**Int. J. Dev. Biol. 49: 301-308 (2005)**
doi: 10.1387/ijdb.041953am

# Sclerotome development and morphogenesis: when experimental embryology meets genetics

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**ABSTRACT The vertebra develops from the ventral part of the somite, the sclerotome. Sclerotome progenitors are subject to multiple signaling molecules secreted by the adjacent tissues that control their fate. The aim of this article is to discuss the mechanisms of sclerotome induction, chondrogenesis and morphogenesis. By integrating the results from classical studies and recent molecular advances, this will illustrate how the powerful combination of experimental embryology and genetic approaches has recently illuminated the multiple steps of vertebra formation.**

KEY WORDS: *sclerotome, BMP, sonic hedgehog, Nkx3.2, Pax, Msx, Sox9*

## Introduction

The vertebral column is an essential element of support and motility of vertebrate body. It is also one of the most obviously segmented structures in the adult, together with its associated ribs or spinal nerves. First descriptions and analysis of the vertebral progenitors, the somites, were reported in the early chick embryo during the 17th and 18th centuries, by the famous embryologists Malpighi, von Baer and His (quoted by (Brand-Saberi and Christ, 2000). Since then, the chick embryo has remained a model of choice for the studies of somitogenesis and chondrogenesis, although other model organisms such as mouse or zebrafish have recently provided useful mutants. Hence, the chick model, which is also the main experimental system used in the Nogent Institute, will be the organism of reference for this discussion. The early steps of somite formation and the segmentation process are analyzed by O. Pourquié in this issue (Maroto *et al.,* 2005). This report focuses on early sclerotome specification, the control of its chondrogenesis and some molecular mechanisms that may be involved in its morphogenesis. We will analyze classical studies, many of which have been performed in the Nogent Institute, in the light of the more recent contributions deciphering the molecular mechanisms implicated in vertebral chondrogenesis.

## Sclerotome formation from the somite

As gastrulation proceeds, the axial mesoderm is deposited at the level of Hensen's node and forms the notochord in the midline. The relationships between the notochord, the floor plate of the neural tube and the dorsal endoderm are described and discussed by M. Catala and colleagues in this issue (Charrier *et al.,* 2005). Posteriorly along the primitive streak, the ingressing cells aggregate and form the paraxial, intermediate and lateral mesoderm (Catala *et al.*, 1996, Christ and Ordahl, 1995). Thus, the paraxial mesoderm flanks the notochord and underlies the neural plate as soon as it is formed (Figure 1A-C). During neurulation, the neural tube rolls up and closes, the paraxial mesoderm matures, segments into epithelial somites located on both sides of the neural tube (Figure 1A-B), (Christ and Ordahl, 1995, Palmeirim *et al.*, 1997, Pourquie, 2003) The somites develop according to an anterior-to-posterior gradient, the anterior-most somites being the more mature ones. Somites are staged according to their position respective to the unsegmented paraxial mesoderm, somite I being the last somite segmented (Figure 1D) (Christ and Ordahl, 1995). Fate-mapping studies have been performed to define the progeny of the different parts of the somite, at different stages of its development. Many detailed experiments were performed in the chick embryo and consisted in the heterospecific and orthotopical replacement of portion of a somite using the nucleolar quail-chick marker developed by N. Le Douarin (Le Douarin, 1969). The somite can be divided into several domains forming sequentially as its maturation proceeds. First, two main compartments are formed. The dermomyotome arises from the dorsal-lateral half and includes the progenitors of the trunk mucles and back dermis (Ordahl and Le Douarin, 1992). The sclerotome forms from the ventral part

*Abbreviations used in this paper:* E, ectoderm; EMT, epithelial-mesenchymal transition; ES, epithelial somites; N, notochord; NT, neural tube; SHH, Sonic Hedgehog; UPM, unsegmented paraxial mesoderm.

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0214-6282/2005/$25.00

Printed in Spain
**www.intjdevbiol.com**

and comprises vertebral and proximal ribs precursor cells (Christ *et al.,* 2000). Cells present in the somitocoele participate to the sclerotome (Huang *et al.,* 1994). It should also be mentioned that thoracic dermomyotomal cells contribute to the formation of the scapula (Huang *et al.,* 2000a). Each compartment is further divided into more specific areas. The sclerotome can be partitioned into cranial and caudal halves separated by the von Ebner's fissure (Christ and Wilting, 1992); the two halves express different molecular markers and giving rise to different elements. The anterior half is invaded by neural crest cells and motor axons (Bronner-Fraser, 2000). The medial quadrant of the anterior somitic half forms the vertebral body whereas the medial and posterior quadrant gives rise to the intervertebral disc; the posterior and lateral quadrant forms the neural arches, the pedicles of the vertebra and the ribs (Christ and Wilting, 1992). Controversy about the extent of somite contribution to rib formation is discussed in a few articles (Huang *et al.*, 2000b, Kato and Aoyama, 1998). Somitocoele cells participate in intervertebral disc and rib formation (Huang *et al.*, 1996, Huang *et al.*, 1994).

