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Human limb malformations; an approach to the molecular basis of development

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ABSTRACT Analysis of human inherited limb malformations and of mouse mutants copying individual human mutations team up to promote the understanding of vertebrate limb development as a model for molecular regulatory interactions in animals. The strength of the human genetic contribution lies in the increasingly complete information on the human genome, transcriptome and proteome, as well as in the wealth of individual mutations interfering with limb development available for study. Based on the strong fundament of the human genome project, mapping and identification of novel genes associated with limb defects extends considerably the range of candidates beyond the repertoire of developmental genes and pathways known from animals. Attempts to correlate genotype and phenotype uncover a very broad range of genetic heterogeneity, i.e. different genes underlying the same phenotype, or allelic heterogeneity between families, i.e. clinically distinct phenotypes associated with mutations affecting the same gene. Mechanisms other than simple Mendelian inheritance have to be taken into consideration. Phenotypic variability within families might be explained by different modifying genes or environmental influence, whereas asymmetry of limb defects within one patient may be caused by epigenetic factors, such as somatic mosaicism or X-inactivation, or by non-genetic factors. The intimate knowledge of the genes and events governing limb pattern formation in humans and animals will elucidate the regulatory interactions underlying normal and pathological development, homeostasis, and repair, and thus propose targets for preventive measures and novel approaches to therapeutic intervention in the new era of molecular medicine.

KEY WORDS: *human limb malformations, comparative genetics, polydactyly, genetic heterogeneity, allelic heterogeneity, asymmetry*

The correlation of specific steps in embryo development with gene expression and functional analysis is unveiling the fundamental cellular pathways that pattern tissues and organs to build a whole organism. The analysis of disturbed function in rare human genetic disorders or developmental malformations can provide valuable clues to the normal role of the genes and their products. For human medicine, genes governing development, homeostasis, and repair / regeneration are considered as targets for the development of novel cures for diseases with high prevalence, such as malignancies, rather than specifically for treatment of rare genetic disorders. Functional defects detected in human disorders can be studied in detail in animal models because of the remarkable functional conservation of many of the key patterning molecules and mechanisms between animal and human development.

In humans and experimental animals, studying limb development to identify conserved molecular and cellular interactions which direct the differentiation of cells and tissues has a number of advantages. Genetic developmental malformations of the limbs, in man, in many instances do not interfere with reproductive

Abbreviations used in this paper: BBS, Bardet-Biedl syndrome; CDPX2, chondrodysplasia punctata2, X-linked dominant; CHILD, congenital hemidysplasia with ichthyosiform nevus and limb defects; CHOL, cholesterol; EPB, emopamil binding protein; Ci, cubitus interruptus; GCPS, Greig cephalopolysyndactyly syndrome; Hh, hedgehog; MKKS, McKusick-Kaufman syndrome; NSDHL, NAD(P)H steroid dehydrogenase-like protein; PAPA/B, postaxial polydactyly A/B; PHS, Pallister-Hall syndrome; PKA, protein kinase A; PPIV, preaxial polydactyly type IV; PTC, patched; SHH, sonic hedgehog; SLOS, Smith-Lemli-Opitz syndrome; SMO, smoothened; ZFD, zinc finger domain.

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fitness, yet they are likely to be recognized and reported by a physician. The medical literature is abound of careful descriptions of sporadic or familial phenotypes with developmental malformations of the limbs.

Significant advances in the analysis of genetic determinants for limb morphogenesis have been made in model systems, which has accelerated progress in elucidating the genetic causes underlying a fast growing list of heritable limb malformations in humans. In laboratory animals such as Drosophila, chick or mouse these structures are accessible for functional analysis via genetic or embryologic manipulations, without influencing the viability of the embryo, yet many of the emerging principles can be applied to understanding earlier developmental events, such as specifying the main body axes, and many of the developmental genes are involved in tumourigenesis. In addition, the analysis and comparison of limb development in diverse species has provided much insight into the evolutionary mechanisms through which exchanges in developmental pathways have led to the extraordinary diversity of limbs.

