4 DISCUSSION

4.1 Genotype-phenotype correlation in the patient with a translocation t(2;10)(q31.1;q26.3)

In this study, the translocation t(2;10)(q31.1;q26.3) present in a male patient with skeletal abnormalities and mental retardation has been investigated. Cytogenetic and molecular analysis indicated that the breakpoint on chromosome 10 disrupts the *MGMT* gene, whereas on chromosome 2 the break is localised approximately 390 kb centromeric to the *HOXD* cluster. Moreover, there was no indication that any gene might be disrupted on chromosome 2. The relevance of both breakpoints for the patient's phenotype will be discussed in the following sections.

4.1.1 The *MGMT* gene on chromosome 10 disrupted by the breakpoint does not seem to be responsible for the limb phenotype of the patient

The methylguanine-DNA methyltransferase (*MGMT*) gene disrupted in the patient encodes an enzyme involved in the repair of O^6-alkylguanine-containing DNA. The O^6-alkylguanine-DNA adducts are potent pre-mutagenic lesions, since the modified guanine preferentially mispairs with thymine, instead of building a pair with cytosine. As a result, the G:C → A:T mutations may appear in the DNA. MGMT removes the alkyl group from the DNA by transferring it to its own cysteine residue. This process irreversibly inactivates the protein and the alkylated form of MGMT accumulates as a dead-end product. Therefore, the capacity to repair the O^6-alkylguanine residues is limited by the number of MGMT molecules present in the cell.

In the 1990s two groups created *MGMT* knockout mice in order to study the gene function. Tsuzuki *et al*. showed that the *MGMT* $^{-/-}$ mice were normal, except for a slight growth retardation (Tsuzuki et al. 1996), whereas Glassner *et al.* did not observe any pathologies and abnormalities during development of the knockout mice (Glassner et al. 1999). Therefore, it is rather unlikely that *MGMT* contributes to limb formation. On the contrary, recent data suggest a link between MGMT and cancer. Downregulation of the *MGMT* gene might participate in tumour formation, whereas its upregulation seems to prevent this process

(Sakumi et al. 1997; Nakamura et al. 2001; Oue et al. 2001; Reese et al. 2001; Zhou et al. 2001; Smith-Sorensen et al. 2002). Thus, all these data suggest that disruption of the *MGMT* gene probably did not cause the limb malformations present in the patient.

4.1.2 Could the disruption of *MGMT* have influenced the mental status of the patient?

MGMT is ubiquitously expressed in adult human tissues, as shown in this study in Northern blot experiments. However, different tissues express this gene at various levels, with the highest expression in liver and a very low expression in brain. A similar situation can be seen in foetal tissues. The amount of mRNA of the *MGMT* gene in different cell types correlates with the enzymatic activity of the protein measured by several groups (Grafstrom et al. 1984; Wiestler et al. 1984; Pegg et al. 1985). However, during embryonic development a critical factor can be the rate of DNA repair relative to that of DNA replication. Therefore, some authors compared the activity of MGMT to the activity of DNA polymerase in extracts from different tissues (Krokan et al. 1983). Measured in this way, the lowest activity of MGMT was found in brain of most foetuses. These data suggest that the brain might be more exposed to the risk of DNA mutations than other organs.

Quantitative analysis of *MGMT* expression revealed that in lymphoblastoid cell lines derived from the translocation patient, the level of *MGMT* mRNA was reduced by approximately 50% compared to the control. This is in accordance with the finding that the translocation in the patient caused the disruption of one *MGMT* allele. Decrease of *MGMT* expression in patient's brain during embryogenesis might have led to accumulation of DNA mutations in neuronal tissue, which might have influenced the development of cognitive functions in the patient.

4.1.3 There is no evidence for any gene disrupted on chromosome 2 in the patient with the translocation t(2;10)(q31.1;q26.3)

The results of RT-PCR experiments suggest that no gene is disrupted on the chromosome 2 in the translocation patient. However, recent sequence data available from the UCSC Genome Browser Gateway (May 2004 assembly) indicate that much more ESTs are present on the breakpoint-spanning BAC RP11-538A12 and on the neighbouring BACs than thought before. Since these ESTs are either unspliced or repetitive, it is rather unlikely that any of them might represent a gene.

