressed earlier during embryogenesis than their 5' neighbours. Although extensive overlap of function seems to exist among Hox genes, mutation induction in mice have provided relevant information as to their role. A null mutation of Hoxc-8 lead to transformation of the first lumbar vertebra into a 14th thoracic vertebra, and the eight rib became attached to the sternum. Loss of function alleles of Hoxb-4, Hoxa-2 and Hoxd-3 also induce vertebral transformations supporting the idea that Hox genes are responsible for modifying a common vertebral module thereby defining the identity of each vertebrae.

III. Development of the limbs (appendicular skeleton)
Limb development begins in human at day 24 when cells from the lateral plate mesoderm (LPM) and cells from the lateral edges of nearby somites migrate to the presumptive limb field. The upper limb buds appear as small bulges protruding from the lateral body wall at the level of sclerotomes C4 to C8. The lower limb buds appear at the end of the 4th week. Each limb bud consists of a mesenchymal core of mesoderm covered by an ectodermal cap. The ectoderm at the tip of the bud thickens to form a specialized structure called the apical ectodermal ridge (AER). This structure maintains continuous limb bud outgrowth along the proximo-distal (P-D) axis (shoulders to digits). Concomitant to its elongation along the P-D axis, the limb becomes flattened along the dorso-ventral (D-V) axis (back of hand to palm) and asymmetric along the antero-posterior (A-P) axis (thumb to little finger). Differentiation becomes morphologically apparent as the mesenchymal cells condense to form the primordia of individual skeletal elements. The most proximal elements (stylopod) begins to differentiate first, followed by the progressive differentiation of more distal structures (zeugopod and autopod). This outgrowth and patterning depends on the establishment and maintenance of three signaling centers within the limb bud: 1) the AER, an epithelium that runs from anterior to posterior at the distal margin of the bud; 2) the zone of polarizing activity (ZPA) in the mesenchyme at the posterior margin of the bud with no distinguishing morphological features; and 3) the non ridge ectoderm of the bud.

III.1 Limb bud differentiation with respect to three axes
The limb buds in vertebrates grow with respect to the proximodistal, dorsoventral and craniocaudal (antero-posterior) axes and require positional signaling molecules. Some of the secreted molecules responsible for the activities of the different limb bud signaling centers have been identified. Members of the fibroblast growth factors
(FGF) family produced by AER cells perform the functions of the ridge that are required for P-D outgrowth. FGF signals are responsible for keeping the underlying undifferentiated mesenchymal cells located in a region known as the **progress zone**, in an undifferentiated, rapidly proliferating stage. Sonic hedgehog (Shh) produced by ZPA cells is expressed in the posterior mesenchyme and represents the key mediator of the polarizing activity that regulates patterning along the A-P axis. Specification of the D-V axis seems to be mediated by En1 (a vertebrate homologue of the Drosophila transcription factor engrailed) Wnt7a and Lmx1. Wnt7a (an homolog of wingless in Drosophila) is produced exclusively by dorsal ectodermal cells and act through its downstream target Lmx1 (a Lim homeobox gene) expressed in the underlying mesenchyme. These different signals molecules are interdependent. Regulatory interactions between the signaling centers and their products cooperatively regulate and coordinate limb outgrowth and patterning along all three axes.

### III. 2 Role of FGF and their receptors in limb development

The FGF family comprises at least 24 members. The proteins encoded by the 24 different genes are variable in length (155-268 amino-acids) and contain a conserved 'core' sequence of ~ 120 amino-acids that confers ability to bind heparin or heparan sulfate proteoglycans (HSPG). Secreted FGF are able to bind to HSPG (low affinity receptors) such as syndecans, glypican and perlecan located at the cell surface which restrict their ability to diffuse far from the cells. This also allows their binding to high affinity receptors, the Fibroblast Growth Factor Receptors (FGFRs) which form a family of 4 transmembrane protein tyrosine kinases. The binding of FGF to monomers of FGFR induces receptor dimerization and activates their tyrosine kinase activity that itself triggers signal transduction. At least five FGF (FGF 2, FGF 4, FGF 8, FGF 9 and FGF 10) and two FGFR (FGFR1 and FGFR2) are expressed during limb bud initiation. FGF 2, 4, 8, 9 and FGFR 2 are found in the ectoderm and AER while FGF 10 and FGFR 1 occur in the underlying mesenchyme (table 1).