Progress in our understanding of sclerotome and vertebral development has depended upon the availability of lineage-specific markers: the pioneer studies have focused on cell morphology, cartilaginous matrix formation and chondrocytes differentiation whereas refined and step-wise analysis of early somite and sclerotome formation was allowed by the more recent discovery of precocious stage-specific molecular markers. While the entire paraxial mesoderm expresses *Paraxis, Pax3* and *Pax7* genes before segmentation, these genes are restricted to the dorsal part of the somite later on (Barnes *et al.*, 1997, Goulding *et al.*, 1993). Cells from the ventral somite and somitocoele start expressing *Pax1* as soon as somite stage III (Ebensperger *et al.,* 1995). Later on, during sclerotome formation, genes such as *Pax9* and *Msx1/2* are activated in sub-domains of the sclerotome, prior to cartilage differentiation (Muller *et al.*, 1996, Takahashi *et al.*, 1992). The onset of these genes will be detailed below (Figure 1D).

## Somite survival depends upon the neural tube and the notochord

The entire somite, irrespective of its prospective sclerotomal or dermomyotomal fate, depends upon the activity of the neural tube and notochord for its survival. Although this aspect has been understated in the first studies on cartilage formation, early analyses already mentioned that somites grafted on the chorioallantoid membrane (CAM) of a chick host do not survive well alone, but they are consistently recovered when they are cultured in the presence of a notochord (Murray and Selby, 1933, quoted by (Dockter, 2000)). Similarly, removal of both notochord and neural tube *in vivo* in early stage embryos results in the absence of both cartilage and vertebral muscles (Strudel, 1955).

This role of the axial organs, the notochord and the neural tube, was examined into more details with molecular markers. First, the early ablation of both the notochord and the neural tube does not prevent paraxial mesoderm segmentation but sclerotome derivatives (expressing *Pax-1* ) as well as epaxial muscles (expressing *Pax3*, the early muscle marker *13F4* or *MyoD*) undergo apoptosis, whereas the hypaxial muscles develop normally (Hirano *et al.*, 1995, Rong *et al.*, 1992, Teillet *et al.*, 1998, Teillet and Le Douarin, 1983). This effect is observed as soon as 6 hours post-surgery and was followed by the death of the neural crest cells when they had already migrated into the somite (Teillet and Le Douarin, 1983). Moreover, reimplantation of either the neural tube or the notochord is sufficient to rescue the effect of the ablation. Interestingly, a similar rescuing effect was observed with a fragment of cartilage but not with several other embryonic tissues. The duration of the contact between the paraxial mesoderm and the axial organs required to sustain somite survival is of about 10 hours post-segmentation. Finally, the secreted molecule Sonic Hedgehog (SHH) has been described as a candidate for mediating several aspects of the activity of the axial organs (Chiang *et al.*, 1996, Johnson *et al.*, 1994, Marti *et al.*, 1995). *Sonic Hedgehog* is expressed in the notochord and the floor plate of the neural tube shortly after gastrulation (see also Charrier *et al.,* 2005 in this issue). As far as survival of the somite is considered, implanting cells that secrete Sonic Hedgehog, following axial organ ablation, rescues the apoptosis, both in sclerotome and myotome progenitors (Teillet *et al.,* 1998). Sonic Hedgehog mutant mice present defective cartilage and epaxial muscle development (Chiang *et al.,* 1996). These mutants also exhibit elevated early apoptosis in the ventral somite, although SHH does not seem to be required for dorsal somite survival in the mouse (Borycki *et al.,* 1999). This anti-apoptotic activity of SHH could act by antagonizing the NGF-p75 pathway and complements SHH mitogenic effect (Cotrina *et al.*, 2000, Marcelle *et al.*, 1999).