4.1.4 *HOXD* genes located close to the breakpoint on chromosome 2 are good candidates for the limb phenotype in the patient

The translocation patient presented in this study shows a phenotype similar to that of SPD patients with mutations in the *HOXD13* gene. However, sequence analysis of *HOXD13* in the patient showed no abnormalities within this gene, indicating that the SPD phenotype must have been caused by other defects, most likely by effects of the translocation.

Experiments performed in mice indicate that four posterior *Hox* genes, *Hoxa13, Hoxd11*, *Hoxd12* and *Hoxd13* can control digit development (Dolle et al. 1993; Davis and Capecchi 1996; Kondo et al. 1996; Zakany et al. 1997; Kondo et al. 1998). Progressive reduction in the dose of these genes results in adactylous limbs in mutant mice. Interestingly, the intermediate stage in the pentadactyl to adactyl transition is characterised by polydactyly. The latter phenotype is present in a triple *Hoxd11*/*Hoxd12*/*Hoxd13* knockout mouse, suggesting that SPD is caused by a loss of function of the *HOXD11-13* genes, rather than inactivation of *HOXD13* alone. Therefore, it is very likely that in the translocation patient described in this study, the breakpoint located in the vicinity of the *HOXD* genes affected their proper expression leading to the SPD phenotype. Various hypotheses for the putative deregulation of the *HOXD* genes are discussed in the following sections.

4.1.5 Chromosomal rearrangements can cause disorders in humans and mice via position effect

Balanced chromosomal rearrangements may be associated with pathological phenotypes in humans. One possible mechanism for this is disruption of a gene caused by a breakpoint. However, during the past few years several patients with balanced chromosomal rearrangements and a disease phenotype, who do not carry a disrupted gene, were reported (Kleinjan and van Heyningen 1998; Marlin et al. 1999; Di Paola et al. 2004; Kleinjan and van Heyningen 2004; Lower et al. 2004; Muncke et al. 2004; Tadin-Strapps et al. 2004). This phenomenon can be explained by a so called position effect, which is defined as a deleterious change in the level of gene expression caused by a change in the normal chromosomal environment of the gene (Kleinjan and van Heyningen 1998).

There are several known cases in humans, where breakpoints involving chromosomal band 2q31 are thought to cause improper development of skeletal structures via position effect. One example is a family with mesomelic dysplasia and vertebral defects, carrying a balanced

translocation t(2;8) (Spitz et al. 2002). In addition, a female patient reported with a pericentric inversion inv(2)(p15q31.1) showed bilateral aplasia of radial, ulnar and fibular bones, hypoplasia and dislocation of both tibiae and defects in metacarpals and phalanges (Dlugaszewska et al. 2005). Another known case is a female patient with a balanced translocation t(2;10)(q31.1;q23.3) showing shortening and aplasia of upper limb structures, affecting zeugo- and autopods, and with a slight dextro-convex scoliosis (Dlugaszewska et al. 2005). In all these cases the breakpoints on the long arm of chromosome 2 have been mapped to regions telomeric to the *HOXD* cluster. It is believed that these rearrangements cause position effects, resulting in misregulation of *HOXD* gene expression.

An additional support comes from the analysis of the X-ray induced mouse mutant *ulnaless* (*Ul*), which shows a very severe phenotype, with an affected zeugopod and almost complete absence of ulnae (Herault et al. 1997; Peichel et al. 1997). Recently, it has been shown that an inversion occurred on chromosome 2 in the *ulnaless* mouse, with the breakpoints surrounding the *Hoxd* cluster (Spitz et al. 2003). It is very likely that this rearrangement is the reason for misregulation of posterior *Hoxd* expression observed in limb buds of the *Ul* mouse (Peichel et al. 1997) and therefore the cause for the limb phenotype.

