1 INTRODUCTION

1.1 Origin of tetrapod limbs

Tetrapods (four-footed, from Greek: *tetra* four + *pod-, pous* foot), i.e. vertebrates with two pairs of limbs, have a very long history, starting in the Devonian period, meaning at least 360 million years ago. At that time, the transition between fishes and tetrapods occurred, often referred to as the fin-to-limb transition.

It is believed that limbs evolved to facilitate exploiting a shallow-water environment and they were originally used horizontally as props or/and paddles (Lebedev 1997). Their proximal parts share homology with fish fins, however more distal limb structures, including digits, are present in tetrapods only. The first known digited tetrapods living in the late Devonian, *Ichthyostega*, *Acanthostega* and *Tulerpeton*, were polydactylous and probably aquatic (Coates and Clack 1990; Lebedev 1997; Clack 2002). Spreading over the land, achieved during tetrapod evolution, was associated with profound limb changes including reduction in the digit number (to maximal 5) and increase in the number of carpus and tarsus bones. This process was followed by other morphological, anatomical and physiological changes allowing adaptation to the terrestrial lifestyle.

1.2 Limb development in embryogenesis

1.2.1 Anatomical view

Limb development follows the same sequence of events in all higher vertebrates. Limb buds are derived from specific regions of the so-called lateral plate mesoderm (LPM), which comprises two stripes of tissue that run along the length of the main body axis. Positioning of the limb field (group of cells which give rise to the limb bud) is dependent on complex signalling and can be initiated only if the prospective limb-forming region responds properly to the signals from more medial tissues. After the limb field has been specified, the limb bud induction and its outgrowth along three major axes, proximodistal (PD), anteroposterior (AP) and the dorsoventral (DV) starts. In respect to the limb structure, proximal-to-distal patterning refers to the formation of the stylopod (upper arm or thigh), through the zeugopod (forearm or shank) up to the autopod (wrist and hands or ankle and feet), respectively. The

anterior part of the limb is defined by the location of the digit number 1 (thumb), whereas the posterior part corresponds to the location of the digit number 5 (little finger) in pentadactylous tetrapods. Due to thorough studies in the past decades, many factors and pathways responsible for limb patterning processes along each of the axes have been determined. Most studies were done in chicken or mouse models. Especially, transplantation experiments, injection of signalling molecules into animal embryos, transgene introduction or knockout technology were useful in these complex analyses.

1.2.2 Molecular basis of limb patterning

1.2.2.1 Establishment of the proximodistal axis in the limb

Limb outgrowth along the PD axis is dependent on the apical ectodermal ridge (AER), a layer of tissue that covers the rim of the distal tip of the limb bud. It has been observed that the AER function is mediated by different members of the fibroblast growth factor (FGF) superfamily (Niswander and Martin 1992; Niswander et al. 1993) and that FGF signalling seems to be essential for proper limb patterning both at the early as well as at the late stage of the limb bud outgrowth (Summerbell 1974; Rowe and Fallon 1982; Saunders 1998; Lewandoski et al. 2000).

AER initiation is a complex event, depending on synthesis and transport of many different molecules. At the beginning, a very important role is ascribed to FGF and Wnt proteins which are synthesised in the mesoderm underlying the prospective limb bud and which activate target genes (for instance other *FGF*s) in the AER (Capdevila and Izpisua Belmonte 2001; Tickle and Munsterberg 2001; Barrow et al. 2003). Later, the limb bud outgrowth is regulated by FGF or retinoc acid (RA) signalling, depending on the distance to the AER. The distal part of the bud located close to the AER is under the influence of different fibroblast growth factors, which are capable to repress retinoic acid production. Lack of RA, together with the expression of “distal” bone morphogenetic proteins (BMPs) and homeobox (Hox) proteins, blocks activation of the proximising genes *Meis1* and *Meis2* in the distal limb bud. The proximal part of the future limb is further away from the AER, therefore it lacks FGF molecules. Instead, RA signalling is activated and can induce expression of *Meis1* and *Meis2* (Capdevila et al. 1999; Mercader et al. 1999; Mercader et al. 2000; Capdevila and Izpisua Belmonte 2001).