## The timing of sclerotome determination

Whereas the global anterior-posterior patterning of the paraxial mesoderm is determined before segmentation along the body axis, the two last somites formed by segmentation from the unsegmented paraxial mesoderm are not determined along their medial-lateral and dorsal-ventral (DV) axis (somites I-II) (Aoyama and Asamoto, 1988, Burke, 2000, Kieny *et al.*, 1972). Rotation of the epithelial somites I-II along the DV axis results in normal dermomyotome and sclerotome positioning (Aoyama and Asamoto, 1988, Ordahl and Le Douarin, 1992). In contrast, after dorsal-to-ventral rotation, somite III gave rise to mesenchymal cells located between the ectoderm and the dermomyotome: this was interpreted as the formation of sclerotome dorsally (Aoyama and Asamoto, 1988). This interpretation was recently challenged by the observation that these cells do not express the sclerotome marker *Pax1* at E3, suggesting that they might not maintain their sclerotome fate (Dockter and Ordahl, 2000). However, during normal sclerotome development, as soon as E3, some sclerotome cells migrate dorsally around the neural tube, stop expressing *Pax1* and *express Msx 1/2* genes and form dorsal vertebral cartilage (Monsoro-Burq and Le Douarin, 2000). The *Foxc2/MFH1* and *Zic* genes are also expressed in a larger sclerotome domain than *Pax1* (Aruga *et al.*, 1999, Furumoto *et al.*, 1999). By a more extensive marker analysis, it would be interesting to know whether the ventral part of somite III, when placed under the ectoderm as in Dockter and Ordhal (Dockter and Ordahl, 2000), became a "dorsal" sclerotome type or was diverted towards another somite fate (dermis for example) (Houzelstein *et al.*, 2000, Scaal *et al.*, 2001). Alternatively, if the grafted sclerotome was determined and could not be diverted towards another fate, it may not follow further differentiation if secondary signals required

to complete cartilage differentiation are not found in the novel environment (see below). Alternatively, negative signals from the ectoderm could prevent chondrogenesis in this dorsal location (Kenny-Mobbs and Thorogood, 1987). In both cases, the cells would then remain as an undifferentiated mesenchyme as observed in (Dockter and Ordahl, 2000).

When complete cartilage differentiation is used as a diagnostic of sclerotome determination, differences arise between *in vitro* and *in vivo* studies. The *in vitro* culture of somites explanted at different stages of their maturation evidences their ability to give rise to cartilage autonomously only after stage XXVII; somites I-XIV do not form cartilage *in vitro*, even if they are cultivated in the presence of embryonic extract (Strudel, 1962, Strudel, 1963). Grafts of explants on the CAM of an older host, which allow better long-term survival of the explants, give similar results (Watterson *et al.,* 1954). Recently, the timing of determination of the sclerotome has been refined by performing ectopic implantation of quail sclerotome cells under the ectoderm of a chick host and assessing their ability to form cartilage *in vivo*: in this dorsal environment where undetermined somite cells are expected to give rise to dermis and muscle, cartilage formation from the grafted cells is observed if the sclerotome comes from stage XII or older somites (Dockter and Ordahl, 1998). Thus, the complete process of cartilage determination is engaged around 18 hours post-segmentation, at a time when *Sox9* is already expressed (see below, (Zeng *et al.,* 2002).

## Sclerotome patterning by the surrounding tissues

After segmentation, sclerotome induction in somites I-II depends on positive and negative signals from released by the axial organs medially, the somatopleura laterally and the ectoderm dorsally. When the notochord, the neural tube, or soluble extract prepared from notochord and neural tube, were added to the culture of stage I-XIV somites, they consistently formed cartilage and muscle (Strudel, 1962). It is worth to mention that these cultures were supplemented with serum and embryonic extract. Ectopic grafts of notochord or floor plate dorsal to the epithelial somites result into the recruitment of dorsal somite cells into vertebral cartilage *in vivo* (Brand-Saberi *et al.*, 1993, Pourquie *et al.*, 1993). This activity parallels the ventralizing activity of these tissues during neural tube DV patterning (Hirano *et al.*, 1995, Pourquie *et al.*, 1993, van Straaten and Hekking, 1991). Conversely, the ectoderm inhibits chondrogenesis and promotes dermomyotome formation *in vitro* as well as *in vivo* (Kenny-Mobbs and Thorogood, 1987, Marcelle *et al.*, 1999) whereas the lateral plate mesenchyme controls formation of lateral somite types (Pourquie *et al.*, 1996, Tonegawa *et al.*, 1997). The first molecular markers appearing when the prospective sclerotome is induced are *Pax1, Nkx3.1* and *Nkx3.2/Bapx1. Pax1* appears in the ventral epithelium and somitocoele of somite III, while *Pax3* expression becomes restricted to the dorsal part of the somite (Ebensperger *et al.,* 1995). *Pax9* appears slightly later on, in the sclerotome of stage IV-V somites and displays redundant activity with Pax1 (Muller *et al.,* 1996). In the Pax1/Pax9 double mutant mice, the development of the ventral part of the vertebra –but not of the neural arches- is strongly impaired (Peters *et al.,* 1999). *Nkx3.2/Bapx1* is first detected at similar stages as *Pax1*. Its expression does not appear in the Pax1/Pax9 double mutants and can be activated ectopically by Pax1 over-expression *in vivo* (Rodrigo *et al.*, 2003, Tribioli and Lufkin, 1997). *Pax1* is expressed normally in the Nkx3.2/Bapx1 mutant, but later steps of vertebral differentiation are severely defective (Tribioli and Lufkin, 1999). *Nkx3.1* appears in the newly formed somites but the mutant mice