4.1.5.1 Mechanisms leading to a position effect

In general, different mechanisms can lead to a position effect. First, a chromosomal rearrangement might separate regulatory elements from the gene, thus resulting in its misexpression. Secondly, a gene and an enhancer element from another transcriptional unit could be juxtaposed by a rearrangement. Thirdly, one gene together with its regulatory elements might be placed next to a second gene, and the competition for the same regulatory elements between both of them could change the expression level of the first gene. Lastly, the rearrangement could lead to position effect variegation. Assuming that the translocation in the patient presented in this study led to a change in *HOXD* gene expression, it would be interesting to find out which of these mechanisms contributed to the disease phenotype.

It has been suggested that expression of the posterior *Hoxd* genes in limb buds is dependent on both local regulatory elements lying in the direct vicinity of these genes as well as on enhancers lying outside of the cluster. Especially, two global elements, a putative early limb control region and the digit enhancer, lying respectively 3' and 5' of the *Hoxd* genes are involved in the regulation of the whole complex (Deschamps 2004). Since the breakpoint in the translocation patient occurred 5' to the *HOXD* complex, the important question to answer

is, whether 5' regulatory regions are affected by the rearrangement. Recently, the position of the human digit enhancer has been narrowed down to a 40 kb long sequence present on BAC clone 504O20 (Spitz et al. 2003). In the translocation patient, this region is neither disrupted nor separated from the *HOXD* cluster by the rearrangement. However, it is still possible that other regulatory elements responsible for *HOXD* expression may also be present further upstream to this digit enhancer. This hypothesis is supported by the fact that the region 5' to the 2q31.1 breakpoint in the translocation patient was shown to be highly similar to the corresponding region in mouse. It is broadly accepted that sequence conservation maintained during evolution, reflects an important role of the conserved elements. Since there is no evidence for a gene in this region, the presence of regulatory elements is one possible explanation for the sequence similarity close to the breakpoint. If this theory was true, it would be possible that these, till now unknown, regions could be affected by the translocation.

Another mechanism leading to the position effect suggests that the rearrangement, which brings together two different chromosomes, could place the *HOXD* cluster under the influence of another regulatory element located on chromosome 10 or that the impact of the digit enhancer on the *HOX* genes could be reduced by another transcriptional unit lying on the centromeric side of the breakpoint. The first suggestion is possible, however at the present state of knowledge it has a very speculative character. Nothing is currently known about regulatory elements of *MGMT* or other genes in the vicinity of the breakpoint at 10q26.3. The second mechanism is rather unlikely, since *MKI67*, the closest gene located approximately 1.4 Mb centromeric to the chromosome 10 breakpoint, lies probably too far away to be able to compete with *HOXD* genes for the digit enhancer.

The last mechanism, the classical position effect variegation causes silencing of a gene by inserting it into or nearby a heterochromatic region. Alternatively, a long-range insulator or another boundary element may be removed by the rearrangement, which results in spreading the heterochromatin and inactivating of the whole locus. However, it is rather unlikely that one of these mechanisms caused the putative misregulation of the *HOXD* genes in the patient, since the whole *HOXD* cluster has been placed into the middle of the transcriptionally active region at 10q26.

4.1.5.2 HOXD gene expression may be influenced by the accessibility of the entire cluster for transcription factors

Regulation of posterior *Hoxd* genes in limb buds is most likely regulated by the interplay between *cis*-acting elements and *trans*-acting factors. Till now two groups of genes, *Polycomb* (*PcG*) and *trithorax* (*trxG*) have been implicated in maintenance of the active or the silent state of *Hox* genes in *Drosophila* (Simon 1995). Later, *PcG* and *trxG* homologues were also found in mammals (Schumacher and Magnuson 1997). Different *Polycomb* or *trithorax* mouse mutants showed homeotic transformations in the vertebral skeleton which corresponded to the shift in *Hox* gene expression domains (Alkema et al. 1995; Akasaka et al. 1996). These observations suggested that the role for *Polycomb* and *trithorax* genes in the regulation of *Hox* gene expression along the main body axis is conserved between *Drosophila* and mammals.