The maintenance of the AER occurs via two positive feedback loops (Laufer et al. 1994; Niswander et al. 1994; Zuniga et al. 1999; Capdevila and Izpisua Belmonte 2001; Panman and Zeller 2003). The first one is established between Fgf10, expressed in the limb bud mesoderm, and Fgf8 from the AER. In the second loop, FGF from the AER activates *Sonic hedgehog* (*Shh*) expression in the posterior distal mesenchyme. Shh, acting via *Formin* and *Gremlin* genes, switches off BMP signalling, which in turn enables FGF activation in the AER.

Up to now two models explaining the mechanism of proximal-to-distal patterning in the developing limb bud have been proposed. The older one, called progress zone model, assumes that an internal clock controlled by the AER determines the fate of the cells lying underneath, in the so-called progress zone. While proliferation proceeds, older cells leave the progress zone and escape from the influence of the AER. Cells, which left the progress zone earlier, give rise to the more proximal parts of the limb, whereas cells, which stayed longer under the AER control, contribute to the distal parts of the limb (Summerbell et al. 1973). Recently, the progress zone model has been questioned and a new hypothesis has been proposed (Dudley et al. 2002; Sun et al. 2002). According to it, cells are "labelled" as proximal or distal very early, and the limb development corresponds to the outgrowth of the pre-specified domains.

1.2.2.2 Establishment of the AP axis

Establishment of the anterioposterior axis in the limb bud is tightly connected with the AER. Fgf4 and other FGFs expressed in the AER are able to activate *Shh* in the posterior part of the limb bud, called zone of polarising activity (ZPA). However, the induction of *Shh* is only possible in the presence of posteriorising factors like RA and Hox proteins (Johnson et al. 1994; Niswander et al. 1994; Knezevic et al. 1997; Mackem and Knezevic 1999; Catala 2000; Capdevila and Izpisua Belmonte 2001; Panman and Zeller 2003). AP asymmetry in the limb bud is subsequently established by the negative feedback loop between Shh and the repressor form of Gli3 (Gli3R). The interplay between these two proteins results in formation of the Shh gradient along the AP axis. Lack of Shh signalling in the anterior part of the limb bud results in expression of specific genes, which give anterior identity to the mesenchyme. Posterior mesenchyme is specified by the positive feedback loop between 5' *Hox* genes, *Shh* and *dHand* (te Welscher et al. 2002a; te Welscher et al. 2002b; Panman and Zeller 2003; Zakany et al. 2004). A schematic representation of these pathways can be seen in Fig. 1.

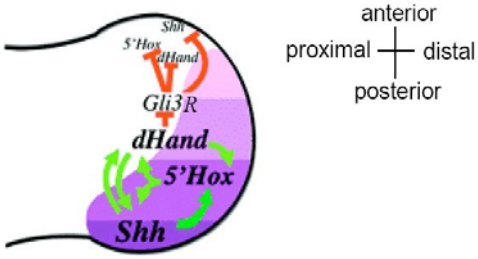

Fig. 1 Establishment of the anterioposterior (AP) asymmetry in the limb bud. Only the most important players are shown. 5' *Hox* genes are expressed in the posterior part of the limb bud and activate Sonic hedgehog (Shh) and the transcription factor dHand, which subsequently activate each other. All these proteins inhibit accumulation of transcriptional repressor Gli3R in the posterior part of the limb bud. Therefore, Gli3R is present only in the anterior structures, where it suppresses transcription of 5' *Hox, dHAND* and *Shh.* The resulting Shh gradient (purple zones) along the AP axis drives expression of different genes in the anterior and the posterior parts of the limb bud. Adapted from Zakany *et al.* 2004.

1.2.2.3 Limb patterning along the dorsoventral axis

Establishment of the DV axis of the limb bud is strictly dependent on the formation of the DV boundary at the mid-point of the AER. Wnt/β-catenin signalling from the ectodermal ridge activates BMPs in the ventral ectoderm, which subsequently induce expression of the *Engrailed 1* gene (*En-1*), coding for a homeobox-containing transcription factor. Presence of En-1 proteins specifies ventral ectoderm and blocks expression of *Wnt7a*, which is active only in the En-1-free dorsal cells of the distal limb bud. Wnt7a signalling from the dorsal ectoderm induces expression of the LIM-homeodomain factor Lmx1b in the same tissue. Thus, both Wnt7a and Lmx1b are responsible for the establishment of the dorsal pattern (Capdevila and Izpisua Belmonte 2001). In addition, it is known that *Radical fringe* (*Rfng*), expressed in the dorsal ectoderm and in the whole AER of chicken limb buds, might be also involved in the DV patterning (Tickle and Munsterberg 2001).