There is good evidence that FGFs produced in the AER serve at least two major functions. One is to stimulate the proliferation of cells in the progress zone due to their mitogenic activities for limb bud mesenchyme and thus produce the new cells required for limb outgrowth. Another function of FGFs is to maintain Shh expression in the ZPA. FGF 4 although not required to induce Shh expression is largely responsible for maintenance of its expression as the limb elongates. The regulatory interaction between FGF4 and Shh could be reciprocal as Shh produced in the ZPA induces and maintains FGF 4 expression in the AER. This positive feedback loop between FGF 4 and Shh could be one of the mechanisms by which outgrowth and patterning of limb would be coordinate regulated, although additional molecules such as Wnt7a are likely to play a role in regulating Shh expression. One of the target for FGF signaling from the AER is FGF 10 which is expressed in the distal limb bud mesenchyme. This factor is able to interact with FGF 8 and there might be a positive feed-back loop between FGF 10 and FGF 8. This reciprocal regulation is likely to be mediated by two isoforms of FGFR 2, FGFR 2b (that binds FGF 10 exclusively) and FGFR 2c (that binds FGF 8). A recent model has been proposed in which FGF 10 made in the mesenchyme of the limb field diffuses in the ectoderm where it binds FGFR 2b and induces FGF 8 in the ectoderm. The FGF 8 in turn diffuses into the mesoderm and activates FGFR 2c which causes the upregulation of FGF 10. The FGF 10 then continues the loop and results in limb bud induction. Hence FGFR 2 appears to be essential for limb bud initiation whereas FGFR 1 seems to play an essential role at several stages of limb development. This assertion is based on the study of mouse models and expression patterns which have revealed an important function of FGFR 1 in specification of P-D axis formation. FGFR 1-mediated signals are required for maintaining ZPA and progress zone activities.
III. The role of Hox and BMP genes in the limb bud development

Some of the FGF in conjunction with Shh can affect expression of the bone morphogenetic protein (Bmp-2 and 7) and Hox genes, mostly Hoxd-12 and Hoxd-13. These latter genes are members of the Hoxd complex and are expressed within the distal wrist (Hoxd 12) and within the hand and fingers (Hoxd 12 and 13). The role of the Hoxd 13 gene in the proximodistal differentiation of limb segments has been illustrated by the demonstration that mutations in the human gene transforms the metacarpals to carpals and metatarsals to tarsals. Likewise, overexpression of Hoxd13 in chick limb bud resulted in the transcriptional repression in the proximal part of the limb of Meis, the vertebrate ortholog of an homeo-box containing gene in *drosophila* called homothorax (hth) that is required for proximal leg development.

The skeletal elements of the limb develop from a column-like mesodermal condensation that appears along the long axis of the limb bud during the fifth week of gestation in human. With the exception of clavicle, the bones of the limbs form by ossification of a cartilaginous precursor according to a process called endochondral ossification. Mesenchymal cells from the lateral plate condense to form prechondrogenic elements in the proximal region of the limb, giving rise to the anlagen of the humerus (or femur). Distal extension of the process results in the formation of the ulna and radius (or fibula and tibia) which further branches and segments to form the posterior proximal carpal (tarsal) element as well as the digital rays of digits IV-II. Prechondrocytes in the prechondrogenic condensations differentiate into chondrocytes in response to growth factors and secrete molecules characteristic of the extracellular matrix such as collagen type II and aggrecan (a large proteoglycan). The initial phase of chondrification results in the formation of a cartilaginous envelope, the perichondrium. This perichondrium in which bone morphogenetic protein 2, 4 and 7 (BMP 2, 4 and 7) and parathyroid hormone/parathyroid hormone-related peptide receptor (PTH/PTHrPR) are expressed, inhibits chondrocyte proliferation and maturation thereby helping to control the growth and differentiation of the forming cartilage elements. As the cartilage elements grow different zones can be distinguished that demarcate the progressive differentiation of the chondrocytes. Cells at the ends of the elements are immature and undergo rapid proliferation. Adjacent to the proliferation zone are the larger and more sparsely distributed pre-hypertrophic chondrocytes that express *Indian hedgehog* (Ihh), PTH/PTHrPR, BMP 6 and BMP receptor IA (BMPRIA). The terminally differentiated hypertrophic cells express a unique form of collagen, type X collagen, and eventually undergo programmed cell death and are replaced by osteoblasts. Defective cartilage growth occurs in a wide spectrum of disorders called chondrodysplasias that usually result in dwarfishs of variable severity. The most common of these disorders is achondroplasia, a dominant genetic disease caused by a recurrent activating mutation in the transmembrane domain of FGFR3 affecting chondrocyte proliferation and differentiation. The process of bone ossification begins in a region called the primary ossification center. Mesenchymal cells in the perichondrium differentiate into osteoblasts that secrete the calcium salt matrix of mineralized bone and form a primary bone collar around the bone which thickens as osteoblasts differentiate. In addition to chondrocytes and osteoblasts, a third cell type of hematopoietic origin, the osteoclasts contribute to skeletal remodeling throughout development. Indeed, the function of osteoblasts and osteoclasts is intimately linked since osteoblast synthesize and secrete molecules that control osteoclast differentiation.