Results published during the last few years provided evidence that PcG genes may also play a role in proper expression of posterior *Hoxd* genes in mouse limb buds (Barna et al. 2000; Barna et al. 2002). According to these studies, Bmi-1 belonging to the Polycomb group proteins binds Plzf, a nuclear zinc finger protein. Plzf can recognise and bind different regulatory sequences within the *Hoxd* locus, and it can remodel the chromatin by histone deacetylation, which results in *Hoxd* repression. In addition, Plzf can mediate long-distance interactions between *cis* regulatory elements within the *Hoxd* locus. Therefore, Plzf together with its interacting partner Bmi-1 are excellent candidates for factors which could integrate both local and global regulatory mechanisms in order to mediate the correct expression of posterior *Hoxd* genes. It is very likely that they are not the only players in this complex system and that other regulatory proteins will be discovered soon.

Binding of transcriptional regulators to DNA might be dependent on proper chromatin architecture (Kornberg and Lorch 1992; Nourani et al. 2004). Since it has been proposed that the chromatin structure of any locus can be determined by the combination of *cis*-acting elements and by the wider chromosomal and nuclear environment (Kleinjan and van Heyningen 2004), it is plausible that chromosome rearrangements could alter the chromatin architecture. In fact, changes in chromatin structure have been proposed following insertion of some transgenes (de Graaff et al. 2003) or in case of small deletions (Jiang et al. 2003). Hence, it is possible that the translocation, which has occurred in the patient presented here, changed the chromatin structure around the *HOXD* locus. This event might have modified the access to the chromatin for transcription factors, disturbed the interplay between *cis*- and

trans-acting regulatory elements and resulted in deregulation of *HOXD* gene expression and the limb phenotype in the patient.

4.1.6 Are others genes possibly involved in the patient's phenotype?

Interestingly, changes in the global chromatin structure might also influence expression of other genes near the chromosome 2 breakpoint. The closest gene, located only 220 kb telomeric to the breakpoint in the translocation patient, is *KIAA1715*. It spans over 75 kb of the genomic sequence at 2q31 and it is transcribed from the opposite strand compared to the *HOXD* cluster. Another candidate, *EXV2*, is lying 380 kb telomeric to the breakpoint and has the same orientation as *KIAA1715*. Mouse homologues of both genes, *Lnp* and *Evx2*, show the same expression pattern in limb buds and external genitalia as *Hoxd13*, which suggests that all three transcription units are under the control of the same regulatory sequences. In addition, *Lnp* is also expressed in the developing central nervous system in a highly similar pattern to that of *Evx2*, and it has a specific expression domain in the eyes, the heart and the forebrain (Spitz et al. 2003). The neural enhancer that may activate *KIAA1715* and *EVX2* is located in part within the same 40 kb region as the digit enhancer mentioned earlier. Therefore, it is possible that both the limb and the neuronal expression domains of *KIAA1715* and *EVX2* have been affected by the translocation via position effect. Since the translocation patient has cognitive deficits in addition to limb abnormalities, it is tempting to link the central nervous system phenotype with disturbed expression of *KIAA1715* or *EVX2*.

4.2 Search for interaction partners of Hoxd13 protein

The second part of this study focused on the search for Hoxd13 interaction partners in order to shed more light on the molecular basis of limb development. It has been suggested that Hox proteins act in complexes (see also section 1.3.7), however little is known about Hox cofactors playing a role in the development of distal limbs. To address this question, a yeast two-hybrid screen was performed, and in this approach many putative Hoxd13 interaction partners were identified. Several candidates were analysed in more detail, and the results of these studies will be discussed in the following sections.

4.2.1 Peg10 is a putative Hoxd13 binding protein

Peg10, the paternally expressed gene 10, has been mapped to an approximately 1 Mb long cluster of imprinted genes on mouse chromosome 6 (Ono et al. 2003). It is highly conserved between species, with homologous sequences in humans, cow, rat, mink, pig, rhesus and pufferfish. The presence of two long overlapping open reading frames (called ORF1 and OFR2) and the similarity of their predicted amino acid sequences to retroviral proteins Gag and Pol suggested that *Peg10* is a retrotransposon fossil in the mammalian genome (Ono et al. 2001; Shigemoto et al. 2001). Similarly as for other viral genes, a single *Peg10* transcript gives rise to two partially different proteins (Shigemoto et al. 2001; Lux et al. 2005).