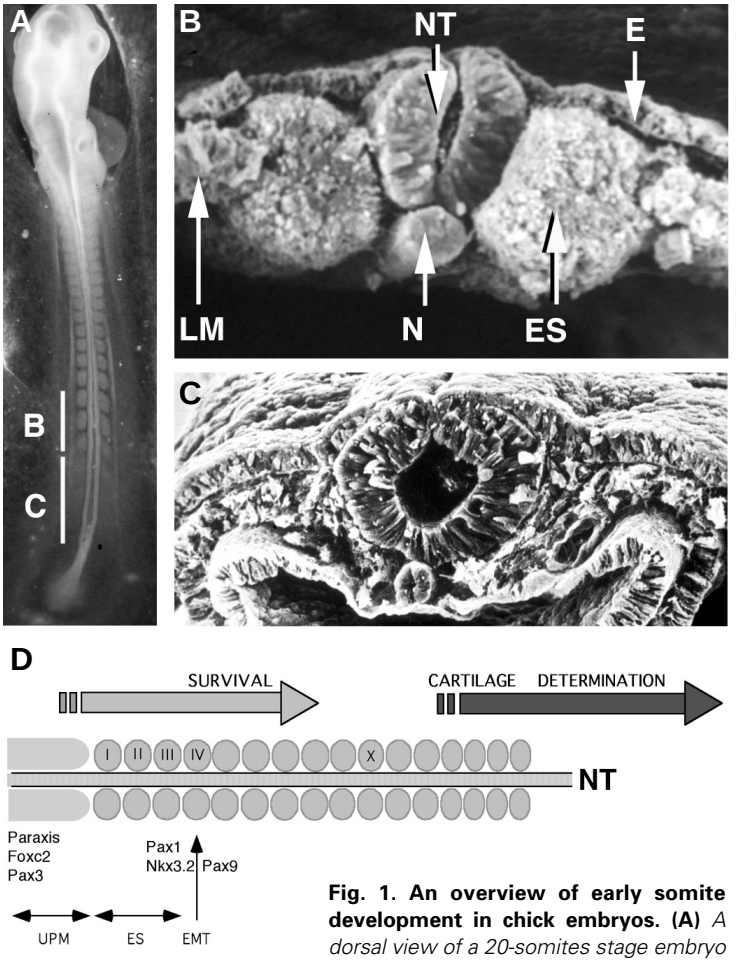


**Fig. 1. An overview of early somite development in chick embryos. (A)** *A dorsal view of a 20-somites stage embryo shows the positioning of the somites along the antero-posterior axis of the embyo. Posteriorly lies the unsegmented paraxial mesoderm (UPM). In front of the UPM, the somites segment as epithelial somites (ES, somites I, II, III). More anteriorly, the somites mature and differentiate.* **(B)** *A transverse view by scanning electron microscopy evidences the vicinity of the epithelial somites (ES) to the neural tube (NT), the notochord (N), the ectoderm (E) and the lateral and intermediate mesoderm (LM).* **(C)** *View of the unsegmented paraxial mesoderm (UPM) on transverse section by scanning electron microscopy (SEM images are from the Nogent Institute collection).* **(D)** *A few landmarks in sclerotome development. On top of the scheme are indicated the periods when the axial organs are critical for somite survival (Survival) and when the cartilage is determined (Cartilage Determination). Below are indicated some genes expressed in the UMP (Paraxis, Pax3 and Foxc2) and the time of onset the first sclerotome markers, in the epithelial somites (Pax1 is activated in somite III and Nkx3.2 shortly afterwards) and after the epithelial-to-mesenchymal transition (EMT, Pax9). Progessively, the various scletotome markers (e.g. Gli2, Gli3, Zic1, Sox9 etc) are activated but the exact somite stage of their onset in chick embryos has not been recorded yet.*

do not exhibit skeletal defects (Kos *et al.*, 1998, Schneider *et al.*, 2000). Together, these data suggest that Pax1 (supported by Pax9 later on) act upstream of Nkx3.2 at the initiation of sclerotome development. Moreover, Pax1 is able to activate the expression of early chondroblast markers (aggrecan) in chick presomitic mesoderm cultivated *in vitro* (Rodrigo *et al.,* 2003). This suggests that the activation of *Pax1* is the key event that triggers sclerotome formation during development.