1.3 *HOX* genes in limb development

1.3.1 Chromosomal clustering of *Hox* genes is linked to a specific expression pattern during embryogenesis

Hox genes code for a conserved family of homeobox-containing transcription factors. They are usually clustered and can be found in genomes of different organisms, for instance cnidarians, nematodes, arthropods, echinoderms, cephalochordates and vertebrates (Martinez et al. 1999; Aboobaker and Blaxter 2003; Hill et al. 2003; Wagner et al. 2003). In mammals *Hox* genes are organised in four clusters named A, B, C and D located on different chromosomes. Each cluster consists of 9–11 genes, which are expressed according to the spatio-temporal collinearity rule along the primary body axis. This means that the anterior genes, located at the 3' extremities of the complexes, are expressed earlier and more anterior in the embryo than the posterior genes, located at the 5' ends of the clusters (Duboule and Dollé 1989; Izpisúa-Belmonte et al. 1991). The pattern of *Hox* gene expression is evolutionary conserved and can be observed not only in vertebrates but also in invertebrate species, for example in *Drosophila*, which contains a single homeobox cluster (HOM-C complex) (Fig. 2).

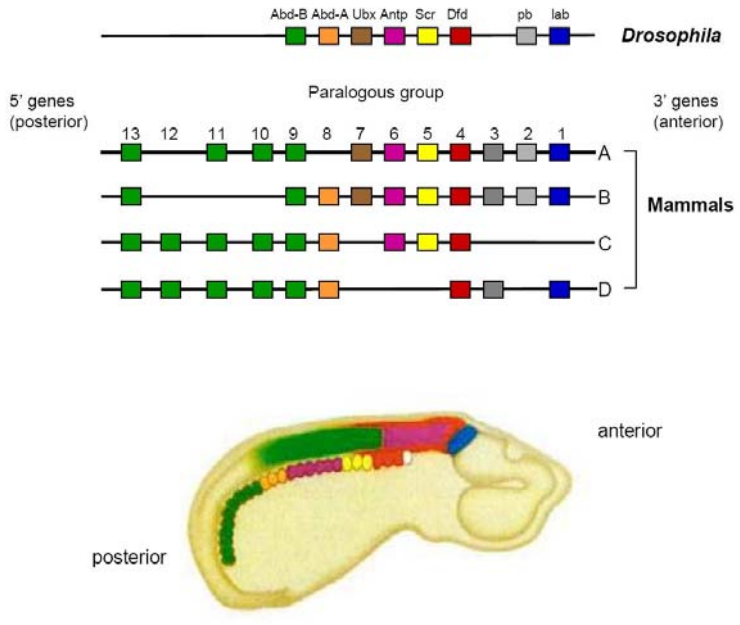

Fig. 2 Organisation of the *Drosophila* HOM-C complex and of the corresponding four *Hox* complexes in mammals. Genes marked with the same colour exhibit the highest homology, thus correspond to the same paralogous groups. Below, expression pattern of *Hox* genes along the main body axis in the mouse embryo. Different colours correspond to expression domains of various *Hox* genes, as shown in the upper panel. Adapted from Carroll 1995.

In addition, in certain tissues *Hox* genes are expressed in a quantitative order. For instance, in limbs of higher vertebrates *Hoxd* genes follow the so called third collinearity rule in such a way that the most posterior gene *Hoxd13* is expressed very strongly, whereas genes located towards the 3' end of the cluster, have a progressively reduced expression level (Kmita et al. 2002a).

1.3.2 *Hox* genes and pre-patterning of the embryo

Nested *Hox* gene expression along the primary body axis forms a pre-pattern, which can define prospective organ regions. For example, anterior expression boundaries of *Hoxc6*, *Hoxc8* and *Hoxb5* in the lateral plate mesoderm of vertebrate embryos fit exactly to the the regions where the forelimb fields are specified, thus suggesting a role of these *Hox* genes in the determination of these particular regions in the embryo (Nelson et al. 1996). In addition, it has been shown that *Hoxb5* knockout mice develop the shoulder girdle shifted, which corresponds to the shift in *Hox* expression domains compared to the wildtype mice (Burke et al. 1995; Rancourt et al. 1995; Gaunt 2000). It is possible that also other *Hox* genes influence the pre-specification of the limb fields. For example, it has been observed that ectopic expression of the *Hoxb8* gene in the anterior part of the limb bud induces an additional ZPA. Furthermore, the lack of limbs in snakes correlates with specific changes in *Hox* expression domains (Cohn and Tickle 1999).