Comparison of human gene maps and the genome draft sequence with genome and sequence data from other organisms, in particular vertebrates such as the pufferfish Fugu or the mouse, has detected an astonishing conservation both in sequence, structure and function of individual genes, and with respect to their map order. Comparative mapping, therefore, can form a valid basis for the integration of genomic and functional knowledge from different organisms. High throughput approaches to catalogue in humans and mice the “transcriptome”, all normal and pathologic gene expression, as well as the “proteome”, all translation products, the results of their posttranslational modification, and their functional interactions are under way. When these projects are finished successfully, detailed understanding of all interactions of genes, regulatory elements, transcription factors and signalling molecules towards patterning during development should be upfront.

Meanwhile, on the common ground of comparative genomics, the study of human congenital limb defects and of limb development in animal models, mutually drives progress towards elucidating at the cellular and molecular level interactions that mediate patterning and the genes directing these processes. This review will describe resources and focus on conclusions which to date the study of human limb malformations contributes to the comparative study of limb development.

Finding Developmental Genes by Comparative Genetics

The mouse has become the pre-eminent mammalian model animal because of sufficient biological similarity with human, the extensive comparative linkage map information, and the ability to manipulate its genome in a targeted or random way (Denny and Justice, 2000). Two conceptually different approaches are followed in developing mouse models for the study of human genetic defects, a disease driven direct genetic approach and a mutagenesis driven, non directed approach.

The first starts from human disease genes in which the causing mutations are identified, and tries then to find the corresponding gene in the mouse and to create either the exact copy of a mutation or a functionally similar situation by altering the mouse genome. The alternative approach, essentially, screens the offspring of mutagenized mice for mutants and tries then to map, identify and characterize the mutated gene (Hardouin and Nagy, 2000). Several ongoing high throughput mutagenesis projects add a wealth of new mutants to the valuable resources generated over many years by classical genetic modification and breeding experiments (review and URLs in Denny and Justice, 2000).

High speed genotypic approaches should soon eliminate a bottleneck in breeding experiments necessary to determine the genetic map positions of the mutated genes in the mouse (Lindblad-Toh *et al.,* 2000).

Irrespective of the approach followed to generate the mouse models for genetic diseases, drawing conclusions on the human situation will depend on the parallel genomic and functional analysis of the orthologous genes in humans and mice. For this comparative approach, human genetics provides both an ordered, almost complete human genome sequence annotated with a rapidly growing resource of functional information, and, for an increasing number of syndromes, comparison of genotype and phenotype in many patients and families representing a great variety of mutations.

Human genetic analysis of limb malformations takes advantage both of the advanced status of the human genome project and the wealth of individual case reports in the medical literature. Various efficient strategies allow to identify a human gene associated with a limb malformation without prior comparative mapping information. The phenotype can be linked to a genetic map interval by segregation analysis in families. Genetic and physical maps such radiation hybrid or cytogenetic maps are well integrated and aligned with the annotated genome sequence. Candidate transcription units in the relevant map segment with the expected expression pattern and predicted functional properties are scrutinized for mutations which might explain the functional deficiencies observed in the patients and thus identify the underlying gene.

When sporadic cases are studied, where linkage studies are not possible, chromosome breakpoints in human cytogenetic rearrangements associated with limb malformations are a valuable help to focus the search for the responsible gene. Candidate genes can also be selected from the fast growing list of factors involved in pattern formation in model organisms. Human orthologues can be pinpointed at all levels of comparative genomics or proteomics.

In support of the search for human genes governing limb development by any of these approaches, biological resources are being collected and distributed, and genomic data, information on mutations, or phenotypic descriptions of syndromes are freely available in public databases (Table 1).