The notochord and the ventral part of the neural tube -but not the roof plate- activate *Pax1* both *in vivo* and *in vitro* (Brand-Saberi *et al.*, 1993, Ebensperger *et al.*, 1995, Koseki *et al.*, 1993, Muller *et al.*, 1996). This activity is more potent in the caudal part of the notochord than in more rostral parts (Muller *et al.,* 1996). This observation may also infer that some notochord signals could be required only transiently during sclerotome development. The signaling molecules secreted by the axial organs that pattern the somite also regulate the onset of *Pax1* and *Nkx3.1* expression (Brand-Saberi *et al.*, 1993, Schneider *et al.*, 2000). Noggin and Sonic Hedgehog are two notochord-derived secreted factors critical for *Pax1* initiation and early maintenance. Noggin is expressed in the notochord as early as Hamburger and Hamilton stage 4-5 in chick (Streit and Stern, 1999). In Noggin mutants, *Pax1* onset is delayed, whereas Noggin can induce low levels of *Pax1* in presomitic mesoderm *in vitro* (McMahon *et al.,* 1998). This suggests that antagonizing BMP activity is critical to initiate sclerotome development. However, alternative factors can partially compensate, after some time, for the lack of Noggin activity *in vivo* since in the Noggin mutant mice, *Pax1* onset eventually occurs and only a few vertebrae are missing (McMahon *et al.,* 1998).

In addition to its activity on promoting survival of the entire somite, SHH further sustains sclerotome development (Borycki *et al.*, 1999, Fan and Tessier-Lavigne, 1994, Johnson *et al.*, 1994, Teillet *et al.*, 1998). The fact that mice lacking SHH display a normal but transient expression of *Pax1* initially, suggests that SHH is involved in the maintenance of the induction *in vivo* (Chiang *et al.*, 1996, Marcelle *et al.*, 1999). *In vitro*, *Pax1* is initiated in the presomitic mesoderm early on, independently of SHH signals, but is not maintained long (Murtaugh *et al.,* 1999). Addition of SHH sustains and enhances *Pax1* and *Nkx3.1* expression in unsegmented paraxial mesoderm maintained in culture (Fan *et al.*, 1995, Fan and Tessier-Lavigne, 1994, Kos *et al.*, 1998, Munsterberg and Lassar, 1995). The effect of SHH requires the activity of Gli2 and Gli3 genes (Buttitta *et al.,* 2003). Shortly before *Pax1* activation by SHH, members of the SHH pathway are activated in the presumptive sclerotome (Borycki *et al.,* 1999). Noggin and SHH synergize in sclerotome induction and apparently do so by using parallel signaling pathways (McMahon *et al.,* 1998).

The activity of SHH is blocked in the presence of two classes of soluble molecules secreted by the somite environment. Firstly, as suggested by the importance of Noggin activity, BMP4, expressed in the lateral plate and the roof plate of the neural tube, antagonizes SHH ventralizing activity both in the neural tube and in the somite, *in vitro* and *in vivo* (Liem *et al.*, 2000, Liem *et al.*, 1995, Monsoro-Burq *et al.*, 1996). The epithelial somites subjected to ectopic BMP2/4 signals are transformed into lateral somite expressing *Sim-1* or lateral mesoderm (Pourquie *et al.*, 1996, Tonegawa *et al.*, 1997). As a result, the vertebral bodies do not develop at the level of the graft (Monsoro-Burq *et al.,* 1996). This supports the model of the requirement for an initial BMP antagonism provided by Noggin, to prevent BMPs secreted from the lateral plate to bind to their cellular receptors in the somites and to allow SHH patterning activity (McMahon *et al.,* 1998). Secondly, WNT molecules produced by the ectoderm also prevent *Pax1* activation (Capdevila *et al.,* 1998).