4.2.1.1 Parts of the Peg10 protein bind Hoxd13-HD in yeast

In the yeast two-hybrid screen performed with the LexA_Hoxd13-HD bait, eight different *Peg10* clones were identified. Five of them contained the 5' part of the *Peg10-ORF2*, whereas three other clones were very similar to each other and covered the 3' end of the *Peg10-ORF2*. Binding to Hoxd13 lacking the homeodomain was confirmed in the LexA and GAL4 yeast systems for five positive clones originating from both the 5' and the 3' ends of the ORF2. Therefore, it seemed plausible that the full length Peg10 and Hoxd13 proteins could be real binding partners. Moreover, the results suggested that the N-terminus of Hoxd13 is sufficient for the binding. For Peg10, the putative interacting regions are located at the N- and the C-terminus of the Peg10-ORF2, and are separated by approximately 150 amino acids. However, it is possible that these two regions could be brought into proximity by protein folding and in this way they might be both responsible for binding to Hoxd13.

4.2.1.2 Interaction between Peg10 and Hoxd13 in mammalian cells

In spite of intensive attempts it was not possible to clone repetitive sequences present in the *Peg10* gene, therefore for further experiments a partial *Peg10* clone containing the ORF2-encoding sequence identical to GenBank entry AB091827 was used. Subsequent overexpression experiments showed that Peg10-ORF2 perfectly co-localises with the Hoxd13 protein lacking the homeodomain in the cytosol of COS1 cells. Moreover, similar studies have been performed for the wildtype Hoxd13. In the COS1 cells transfected with the Peg10-ORF2 construct, the overexpressed protein showed solely the cytosolic localisation. Co-expression of wildtype Hoxd13 and Peg10-ORF2 induced in some cells a clear change in

Peg10 localisation, from the cytosol to the nucleus. This alteration in cellular localisation suggests that Peg10 might interact with Hoxd13. However, it is not clear, why the co-localisation could not be seen in every double transfected cell. One possible explanation is that Hoxd13 and Peg10 bind each other only in a specific phase of the cell cycle, similarly as it is known for several proteins involved in the DNA-repair or the DNA-replication (Taniguchi et al. 2002; Fan et al. 2004).

The results of the coimmunoprecipitation assay clearly show that Peg10-ORF2 binds to full length Hoxd13. Therefore, it is very likely that the long version of Peg10 containing both ORF1 and ORF2 could also interact with Hoxd13.

Further experiments are needed to answer the questions, whether Hoxd13 binds Peg10 in the direct way and whether this interaction is dependent on DNA binding. In several reported cases, Hox proteins have been shown to interact directly with various proteins, for instance with Pbx or Meis (Shen et al. 1996; Shen et al. 1997), whereas other cofactors like Prep or Sp1 might be bound to Hox in the indirect way (Fognani et al. 2002; Suzuki et al. 2003). Moreover, in the same reports it has been shown that formation of complexes between Hoxd13 and Pbx or Meis is dependent on DNA binding. Interestingly, in all these cases interactions between Hox and other proteins require only the N-terminus of the Hox protein. The same seems to be true for Peg10 binding, since Hoxd13 lacking the homeodomain co-precipitated with Peg10-ORF2. In contrast, binding to Gli3, occurs via the homeodomain of Hoxd13 (Chen et al. 2004). This suggests that Hoxd13 could bind different factors via various domains. Hence, it is possible that Hoxd13, similarly as anterior Hox proteins, could participate in multimeric complexes.

4.2.1.3 Does Peg10 bind Hoxd13 in vivo?

Although it has been shown that Hoxd13 and parts of the Peg10 protein could interact in transformed mammalian cells, the question of much higher biological relevance is whether the binding between Peg10 and Hoxd13 could also occur during mouse embryogenesis. In order to address this question, the expression profile of *Peg10* was examined by whole mount *in situ* hybridisation. Comparison with the known *Hoxd13* expression domains (Albrecht et al. 2002) revealed that at the early stages of mouse development (E10.5) expression of both genes can be detected in similar domains of the distal limb bud. At a slightly later stage (E11.5) expression domain of *Peg10* becomes broader and certainly covers the area expressing *Hoxd13*. At stage E12.5, *Peg10* transcripts can be observed among others in digits

and in the proximal mesoderm, whereas *Hoxd13* is present in the interdigital zones. Different expression domains of both genes were also shown by section *in situ* hybridisation at the later stages of limb development. All these data suggest that Peg10 and Hoxd13 could interact at early stages of development, being expressed in the same regions of limb buds. However, further studies will be needed to confirm the presence of Hoxd13/Peg10 complexes in these tissues. At the later stages of embryogenesis, *Hoxd13* and *Peg10* genes are expressed in different cells and therefore it is rather unlikely that their products can bind each other.