1.3.3 Posterior *Hoxa* and *Hoxd* genes and their role in limb patterning

As previously mentioned, *Hox* genes seem to play a very important role in specification of the limb field, but it is also known that they are essential at later stages of limb development, namely for the establishment of the PD and the AP limb axes.

Expression profiles of the *Hoxd9-13* genes in limb buds differ depending on the stage of development. In the first phase, the posterior *Hox* genes are expressed in the entire limb bud. According to the collinearity rules, more anterior genes such as *Hoxd9* and *Hoxd10* are expressed first, followed by expression of the more posterior genes. A similar profile can be observed for the *Hoxa9-13* genes. In phase II, a clear change in the expression domains is visible, namely the *Hoxd* genes are activated in the posteriorly nested order. It means that the more anterior genes, for instance *Hoxd10*, are expressed in the anterior part of the limb bud, whereas expression of the posterior genes like *Hoxd13* is restricted to the posterior

mesoderm. In the last phase, the *Hoxd* genes are expressed only in the distal part of the limb bud, and there is a switch in the AP expression domains, so that the anterior genes are expressed in the posterior part of the limb bud and *vice versa* (Fig. 3) (Izpisua-Belmonte and Duboule 1992; Duboule 1994; Nelson et al. 1996).

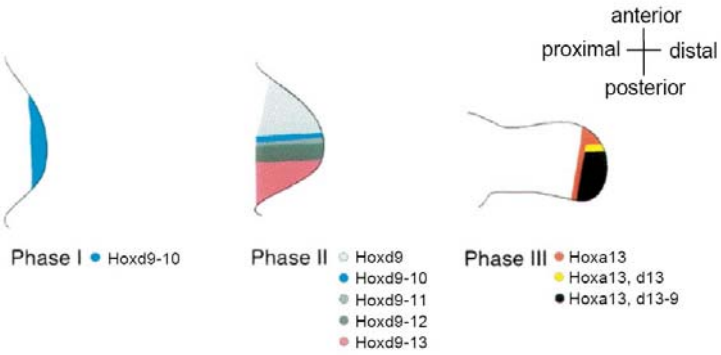

Fig. 3 Three phases of *Hoxd* genes expression in developing limb buds. See text for further explanations. Adapted from Shubin *et al.* 1997.

Overlapping expression domains of *Hox* genes create a dynamic pattern for Hox proteins activity. However, it is known that the region in which few *Hox* genes are expressed, is dominated by the most 5' gene (i.e. the most posterior one). This phenomenon is called posterior prevalence and leads to the situation that in various limb regions, different *Hox* genes at different timepoints play a dominant role. Thus, during phase I, expression of *Hoxd9* and *Hoxd10* specifies the stylopod. Zeugopod patterning is accomplished during both phases I and II, whereas digit formation is dependent on expression of the most posterior *Hox* genes during phases II and III (Johnson and Tabin 1997).

This model has been confirmed by the observation of skeletal defects and *Hox* expression domains in different *Hox* mutants. Thus, *Hoxd9* and double *Hoxa9*/*Hoxd9* knockout mice show forelimb defects, affecting the humerus (Fromental-Ramain et al. 1996), whereas *Hoxd9*/*Hoxd10* double mutants show alterations in the hindlimb skeleton, visible on the border between the stylopod and the zeugopod, which is similar to the defects observed in the single *Hoxd10* mutant mice. In addition, in a small percentage of *Hoxd9*/*Hoxd10* $^{-/-}$ mice the humerus is also deformed (Carpenter et al. 1997; de la Cruz et al. 1999). Inactivation of both *Hoxa10* and *Hoxd10* affects the femur, knee joint and tibia/fibula in mice (Wahba et al. 2001). Moreover, improper development of the thigh and shank has been described in mice lacking either *Hoxd11* or *Hoxa11* genes and in the double mutants (Small and Potter 1993;