Human Genes associated with Limb Malformations

Human loci patterning limb development identified through associated limb defects are listed in Table ESM1 (http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm) according to their cytogenetic position. As a consequence of different approaches to obtain this map information – genetic mapping, candidate gene analysis in patients, comparative mapping – several entries in the same cytogenetic position might represent phenotypes associated with mutations in the same gene. Several syndromes mapping to the same chromosome region might be caused by different mutations in the same human candidate gene identified by an orthologous mouse gene under study. To assist in the search

for phenotypic overlap, a brief summary of the associated limb malformations is listed with each entry in Table ESM1 (http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm). Detailed descriptions of the phenotypes and links to the original reports can be queried from the OMIM and GENATLAS databases (cf. Table 1).

The Obstacle of Nomenclature

In the medical literature there is no generally accepted standard for classification of particular limb malformations such as polydactylies. Based on patterns of ossification and bony alignment of a duplicated digit or ray, several classifications of the polydactylies have been proposed such as the one by Temtamy and McKusick which distinguishes preaxial, central or postaxial polydactylies and discriminates "fully developed" additional digits (Type A) from "rudimentary" ones (Type B)(Temtamy and McKusick, 1969; Temtamy and McKusick, 1978). OMIM adopts this nomenclature. Surgeons favour a more descriptive approach to the elements of duplicated digits, which is considered to be more informative in planning cosmetic or functional reconstruction of the malformation (reviewed by Graham and Ress, 1998).

When a phenotypic entity such as "polydactyly" observed in a mutant animal is used to query OMIM, a startling number of entries will appear. Reports listed under a common syndrome name can combine phenotypes caused by mutations in different genes, such as Bardet-Biedl Syndrome (BBS, MIM 209900). As soon as genetic evidence indicates that different loci are involved, a discriminatory suffix is used (e.g. BBS1 to BBS6) (Table ESM1 at http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm).

Alternatively, several clinically distinct syndromes may turn out to be caused by mutation in the same gene. Neighbouring mutations of the zinc finger gene GLI3, e.g., cause either preaxial polydactyly (PP IV), postaxial polydactyly (PAPA/B), Greig cephalopolysyndactyly (GCPS) with preaxial and/or postaxial polydactyly, and Pallister-Hall syndrome (PHS) associated with central polydactyly (Table ESM1 at http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm). To highlight the affected gene rather than variable morphological details, all these syndromes can be "lumped" using the term "GLI3 morphopathies" (Radhakrishna *et al.,* 1999). However, in order to stress the importance of the morphological phenotype, clinical geneticists tend to prefer a stance of "splitters", allocating different syndrome names to variant phenotypes caused by mutations in the same gene (Biesecker, 1998).

In addition, historically, different denominators have been used for the same clinical entity. OMIM accomodates this incongruency of syndrome designations by listing aliases in addition to a preferred name. In Table ESM1 (http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm) mapped human developmental defects are named according to OMIM.

With regard to gene symbols the confusion caused by variant nomenclature is annoying, as well. HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/) approved gene symbols should be used throughout. Aliases for these official gene symbols can also be searched in GDB and GENATLAS databases.

Polydactylies as Examples of Heterogeneity

A major contribution of human genetics to the studies of limb development is that it extends the view from the individual to the population level. Soon after a candidate gene underlying a specific defect is identified, many unrelated individuals with a similar phenotype become available for study. Generally, a series of different mutations in this gene is detected, the position and the functional consequences of which can elucidate the molecular and cellular mechanisms leading to the phenotype. Searching for mutations in a multitude of patients allows to identify and study heterogeneity, a phenomenon which is relevant both for understanding a disease in the human population and for elucidating the network of interacting factors generating a phenotype. *Genetic heterogeneity* can be invoked, when mutations in a candidate gene are detected only in part of phenotypically identical cases, and *allelic heterogeneity* appears, when mutations in the same candidate gene are associated with several clinically distinct phenotypes. It is specifically the study of phenomena resulting in heterogeneity that human genetics contributes to the analysis of limb development