<table>
<thead>
<tr>
<th>FGF or FGFR member</th>
<th>Expression domain in the chick limb bud</th>
<th>Prospective limb territory</th>
<th>Phenotype of null mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF 2</td>
<td>Throughout AER and dorsal ectoderm</td>
<td>Ectoderm and mesoderm</td>
<td>Neuronal defects; normal development of limbs</td>
</tr>
<tr>
<td>FGF 4</td>
<td>AER, distal and posterior region</td>
<td>Not detected</td>
<td>Epiblast cell defects lethal at E5.5</td>
</tr>
<tr>
<td>FGF 8</td>
<td>Throughout AER</td>
<td>Ectoderm</td>
<td>Primitive streak defect, lethal at E8.5</td>
</tr>
<tr>
<td>FGF 9</td>
<td>Throughout AER</td>
<td>Not detected</td>
<td>Normal development of limbs</td>
</tr>
<tr>
<td>FGF 10</td>
<td>Distal mesenchyme</td>
<td>Mesoderm</td>
<td>Absence of limbs and lung</td>
</tr>
<tr>
<td>FGFR 1</td>
<td>Throughout mesenchyme</td>
<td>Mesoderm</td>
<td>Distal truncation of limb buds, lethal at E9.5-12.5</td>
</tr>
<tr>
<td>FGFR 2 IIb isoform</td>
<td>Throughout ectoderm</td>
<td>Ectoderm and mesoderm</td>
<td>Lethal at E4.5-5.5</td>
</tr>
<tr>
<td>FGFR 2 Iic isoform</td>
<td>Low levels in mesenchyme</td>
<td>Ectoderm and mesoderm</td>
<td>Failure of limb bud initiation</td>
</tr>
</tbody>
</table>

Table 1. Expression domains of FGFs and FGFRs in the chick limb bud and phenotype of FGF and FGFR null mice (according to Martin 1998; Xu et al 1999).
IV. Development of the skull

The skeleton of the head in human is made up of chondrocranium (neurocranium), membrane bones and viscerocranium. The chondrocranium forms primarily from neural crest cells which emigrate from the dorsal neural tube and will give rise to the bones of the skull base. These bones preform in cartilage and ossify by the process of endochondral ossification described above. The four primary areas in the chondrocranium are the occipital, orbitotemporal, otic and ethmoidal regions. The occipital and otic region are chondral and the orbitotemporal and ethmoidal regions are prechondral, reflecting the extent to which the notochord is involved.

Membrane bones that roof the skull are flat bones. The skull consists of a pair of frontal bones, a pair of parietal bones and an interparietal bone. All of them are not preformed in cartilage but ossify directly from ectomesenchyme to give rise to the cranial vault or calvaria. These bones mineralize from several ossification centers and do not complete their growth during fetal life. At 18 weeks of gestation mineralizing bone fronts meet but do not fuse. The soft fibrous sutures that join them permit the vault to deform and skull to enlarge by appositional growth at the suture and deposition of premineralized matrix (osteoid) along the suture margins. Six sutures are present in the human skull, two coronal, one sagittal (between the parietal bones), two lambdoid (between the parietal and interparietal bones) and one metopic (between the parietal bones) Six large membrane-covered fontanelles occupy the areas between the corners of cranial vault bones at birth. The suture is anatomically a simple structure comprising the two plates of bone separated by a narrow space containing immature, rapidly dividing osteogenic stem cells; a proportion of which are recruited to differentiate into osteoblasts and make new bone.