Davis and Capecchi 1994; Davis et al. 1995; Favier et al. 1995; Boulet and Capecchi 2004). Additionally, in *Hoxd11* knock-out mice metacarpals, phalanges and wrist bones are also affected (Davis and Capecchi 1994; Favier et al. 1995). Interestingly, compound mutants for *Hoxa10* and *Hoxd11* show a more severe phenotype, giving the evidence that zeugopod development is dependent on the proper expression of several posterior *Hox* genes from both *Hoxa* and *Hoxd* paralogous groups (Favier et al. 1996; Wahba et al. 2001). Finally, production of various single and compound mutant mice indicated that four posterior genes, *Hoxa13, Hoxd11*, *Hoxd12* and *Hoxd13* regulate digit development in a dose-dependent manner (Dolle et al. 1993; Davis and Capecchi 1996; Kondo et al. 1996; Zakany et al. 1997; Kondo et al. 1998). Moreover, *Hoxa11* and *Evx2*, the latter one being located at the proximal end of the *Hoxd* complex, were also shown to contribute to digit morphogenesis, however to a lesser extend (Zakany and Duboule 1999).

1.3.4 A special role of the posterior *Hoxd* genes in the development of digited limbs and the contribution of different pathways to digit formation

It has been proposed that during evolution *Hox* genes acquired new functions, which enabled development of new structures, for instance digits, which appeared only after the fin-to-limb transition. This hypothesis has been supported by the recognition that the specific expression domains of the 5' *Hoxd* genes known for tetrapods, is not established during development of teleost pectoral fins (Sordino et al. 1995). For a long time it was not clear, what exactly happens on the molecular level and how Hoxd proteins regulate digit formation. However, recent data revealed links between *Hox* genes and pathways which play an established role in digit development.

Until now it was known that digit patterning is dependent on *Sonic hedgehog*, as concluded from the analysis of *Shh* mutant mice (Kraus et al. 2001). Moreover, Shh-signalling was shown to induce various genes, among them *BMPs*. Their expression in the interdigital necrotic zone suggested their contribution to apoptotic events separating prospective digits (Yokouchi et al. 1996; Zou and Niswander 1996; Chen and Zhao 1998; Drossopoulou et al. 2000; Guha et al. 2002). Recently, it has been shown that *BMPs* can also be directly activated by posterior *Hox* genes (Suzuki et al. 2003; Knosp et al. 2004). Furthermore, other experiments indicated that the early posterior repression of 5' *Hoxd* genes is required for the localised expression of *Shh*, which in turn promotes late activation of *Hoxd* genes leading to digit asymmetry (Zakany et al. 2004). Moreover, it has been lately shown that Gli3, the

intercellular mediator of Shh, directly interacts with 5' Hoxd proteins and it was suggested that the varying Gli3:Hoxd ratio across the limb bud is responsible for differential activation of target genes (Chen et al. 2004). Therefore, according to the current knowledge, the interplay between Hoxd, Gli3 and the Shh- and the BMP-signalling is thought to pattern the prospective digit area.

1.3.5 Mechanisms controlling expression of *Hox* genes

The identification of regulatory sequences responsible for gene expression is fundamental to obtain the full knowledge about gene function, its connection to the cellular network and its possible implication in diseases. Thus, a very important role of *Hox* genes for the development and patterning of the embryo led many scientists to investigate regulation of these genes in more detail. Especially, a lot of effort has been put to find promoters and enhancers, as well as to explain the collinear expression of *Hox* genes. Moreover, special attention has been directed on the regulation of the 5' *Hox* genes in developing limb buds.

1.3.5.1 Regulation of single Hox genes

Different studies revealed that several anterior *Hox* genes respond to RA treatment, so it was not surprising that retinoic acid responsive elements (RAREs) have been found in enhancer regions of different *Hoxa*, *Hoxb* and *Hoxd* genes (Maconochie et al. 1996; Morrison et al. 1996; Gould et al. 1998; Packer et al. 1998; Zhang et al. 2000; Oosterveen et al. 2003). In addition to the induction by RA, some of these genes are controlled by other mechanisms as well, for instance autoregulation (Popperl and Featherstone 1992; Packer et al. 1998; Manzanares et al. 2001; Yau et al. 2002), cross-regulatory interactions (Gould et al. 1997; Maconochie et al. 1997; Manzanares et al. 2001; Yau et al. 2002) or activation by other transcription factors (Sham et al. 1993; Manzanares et al. 1997; Manzanares et al. 1999; Manzanares et al. 2002).

In many cases enhancers specific for single *Hox* genes, flanking these from the 3' or the 5' side, were found (Whiting et al. 1991; Eid et al. 1993; Gerard et al. 1993; Knittel et al. 1995; Shashikant et al. 1995; Becker et al. 1996; Morrison et al. 1997; Kwan et al. 2001). Interestingly, these regulatory elements can also be shared between neighbouring genes, as shown for the *Hoxa* and *Hoxb* clusters (Gould et al. 1997; Sharpe et al. 1998; Oosterveen et al. 2003).