## The sclerotome is further subdivided into several domains

Shortly after being induced in the ventral part of the epithelial domain, sclerotome cells undergo an epithelial-to-mesenchymal transition (EMT) from the ventral part of the epithelial somite and migrate around the neural tube, the notochord or more laterally to form the proximal part of the ribs. The EMT is not required for initial sclerotome patterning: *Pax1* appears before EMT, *Pax1* and *Pax9* are activated in mice with epithelialization/segmentation defects (Paraxis and Delta-1 mutants for example)(Barnes *et al.*, 1997, Burgess *et al.*, 1996, Hrabe de Angelis *et al.*, 1997). Conversely, the EMT occurs normally in Pax1/9 or Nkx3.2 mutants (Peters *et al.*, 1999, Tribioli and Lufkin, 1999). Axial organ ablation experiments show that the EMT does not depend on their influence (Teillet and Le Douarin, 1983).

During this second step of its development, several distinct compartments can be defined in the sclerotome population, according to the distinct sets of genes they express. *Pax1* expression becomes stronger in the ventral-medial part of the sclerotome (that will form the vertebral body and the intervertebral disk) whereas *Pax9* is reinforced in the posterior ventral-lateral compartment (progenitors of the neural arch and the proximal part of the rib) (Neubuser *et al.,* 1995). *Pax1* is not expressed by the sclerotome cells migrating dorsally to the neural tube (Monsoro-Burq and Le Douarin, 2000). The *Gli2* gene is strongly expressed around the notochord, whereas *Gli3* is not present around the notochord but is expressed in a broader lateral and dorsal sclerotome domain (Mo *et al.,* 1997). The *Foxc2/MFH1* and *Zic1* genes are expressed in the entire sclerotome (Aruga *et al.*, 1999, Furumoto *et al.*, 1999). The cells that migrate between the ectoderm and the roof plate begin to express *Msx1* and *Msx2* as soon as Hamburger and Hamilton stage 20 (Monsoro-Burq *et al.*, 1994, Monsoro-Burq and Le Douarin, 2000, Takahashi *et al.*, 1992). Thus different parts of the sclerotome are likely to follow both common and specific genetic pathways, related to their common cartilaginous fate and specific morphogenesis processes.

We have studied the fate of the cells migrating dorsally to the neural tube. These cells are forming the dorsal part of the neural arches and the spinous process of the vertebra (Monsoro-Burq *et al.*, 1994, Monsoro-Burq and Le Douarin, 2000, Takahashi *et al.*, 1992). Thus, this population forms cartilage in close vicinity to the ectoderm, despite the inhibitory activity of the ectoderm on chondrogenesis (Capdevila *et al.*, 1998, Kenny-Mobbs and Thorogood, 1987, Marcelle *et al.*, 1999). In order to identify the mechanisms that promote cartilage formation under the ectoderm in this very specific location, a series of studies were carried out to test the activities of the roof plate or the notochord on development of this part of the vertebra. According to its expression pattern in mice embryos, *Foxc2* seems also expressed by those cells (Furumoto *et al.,* 1999). Thus, we can hypothesize that chondrogenic and dermis precursors expressing *Msx* genes

could be discriminated by their expression of *Foxc2* or *Dermo-1* respectively (Houzelstein *et al.,* 2000).

The induction of *Msx1/2* genes in the dorsal sclerotome population is mediated by BMP4 signals emanating from the ectoderm and the roof plate of the neural tube; it is blocked when noggin-secreting cells are grafted dorsal to the roof plate (Monsoro-Burq *et al.,* 1996) and unpublished data). WNT signals, also present in the roof plate, do not participate in this induction (Takahashi *et al.,* 1996). Moreover, the ectopic grafting of a roof plate or of BMP-secreting cells under the lateral ectoderm, above the prospective dermomyotome, induces *Msx* gene activation followed by sub-cutaneous cartilage formation (Takahashi *et al.,* 1992). This effect is not observed after the graft of a floor plate. This effect is very potent if BMP-secreting cells are implanted dorsally at E3, at the time of migration of the sclerotome cells dorsally to the roof plate (Monsoro-Burq *et al.,* 1996). However, the activation of *Msx* genes is not sufficient to sustain full skeletal formation in the dorsal location, since after notochord ablation, a few *Msx2*-positive cells are observed dorsally, but do not develop further into cartilage (Monsoro-Burq *et al.,* 1994). These observations suggested a two-step mechanism, requiring the activity of the notochord and the floor plate early on (E2), followed by the requirement for a roof-plate-mediated BMP activity at E3 (Monsoro-Burq *et al.,* 1996). Moreover, the notochord or SHH-secreting cells could not substitute for the roof plate activity: dorsal implantation of either one resulted in the loss of BMP4 expression in the roof plate, the loss of *Msx* expression in both the neural tube and the dorsal sclerotome and the lack of cartilage differentiation around the graft (Monsoro-Burq *et al.*, 1994, Watanabe *et al.*, 1998). This kind of grafts prevented the normal DV patterning of the neural tube and eventually resulted in the degeneration of the dorsal half of the spinal cord (Monsoro-Burq *et al.,* 1995). These observations were confirmed in mice embryos when SHH was expressed under the control of the *Wnt1* promotor in the roof plate (Rowitch *et al.,* 1999). This suggests that the defect observed in dorsal vertebral formation, after the dorsal graft of a notochord, is secondary to the lack of BMP expression from the roof plate. Finally, in the various studies analyzing the implantation of the notochord dorsally to the somite, the expansion of the vertebral cartilage is always located ventral to the graft: ectopic cartilage does not form between the ectoderm and the ectopic notochord (Pourquie *et al.,* 1993). From these data, we have hypothesized that cartilage formation from committed sclerotome cells can occur close to the ectoderm, provided that BMP signals relieve the inhibitory effect from the ectoderm during a second step of sclerotome development.