TABLE 1

HUMAN MATERIALS AND DATABASES FOR COMPARATIVE GENETICS HUMAN GENES, MAPS AND TRAITS

Online Mendelian Inheritance in Man (OMIM)	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=&DB=omim
Entrez Map Viewer	http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez hum_srch?chr=hum_chr.inf&query
Genome Database (GDB)	http://gdbwww.gdb.org/
Human Gene Mutation Database (HGMD), Cardiff	http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html
Genatlas	http://www.dsi.univ-paris5.fr/genatlas/
GeneClinics	http://www.geneclinics.org/
Genetic Alliance	http://www.geneticalliance.org/
Genes and Disease	http://www.ncbi.nlm.nih.gov/disease/
Search for novel human candidate genes	
UniGene	http://www.ncbi.nlm.nih.gov/UniGene/index.html
Human Genome Browser	http://genome.ucsc.edu/cgi-bin/hgGateway
NIX – identify unknown nucleic sequence	http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/
Comparative mapping	
LocusLink	http://www.ncbi.nlm.nih.gov/LocusLink/
Online Mendelian Inheritance in Animals (OMIA)	http://www.angis.su.oz.au/Databases/BIRX/omia/
Human-mouse Dysmorphology database (DHMHD)	http://www.hgmp.mrc.ac.uk/DHMHD/dysmorph.html
Davis Human/Mouse Homology Map	http://www.ncbi.nlm.nih.gov/Homology/Davis/
Homophila	http://homophila.sdsc.edu
Human cell materials and recombinant clones	
HGMP GenomeWeb Materials & Culture collections	http://www.hgmp.mrc.ac.uk/GenomeWeb/culture-collections.html
Coriell Cell Repositories	http://locus.umdnj.edu/ccr/
IH Centre for Inherited Disease Research	http://www.cidr.jhmi.edu/
Mendelian Cytogenetics Network (MCN)	http://mcndb.imbg.ku.dk
National Human Genome Research Institute (NHGRI)	http://www.nhgri.nih.gov/
HUM-MOLGEN	http://www.hum-molgen.de/
Developmental Studies Hybridoma Bank	http://www.uiowa.edu/~dshbwww/