Recently, several genes for microRNAs have been proposed to lie within *Hox* clusters and to downregulate expression of single genes, as shown *in vivo* for *Hoxb8* and *in vitro* for *Hoxb8*, *Hoxc8*, *Hoxd8*, and *Hoxa7* (Calin et al. 2004; Mansfield et al. 2004; Yekta et al. 2004).
All these data, although valuable for understanding the regulation of *Hox* genes, do not explain their specific nested expression domains thought to result from the clustered organisation on chromosomes. Therefore, it has been proposed that in addition to the regulation of single genes driven by their promoters and local regulatory elements, other regions controlling and regulating the expression of the whole cluster have to be present as well.

1.3.5.2 Global regulation of the Hox clusters

Molecular mechanisms responsible for driving the collinear expression of *Hox* genes have been proposed by several authors (Deschamps et al. 1999; Kmita and Duboule 2003). The first hypothesis suggests that the mechanism of the collinearity is dependent on the progressive accessibility of *Hox* transcriptional units from one end of the cluster to the other. This might involve the process of opening the chromatin structure by transcription of one gene, which would be spread out on to the neighbouring regions. According to this model, expression of the most anterior *Hox* genes is initiated by retinoic acid (Roelen et al. 2002), and upon induction sequential activation of the more 5' genes occurs. The second hypothesis proposes that the collinear activation of the *Hox* genes is driven by local cis-acting elements, which show increasing or decreasing affinity to certain signalling molecules. The existing gradient of these molecules could be "read" along the cluster, allowing expression of the *Hox* genes in the proper way. The third mechanism assumes that a global control region (GCR), located outside the clusters, can regulate several genes in a relatively promoter-unspecific manner. These three mechanisms are not exclusive; on the contrary, they could work in combination with each other, depending on the site and the stage of *Hox* gene expression.

1.3.5.3 Regulation of the posterior Hox genes during limb development

It has been observed that the posterior *Hoxd* genes, *Hoxd10*, *Hoxd11, Hoxd12* and *Hoxd13* show very similar expression domains in presumptive digits (Sordino and Duboule 1996), therefore it has been proposed that these four transcription units are under the control of the same enhancer, which could regulate their spatial and temporal expression in developing limbs (van der Hoeven et al. 1996; Herault et al. 1999). Moreover, it has been suggested that this element (called digit enhancer) is located centromeric to the *Hoxd* complex (Kondo and Duboule 1999; Spitz et al. 2001).

Recently, a conserved region (called region XII) located at the 5' end of the *Hoxd* cluster has been described and it has been shown to be required for the quantitative collinearity of the *Hoxd* genes in limbs (Kmita et al. 2002a; Kmita et al. 2002b). In addition, an approximately 40 kb large segment of human DNA located further 5' to the *HOXD* cluster, has been found to contain the digit enhancer and to control the expression of both *Hoxd* and *Evx2* genes (Spitz et al. 2003). Moreover, a region regulating *Hoxd* gene expression before Shh signalling (**e**arly **l**imb **c**ontrol **r**egion – ELCR) has been lately proposed to be located 3' to the whole complex (Zakany et al. 2004).

Thus, the current model proposes that at the early stage of limb development, the ELCR controls phase II of *Hoxd* gene expression (more 5' genes become activated progressively in more posterior domains). The Hoxd-dependent Shh production probably causes a switch into the later phase of *Hoxd* gene regulation, which is controlled by the global elements located 5' to the complex. The AP expression domains of the *Hoxd* genes change, and at the same timepoint the quantitative collinearity is established by interactions between the remote digit enhancer and local regulatory elements (for instance region XII). The strongest effect, resulting in the highest expression level, is exerted on the most 5' gene (*Hoxd13*) and progressively weaker effects on more anterior genes (Fig. 4) (Deschamps 2004).

Regulation of *Hoxa* expression has not been studied so extensively as that of the *Hoxd* genes. However, recent analyses indicated that over 900 kb long regions upstream of the *Hoxa* and the *Hoxd* clusters are higly conserved. This gave rise to the hypothesis that also for the posterior *Hoxa* genes, limb-specific expression might be dependent on global regulatory elements present upstream of the cluster. Further functional tests partially confirmed this theory (Lehoczky et al. 2004).