## Sclerotome differentiation into cartilage depends upon additional signals

Recently, a series of *in vitro* studies have provided new insights into the step-wise process of cartilage differentiation from the sclerotome. They have established the requirement for a two-step process involving SHH activity early on, which induces the competence of the sclerotome to respond to subsequent BMP signals.

When presomitic mesoderm is cultivated in a defined medium, the addition of SHH or of a combination of SHH and Noggin induces early sclerotome markers but is not sufficient to promote cartilage differentiation (Fan C.-M unpublished, quoted in (Dockter, 2000). However, in a semi-defined medium containing chick embryonic extract (2%), low levels of cartilage formation from presomitic mesoderm is observed when SHH is added; this effect is enhanced in the presence of serum (Murtaugh *et al.,* 1999). These results indicate that SHH can cooperate with yet unidentified factors, present in the chick extract and the serum, to promote chondrocyte differentiation. In particular, without serum, SHH induces *Pax1* and *aggrecan* expression. This is in agreement with the ability of Pax1 to induce *aggrecan* in similar conditions (Rodrigo *et al.,* 2003). However, the addition of serum is needed to achieve more robust expression of *aggrecan* and induction of the later differentiation marker *collagen IX* (Murtaugh *et al.,* 1999).

Importantly, presomitic mesoderm exhibits BMP4 and BMPRs expression after 3-5 days *in vitro* (Murtaugh *et al.,* 1999). BMP4 expression is enhanced and BMP7 is strongly activated after culture during 3-5 days in the presence of SHH. When BMP activity is blocked by Noggin *in vitro* and *in vivo*, no cartilage differentiation occurs even if *Pax1* expression remains unaffected. Finally, this study demonstrates that the exposure of the early presomitic mesoderm to SHH changes its response to BMP signals. As mentioned above, early paraxial mesoderm is lateralized by BMPs, whereas after a 2-day exposure to SHH, paraxial mesoderm explants will chondrify under similar BMP treatment (Murtaugh *et al.,* 1999). This fits with the *in vivo* analysis showing that BMP overexpression would transform the somitic mesoderm into lateral mesoderm at E2 but promote cartilage formation when BMP signals are applied to a region populated by the sclerotome later on, such as in the dorsal sclerotome above the roof plate (Monsoro-Burq *et al.*, 1996, Monsoro-Burq and Le Douarin, 2000, Tonegawa *et al.*, 1997).

However, these results do not resolve the fact that cartilage does not differentiate between the ectoderm and an ectopic notochord or a floor plate, whereas it does so between the roof plate and the ectoderm (Takahashi *et al.,* 1992). In this position, the notochord is not surrounded by a thick extracellular matrix and exhibits an longer secreting activity than when located in more ventral positions (Monsoro-Burq *et al.,* 1998). It may then display an altered signaling activity compared to the endogenous notochord. Alternatively, as hypothesized before, the roof plate or high BMP activity could specifically be required to release ectodermal inhibition on cartilage differentiation locally, in addition to the general SHH-BMP mediated control of sclerotome differentiation.

Lassar and his colleagues have further looked for the genes, induced by SHH and allowing competence to respond to BMP signals by chrondrogenesis. They show that *Nkx3.2/Bapx1* is induced by SHH and maintained by BMP4 and able to confer a chondrogenic response to BMP in the absence of SHH treatment (Murtaugh *et al.,* 2001). Moreover, they show that *Sox9* is activated by SHH and BMPs in a similar sequential manner, that Nkx3.2 activates *Sox9* and that Sox9 supports autonomous chondrogenesis in the explants (Murtaugh *et al.*, 2001, Zeng *et al.*, 2002). These *in vitro* analysis are supported by the phenotype of Nkx3.2/Bapx1 mutants: in these mice, the sclerotome forms but does not differentiate and *Sox9* expression is reduced (Tribioli and Lufkin, 1999). However, *Pax1* is expressed at normal levels in those mutants, in agreement with the model of normal sclerotome initiation followed by defective differentiation.