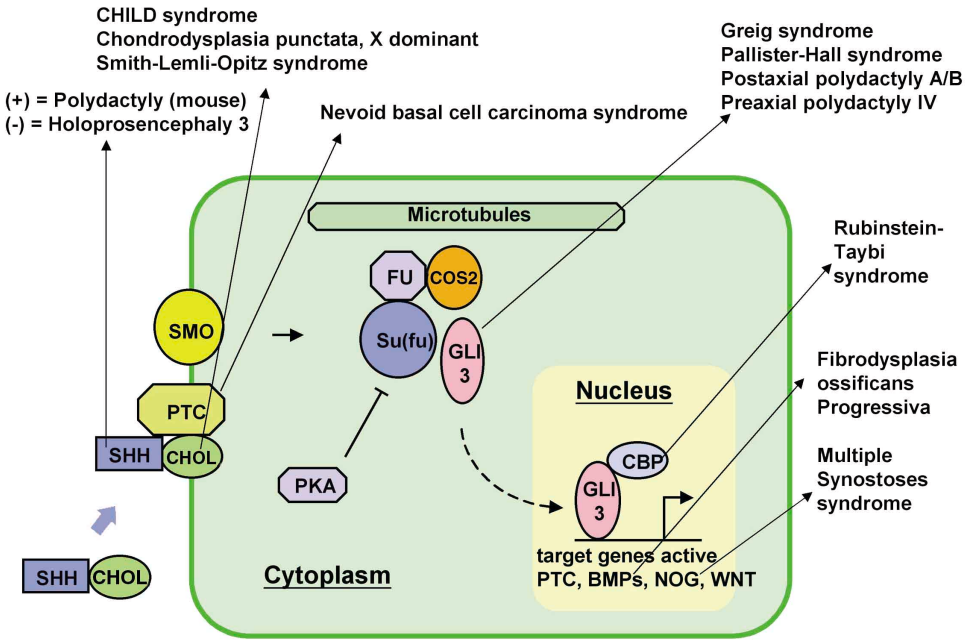

Fig. 1. Schematic presentation of factors involved in Sonic Hedgehog (SHH) signal transduction in a recipient cell, and their association with human developmental defects such as polydactylies. *To generate an active signalling molecule, SHH is cleaved and bound to cholesterol (CHOL). The signal is received and transmitted into the cell by the action of the transmembrane proteins Patched (PTC) and Smoothened (SMO). Signal reception is believed to result in the release of GLI3 from a microtubule bound complex with the proteins FU, COS2, and SU(FU), and transfer into the nucleus where target genes are activated. Protein Kinase A (PKA) appears to be involved in this process (Hammerschmidt* et al., *1997; Ingham and McMahon, 2001) Limb phenotypes of the syndromes are listed in Table ESM1 at http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm*

To review molecular mechanisms underlying heterogeneity, we choose the phenotype "polydactyly".

Polydactylies are associated with disturbances in the network of genes that control anterior/posterior patterning of the limb. The understanding of the genetic interactions that are involved in patterning of vertebrate limbs has been greatly advanced by the observation that many of the developmentally critical genes and genetic pathways of *Drosophila* have been conserved in vertebrates (Platt *et al.,* 1997). Animal development employs localized sources of secreted Hedgehog (Hh) molecules as signals to organize pattern along an anterior/posterior (A/P) axis (reviewed extensively, e. g. by Hammerschmidt *et al.,* 1997; Ingham and McMahon, 2001).

The notion, that the study of polydactylies, disturbances in anterior/posterior limb patterning in humans, can uncover generally used molecular mechanisms attributing specific fates to animal cells during development has gained credibility by the finding, that the genes affected in these disorders are vertebrate homologues of the Hh signalling pathway genes described in *Drosophila*. Our description of mutations in GLI3, a homologue of the Drosophila transcription factor Ci which transforms Hh signals to transcriptional regulation of target genes, to cause Greig syndrome associated with polydactyly in humans (Vortkamp *et al.,* 1991) opened up this research avenue and, at the same time, shed light on the gene family of *GLI* genes as key regulators of patterning. Figure 1 summarizes key players in the regulatory program initiated by sonic hedgehog (SHH) signals from the posterior flank of the vertebrate limb bud resulting in transcriptional control of target genes by GLI1, GLI2, and GLI3 proteins. Human limb malformation syndromes known to be associated with these factors, are indicated.

Comparing mapped genes causing syndromes associated with polydactyly in Table ESM1 (http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm) with the ones shown in Fig. 1 demonstrates that the factors and molecular interactions determining the number of fingers or toes in humans by far can not be accommodated by the simplistic view of an exclusive role of SHH signalling.

Genetic Heterogeneity

In Table ESM1 (http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm) genetic syndromes associated with limb malformations are arranged according to their chromosome map position. When genetic heterogeneity of a phenotype has been observed, different symbols and map positions are listed for the same syndrome (e.g. Bardet-Biedl syndrome, BBS, MIM 209900). BBS is a heterogeneous autosomal recessive disorder characterized by central obesity, pigmentary retinopathy, polydactyly, renal malformations, learning difficulties, and hypogenitalism. Other features that vary in frequency include diabetes mellitus, hypertension and congenital heart disease (Nishimura *et al.,* 2001). The estimated population prevalence varies from 1/13.500 among the Bedouin of Kuwait (Farag and Teebi, 1989) to 1/100.000 in Western Europe (Klein and Ammann, 1969). Six *BBS* loci have been mapped to date: *BBS1* (MIM 209901) on 11q13 (Leppert *et al.,* 1994), *BBS2* (MIM 606151) on 16q21 (Kwitek-Black *et al.,* 1993), *BBS3* (MIM 600151) on 3p13-p12 (Sheffield *et al.,* 1994), *BBS4* (MIM 600374) on 15q22.3-q23 (Carmi *et al.,* 1995), *BBS5* (MIM 603650) on 2q31 (Young *et al.,* 1999), and *BBS6* (MIM 209900) on 20p12 (Katsanis *et al.,* 2000; Slavotinek *et al.,* 2000). A seventh *BBS* locus has been postulated on a few small BBS pedigrees that do not appear to map to any of the known loci (Beales *et al.,* 2001).