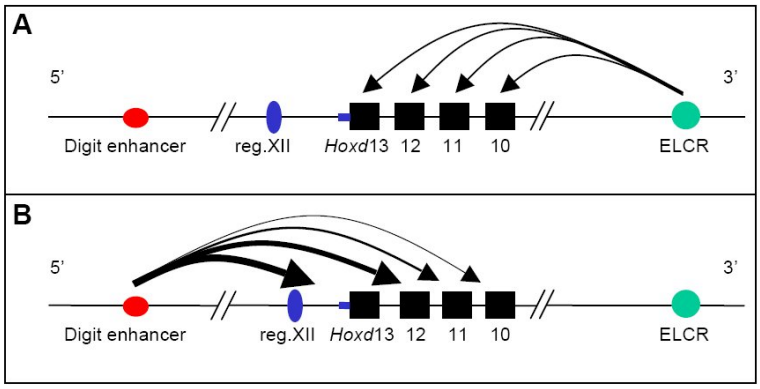

Fig. 4 Schematic representation of 5' *Hoxd* regulation in limb buds. *A*: The hypothetical early limb control region (ELCR) located 3' to the *Hoxd* cluster controls the early phase of *Hoxd* gene expression. *B*: In the later phase, *Hoxd* expression is regulated by the digit enhancer located 5' to the cluster. Local regulatory elements, for instance region XII or other sequences located within the cluster (marked in blue), can co-operate with the globally acting digit enhancer, leading to establishment of the quantitative collinearity. Thickness of the arrows indicates the strength of the enhancement and corresponds to the expression level of a particular gene. Adapted from Zeller and Deschamps 2002, and Deschamps 2004.

1.3.6 *HOX* gene mutations and limb malformations in humans

To date, mutations in four human *HOX* genes, namely *HOXA11*, *HOXA13, HOXD10* and *HOXD13*, have been found. All these mutations, as expected, are associated with limb malformations.

In two families a single nucleotide deletion within the second exon of *HOXA11*, resulting in a frameshift and a premature stop codon, has been found to co-segregate with the proximal radial-ulnar synostosis (Thompson and Nguyen 2000). Different changes in *HOXA13*, including missense and nonsense mutations, polyalanine expansions or small deletions within the promoter region, cause hand-foot-uterus syndrome, a rare dominantly inherited condition affecting distal limbs and genitourinary tract (HFUS, OMIM #140000) (Mortlock and Innis 1997; Goodman et al. 2000), or Guttmacher syndrome (OMIM #176305) (Innis et al. 2002). Recently, a missense mutation in the *HOXD10* gene has been described to be the cause of isolated congenital vertical talus, also known as rocker-bottom feet (CVT, OMIM # 192950), and Charcot-Marie-Tooth disease (CMT, OMIM# 118220) in a big American family of Italian descent (Shrimpton et al. 2004).

The first described mutation within the *HOXD13* gene was an imperfect alanine-coding trinucleotide expansion in the first exon of the gene. This insertion has been linked to

synpolydactyly (SPD, OMIM #186000), a dominant inherited limb disorder affecting exclusively autopods (Muragaki et al. 1996). SPD is characterised by syndactyly of the third and fourth fingers and the fourth and fifth toes, both associated with polydactyly. Subsequent studies revealed that in more than 20 families published up to now, SPD is caused by the same pathological polyalanine tract expansions in HOXD13 protein and that the size of these expansions correlates with the severity of the phenotype (Akarsu et al. 1996; Goodman et al. 1997; Kjaer et al. 2002). Other mutations found in the *HOXD13* gene such as intragenic frameshift deletions, predicted to result in truncated proteins, an acceptor splice site mutation and a missense mutation in exon 2, cause an atypical form of SPD (Goodman et al. 1998; Calabrese et al. 2000; Debeer et al. 2002; Kan et al. 2003). Interestingly, a different missense mutation in the same exon 2 of *HOXD13* has been found in a family with a dominantly inherited combination of brachydactyly and polydactyly (Caronia et al. 2003). The SPD phenotype was also observed in 2 related patients with a microdeletion at the 5' end of the *HOXD* cluster, which removes *HOXD9* to *HOXD13* and extends 85 kb upstream of *HOXD13* (Goodman et al. 2002). In contrast, larger deletions involving chromosome 2q31.1, where the *HOXD* complex is located, have been associated with minor digital anomalies (Nixon et al. 1997; Slavotinek et al. 1999), or with major limb defects (Boles et al. 1995; Nixon et al. 1997; Goodman 2002), or with a combination of severe limb and genital abnormalities (Del Campo et al. 1999).