Finally, the phenotype of various double mutant mice show that multiple activities cooperate to promote sclerotome differentiation and the morphogenesis of different parts of the vertebra, although some genes are not required individually for sclerotome development, nor are they sufficient to elicit chondrogenesis *in vitro*. A few significant examples are detailed below.

In the Pax1/Pax9 double mutants, the derivatives of the medial part of the sclerotome are missing: the vertebral bodies, the intervertebral discs and proximal parts of the ribs are absent, while the Pax1 homozygotes exhibit more restricted defects of the vertebral bodies and intervertebral discs and Pax9 mutant is not affected in its axial skeleton (Peters *et al.,* 1999). Interestingly, the neural arches of Pax1/Pax9 double mutant mice do form and are connected by ectopic elements of cartilage (Peters *et al.,* 1999). Pax1 and Pax9 are thus certainly critical for the formation of skeleton from the medial part of the sclerotome, via *Bapx1* activation, but they do not account for the formation of the entire vertebra (Rodrigo *et al.,* 2003). In contrast, the winged helix transcription factor Foxc2/MFH1 seems critical for the formation of the whole vertebra (Furumoto *et al.,* 1999). *Foxc2* is expressed in the entire sclerotome, including the cells migrating dorsally to the roof plate. Its expression depends on SHH activity and Foxc2 mutants exhibit defective vertebral bodies and neural arches. Double mutants for Foxc2 and Pax1 present an even more severe phenotype, including largely open neural arches and absence of vertebral bodies and intervertebral discs (Furumoto *et al.,* 1999). This phenotype is due to the reduction of proliferation and impaired development of the sclerotome early on (Furumoto *et al.,* 1999).

In contrast to the Pax1/Pax9 mutants, Zic1 mutant mice exhibit skeletal defects primarily in the neural arches (Aruga *et al.,* 1999). The expression of *Pax1* and early sclerotome development does not seem to be significantly perturbed and the other parts of the vertebra are normal. This phenotype resembles the Gli3 phenotype (Mo *et al.,* 1997). Interestingly, *Zic1* is expressed by the dorsal sclerotome cells, some of which do not express *Pax1,* while *Gli3* extends largely over ventral and dorsal sclerotome (Aruga *et al.,* 1999). Zic1/Gli3 double mutants exhibit a more severe phenotype, with fusions of the neural arches laterally while the spinous processes do not form, as observed after the over-expression of SHH lateral to the somite, *in vivo* (Watanabe *et al.,* 1998).

Finally, consistent with the idea that the morphogenesis of the dorsal part of the vertebra could be controlled specifically by signals emanating from the roof plate, some mutants with defective roof plate lack dorsal vertebra at the levels with altered dorsal neural tube (Manzanares *et al.,* 2000).

Together, these complex phenotypes indicate that precise vertebral morphogenesis relies in the integration of these diverse genetic activities in the embryo. The combination of *in vitro* studies, experimental manipulation in the chick embryo and mouse mutants opens the path to uncover these mechanisms.

Progress in identifying the essential regulators of cartilage differentiation opens novel perspectives for orthopedic gene therapy. Bone, cartilage or tendon could potentially be induced in stem cells subjected to the appropriate doses and timing of inducing molecules (reviewed in (Gafni *et al.,* 2004). Adult mesenchymal stem cells seem suitable for such approaches. Most current approaches focus on the activity of BMPs, in particular BMP2, which have now a long history as bone inducers (Baltzer and Lieberman, 2004, Yoon and Boden, 2004). Because Sox9 elicits autonomous cartilage formation in embryonic tissues not fated for chondrogenesis, this gene is likely to focus much attention in the future (Trippel *et al.*, 2004, Zeng *et al.*, 2002). The knowledge of the steps of embryonic chondrogenesis will provide critical informations for reproducing this sequence in the adult.

*Acknowledgements*

*I want to express my gratitude to Pr. Nicole Le Douarin who has been an exceptional mentor from the earliest steps of my researcher life. I want to thank all the people in the Nogent Institute and in particular Dr. Y. Takahashi, who introduced me to this field of embryology. I am very grateful to Dr. M. Catala for his critical reading of this manuscript.*

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