4.2.1.4 A putative role of Peg10 proteins and Hoxd13/Peg10 complexes in limb development

Functional analyses of Peg10 were performed almost exclusively for the protein encoded by ORF1 (Okabe et al. 2003; Tsou et al. 2003). However, the longer version of Peg10 contains both ORF1 and ORF2, suggesting that it could share functional properties with the shorter prtotein. The endogenous *PEG10* has been shown to be upregulated in human hepatocellular carcinoma cells and during liver regeneration in mice. This suggests that the *PEG10* gene product could exert some regulatory function in cell cycle progression. Further experiments supported this hypothesis by showing that overexpression of human PEG10-ORF1 results in an increased rate of G_1 to S transition in 293T cells (Tsou et al. 2003). Moreover, Tsou *et al*. showed that the growth of hepatoma cells is suppressed after their transfection with *PEG10*-specific antisense oligonucleotides. In line with these results, PEG10 overexpression experiments indicated a protective role of this protein in apoptosis (Okabe et al. 2003). All these data are in agreement with the expression pattern of the *Peg10* gene observed in this study in mouse embryonic limbs containing a large number of highly proliferating cells. Interdigital zones of the limb buds, where apoptosis occurs, showed no expression of *Peg10* mRNA.

In vitro studies on the human PEG10-ORF1 protein indicate that it can bind to the activin receptor-like kinase 1 (ALK1) and to other receptors for members of the transforming growth factor beta (TGF-β) superfamily, for instance BMP receptors (Lux et al. 2005). These data provide a link between PEG10 and BMP signalling, which plays an established role in limb development. Binding between diffent receptors and PEG10 assumes that the latter protein must be present in the cytosol, and indeed a few authors report cytosolic localisation of PEG10-ORF1 (Okabe et al. 2003; Tsou et al. 2003; Lux et al. 2005). On the other hand, there are some hints suggesting that PEG10 might be a transcriptional regulator. Sequence comparison revealed that the murine Peg10-ORF1 protein is probably identical to the myelin

expression factor 3 (GenBank acc. number of the nucleotide sequence: AF302691), a brain-derived transcriptional activator containing a predicted nuclear localisation signal (NLS) (Steplewski et al. 1998). Moreover, Okabe *et al.* showed that PEG10-ORF1 can be found throughout the whole cell, thus also in the nucleus (Okabe et al. 2003). Up to now, there are no reports showing cellular localisation of the longer version of PEG10. In this study it was shown that the protein corresponding to the murine Peg10-ORF2 is present in the cytosol, when overexpressed in COS1 cells. However, its localisation can be changed upon co-expression with Hoxd13, and Peg10-ORF2 can co-localise with Hoxd13 in the nucleus. The putative role of Peg10 as a transcription factor can be also supported by the fact that both ORF1 and ORF2 of Peg10 can encode zinc finger domains, which are commonly known DNA-binding motifs.

Considering all these data it is tempting to hypothesise that Peg10 might be involved in BMP and TGF-β signal transduction and it might shuttle between different cell compartments. When present in the nucleus, Peg10 could form complexes with Hoxd13 in order to regulate expression of various target genes during embryogenesis. Since different *Hox* genes, as well as *Peg10*, were described as oncogenes (Okabe et al. 2003; Lawrence et al. 2005), their products could act synergistically by activating other factors responsible for cell proliferation. However, Hoxd13 can also activate genes involved in other processes. For instance, the Hoxd13 protein present in the interdigital zones is thought to induce expression of BMPs which mediate apoptosis, a process necessary for digit separation (Suzuki et al. 2003). Since *Peg10* expression has not been observed in the interdigital zones, it could not co-operate with Hoxd13 in the activation of apoptotic genes. Therefore, various Hoxd13 activities might require numerous interaction partners that would assure the specificity of the DNA binding and would modulate Hoxd13 function.