The viscerocranium is made of five pairs of pharyngeal arches that form in craniocaudal succession on either side of the pharyngeal foregut starting on day 22 of development in human. These elements arise evolutionarily from the pharyngeal arch cartilages which develop from a mesenchymal condensation within each arch consisting of neural-crest derived ectomesenchyme.

IV.1 Signalling molecules involved in craniofacial development

The skeletal elements of the pharyngeal arches are derived from neural crest and lateral plate mesoderm. Proper development of the pharyngeal arches relies on the expression of Hox genes. Gene inactivation of Hoxa-2 result in the replacement of the second pharangeal arch by a duplicated set of proximal first pharangeal arch elements. Hence Hoxa-2 normally permits only endochondral ossification to occur in the second arch whereas both endochondral and membranous ossifications take place in the first arch that gives rise to the middle ear ossicles, squamous bone and tympanic ring. Other factors are implicated in the differentiation of the pharangeal arches including Prxl and 2 (two closely related paired class homeobox genes), homeodomain proteins of the Dlx family (Dlx 1, 2, 3, 5 and 6) and members of the Nkx family of homeobox genes. Analysis of the expression and function of Dlx genes suggests that they specify regional fate of the pharangeal arch ectomesenchyme. Mice lacking Dlx 5 showed craniofacial abnormalities with delayed ossification of the calvaria suggesting multiple roles of this gene in branchial (pharangeal) arches.

The signaling pathways acting to regulate calvarial growth and cranial suture morphogenesis are also beginning to be elucidated. They involve different transcriptions factors including MSX1 and 2 (two Msh-like homeobox genes), Shh, BMPs (BMP2 and 4), TGFβ and Twist but also tyrosine kinase receptors FGFRs and their ligands FGFs. The importance of FGF/FGFR signaling in human skull development has been revealed by the demonstration that premature fusions of the sutures that produce craniosynostoses (Apert, Crouzon and Pfeiffer syndromes being the most common) are often caused by mutations in the FGFR genes. Most mutations are found on FGFR 2 that account for most if not all cases of Apert and Crouzon syndromes. Mutations in FGFR 1 and FGFR 3 have been identified in some cases of syndromic craniosynostoses (Pfeiffer and Muenke syndromes). Most of these mutations would induce ligand independent activation of the receptor but could also alter ligand specificity. Thus FGFR appear to play a key role in the proliferation-differentiation of osteogenic stem cells in the fetal coronal sutures. FGFR2 would be expressed in proliferating cells whereas the onset of differentiation would be accompanied by up-regulation of FGFR 1. Craniosynostosis can also be caused by mutations in MSX 2. Both MSX1 and MSX2 are expressed in the sutureal mesenchyme and induced by BMP 4 and similarly to mice deficient for the FGFR 2(IIIb) isoform, Msx 1 deficiency is associated to defective mandible while disruption of the Msx 2 gene result in a central defect of the frontal bone. Thus correct dosage of Msx 2 seems to be critical for normal osteogenic differentiation in the mammalian skull. Altogether it appears that conserved signaling pathways including BMPs, Msx and FGFR/FGFRs regulate tissue interactions during suture morphogenesis and intramembranous bone formation of the calvaria. The involvement of FGFRs in both craniosynostosis and limb abnormalities (achondroplasia, thanatophoric dysplasia) support the idea that craniofacial and limb development utilize common signaling pathways which, however, remain to be clearly elucidated in human.
V. Conclusion

Great progress has been made recently in understanding limb development which appears as one of the best model of morphogenesis. Nevertheless the specific target genes regulated by Hox genes that influence growth and patterning of skeletal elements remain unknown. Important clues have been provided by the recent demonstration that secreted signaling molecules (FGFs, BMPs, Wnts, Shh) restrict their own activities by inducing antagonists in their responsive cells. Hence activation of the Shh/FGF loop could result from competing combinatorial associations of successively expressed distal Hox proteins with Meis-Pbx complexes. In the coming years, complementary approaches including surgical manipulations, ectopic expression studies as well as targeted gene disruption should provide new insights into the mechanisms of skeletal development.

References


Xu X., Weinstein M., Li C., Deng C-X. Fibroblast growth factor receptors (FGFRs) and their roles in limb development Cell Tissue Res 296: 33-43 (1999)


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