1.3.7 Hox proteins in complexes

The main role of Hox transcription factors is to regulate the pattern of chondrogenic differentiation in limbs, probably by activation a variety of target genes. However, different experiments *in vitro* revealed a poor affinity of single Hox proteins to the DNA and a low specificity of this binding (Gehring et al. 1994; Pellerin et al. 1994; Lu et al. 1995). Thus, it has been suggested that in order to increase the affinity and to generate binding specificity, Hox proteins form multiprotein-DNA complexes. Known Hox-interaction partners are homeodomain-containing proteins Pbx1 and Pbx2 (Lu et al. 1995; Chang et al. 1996; Knoepfler et al. 1996; Shen et al. 1996; Knoepfler and Kamps 1997; Lu and Kamps 1997). Binding of both Pbx molecules is dependent on the YPWM motif in Hox proteins from paralogous groups 1 – 8 and on the specific tryptophane residues in paralogues 9 –10 (Chang et al. 1995; Knoepfler and Kamps 1995; Chang et al. 1996; Shen et al. 1996; Shen et al. 1997b). Furthermore, Pbx proteins have been shown to dimerise with Meis (Chang et al.

1997; Knoepfler et al. 1997), and since Hox paralogues 9-13 bind Meis as well (Shen et al. 1997a), trimeric complexes of Meis, Pbx and Hox can be formed (Shanmugam et al. 1999). It is known that all three classes of proteins are present in the proximal part of a limb bud, therefore it is believed that Meis/Pbx/Hox complexes control development of the proximal limb structures by regulating transcription of downstream targets (Capdevila and Izpisua Belmonte 2001). Furthermore, trimeric complexes between Prep, Pbx and 3' Hox proteins have also been described (Berthelsen et al. 1998). However, in this case Prep proteins do not interact directly with Hox, and the binding occurs via Pbx (Ferretti et al. 1999; Fognani et al. 2002).

Little is known about interaction partners of the most posterior Hox paralogues. As already mentioned, Meis proteins can bind them *in vitro*, but it is rather unlikely that this binding occurs *in vivo* as well, since *Hox* paralogues 11-13 are expressed only in the distal part of the limb, whereas *Meis* expression is inhibited in this region. Recently, it has been shown that the zinc finger transcription factor Gli3, which plays a role in the AP limb patterning, directly binds the homeodomains of Hoxd11, Hoxd12 and Hoxd13 proteins (Chen et al. 2004). Furthermore, it is known that Hoxa13 and Hoxd13 functionally cooperate with Sp1, a GC-box binding transcription factor (Suzuki et al. 2003). However, up to date no other interaction partners for Hoxd13 are known. Thus, it would be interesting to find and define more factors, which co-operate with this and other Hox proteins in order to regulate distal limb development.

1.4 Outline of the project

Human disorders are currently investigated very thoroughly at the molecular level, and many disease-causing genes have been identified so far. However, it became clear that knowledge about the defective gene or even the mutated nucleotide is often not sufficient for prediction of the clinical phenotype or the severity and course of the disease. This is because genes, and most of the proteins which they encode, do not act alone, but they are parts of different pathways and can be regulated or modified by the action of other genes in various ways. To investigate the factors and mechanisms that play a role in the clinical variability of Mendelian disorders, the Collaborative Research Centre in Berlin has been founded. As a part of this research the molecular pathology of *HOXD*-related limb malformations is being studied.

The goal of my project was to investigate at the cytogenetic and molecular level the autosomal translocation t(2;10)(q31.1;q26.3) carried by a male patient presented with synpolydactyly and mental retardation. Systematic analysis revealed that the breakpoint on chromosome 2 is located in the vicinity of the *HOXD* cluster and that it does not disrupt any known gene. The knowledge about the complexity of *HOXD* regulation mechanisms allows us to hypothesise that the translocation might have disturbed these subtle mechanisms by position effect, thus being causative for the limb phenotype in the patient. The second part of the project focused on the search for Hoxd13 interaction partners. A yeast two-hybrid screen has been performed, and afterwards candidate genes were studied using RNA *in situ* hybridisation, immunofluorescence and coimmunoprecipitation methods. The preliminary results presented here give new insights into the molecular mechanisms of limb development and pathogenesis.