4.2.1.5 Ala-stretch mutations within Hoxd13 do not influence the binding to Peg10

Human *HOX*-associated pathologies have been extensively investigated for several years. One of these disorders, synpolydactyly (SPD), is caused by extensions of the polyalanine stretch in the HOXD13 protein (Akarsu et al. 1996; Muragaki et al. 1996; Goodman et al. 1997; Kjaer et al. 2002). Studies performed in mice and in transformed cell lines suggested that a similar mutation in murine Hoxd13 results in a misfolded protein which is either degraded or accumulates in the cytosol and therefore cannot fulfil its normal function (Albrecht et al. 2004). In order to see whether mutations in Hoxd13 change its ability to bind

interaction partners, immunocytochemistry and coimmunoprecipitation studies with Peg10 and mutant Hoxd13 proteins were performed.

The results showed that the binding of Peg10-ORF2 to Hoxd13 is not affected by different lengths of alanine expansions in Hoxd13, since both Hoxd13+14Ala and Hoxd13_2Ala co-precipitate with Peg10-ORF2.

Immunocytochemistry studies showed that Peg10-ORF2 co-localises with wild type Hoxd13 or Hox13_2Ala in nuclei of COS1 cells. However, overexpression of Peg10-ORF2 together with the pathogenic Hoxd13+14Ala mutant, changes the cellular localisation of both proteins. Peg10 becomes incorporated into Hox aggregates and cannot enter the nucleus anymore. This suggests that the normal function of Peg10 might be abolished. Similarly, other authors proposed that co-localisation with aggregates might alter functions of various proteins (Boutell et al. 1999; Steffan et al. 2000; Albrecht et al. 2004). Therefore, it is possible that the aggregate sequestration of Peg10 and other Hoxd13 binding partners might contribute to the severity of SPD.

4.2.2 Other potential Hoxd13 interaction partners

Five other putative Hoxd13 interaction partners identified in the yeast two-hybrid screen were examined in this study. Two of them, Dlxin-1 and Wtip, do not co-localise with wildtype Hoxd13 in COS1 cells. Similarly, Limk1 does not show any clear colocalisation with Hoxd13 lacking the homeodomain. These data suggest that there is no binding between Hoxd13 and these candidates in COS1 cells.

Two other genes, *Limd1* and *Cnot3*, seem to be much more interesting. Both are expressed in limb buds during mouse embryonic development. Furthermore, the partial Limd1 and Cnot3 proteins co-localise with both Hoxd13-HD and wildtype Hoxd13 in mammalian cells. Cnot3, which is a member of a transcription regulatory complex, might modify Hoxd13 function and influence expression of different target genes. Limd1 is a novel gene, therefore more studies would be necessary to uncover its function. In general, for both candidates further experiments are needed, involving cloning of full length ORFs and subsequent co-localisation and binding studies in mammalian cells.

4.2.3 Outlook

HOXD-associated human disorders are being currently investigated in detail. Findings from the recent few years allowed researchers to identify several mechanisms on the cellular and

the DNA level that contribute to the ethiology of these disorders. However, an important step in better understanding of these processes is the identification of Hoxd13 binding proteins. Several potential Hoxd13 interaction partners were presented in this thesis, however only one of them, Peg10, was analysed in more detail. Future studies should be performed in order to confirm Hoxd13/Peg10 interaction *in vivo*. For this purpose, Peg10-specific antibodies were generated. Furthermore, functional analysis of Peg10 protein could be performed, including generation of *Peg10* $^{-/-}$ and *Peg10*/*Hoxd13* double knockout mice in order to observe the genetic interaction between both partners. Moreover, Limd1 and Cnot3 should be further analysed to confirm or to exclude their ability to bind Hoxd13. Preliminary experiments might be performed *in vitro*, similarly as it was done for Peg10. In case the results are positive, further studies would be needed to confirm the potential interactions *in vivo* and to elucidate cellular pathways in which Limd1 and Cnot3 proteins take part.