For three of the syndromes the mutated genes have been identified: BBS6 is caused by mutations in the McKusick-Kaufman syndrome gene (Katsanis *et al.,* 2000; Slavotinek *et al.,* 2000)(MKKS, hydrometrocolpos, post-axial polydactyly, congenital heart defects, Stone *et al.,* 2000). The identification of *MKKS* mutations as a cause for BBS6 underlines the value of the positional cloning approach which is based on map positions as listed in Table ESM1 (http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm). Both syndrome phenotypes mapped to 20p1. Partial phenotypic overlap such as the occurrence of polydactyly in both syndromes, successfully, suggested the MKKS gene as a

candidate for *BBS6*. BBS2 and BBS4 are associated with mutations in novel genes (designated *BBS2* and *BBS4*) with unidentified functions (Mykytyn *et al.,* 2001; Nishimura *et al.,* 2001). The MKKS gene product has sequence homology to the alpha subunit of a prokaryotic chaperonin (Katsanis *et al.,* 2000; Slavotinek *et al.,* 2000) but a functional relationship with the proteins affected in BBS2 and BBS4 is not yet evident, nor is understood how the *BBS* genes might interfere with anterior/posterior signalling in the limb bud.

This example of the mutations in different *BBS* genes resulting in the same phenotype demonstrates, that following up genetic heterogeneity of a human syndrome can guide to a functionally related group of novel genes involved in limb patterning, which were not previously heralded by studies in model organisms.

Triallelic Inheritance

Whereas Mendelian inheritance is mostly taken for granted in the study of developmental genes, mutation analysis of all three *BBS* genes identified to time in a series of BBS families, surprisingly, has provided evidence for a complex inheritance for this syndrome which was thought to be transmitted as an autosomal recessive trait (Katsanis *et al.,* 2001b): Two allelic mutations in one *BBS* gene and a third mutation in another *BBS* gene are required for the disease phenotype to become manifest. The authors call this phenomenon triallelic inheritance. Burghes and colleagues (2001) proposed instead "recessive inheritance with a modifier of penetrance". Triallelic inheritance may represent a transmission model that bridges classic Mendelian disorders with complex traits (Katsanis, *et al.,* 2001a).

Modifiers might be unrelated in sequence to the genes they affect but influence the same function, as in BBS, or else they might be redundant, paralogous genes contributing in a quantitative fashion to the function, as might be the case for *GLI3* and *GLI2* genes.

Allelic Heterogeneity

To outline the phenomenon of allelic heterogeneity of polydactyly syndromes we review the molecular analyses of phenotypes associated with mutations in the GLI3 transcription factor. Vertebrates have three paralogous *Gli* genes, *Gli1, Gli2*, and *Gli3*, the products of which share sequence homology with the Cubitus interruptus (Ci) protein in *Drosophila*. Analysis of the expression pattern and molecular studies of *Gli* genes in vertebrates led to the theory that the dual potential combined in the Ci molecule, transcriptional activation or repression, might be separated in GLI proteins, GLI1 being an activator, but GLI3 preferentially a repressor (Ruiz i Altaba, 1999). GLI1 might lack N-terminal sequences which are implicated in repression by Ci (Ruiz i Altaba, 1997; Liu *et al.,* 1998). Within this gene family only mutations in *GLI3,* so far, appear to be associated with polydactyly in humans: in the Greig cephalopolysyndactyly syndrome (GCPS, MIM175700) showing both pre- and/or postaxial polydactyly (Wild *et al.,* 1997), the postaxial polydactyly, type A/B (PAPA/B, MIM174200) (Radhakrishna *et al.,* 1997), the Pallister-Hall syndrome (PHS,

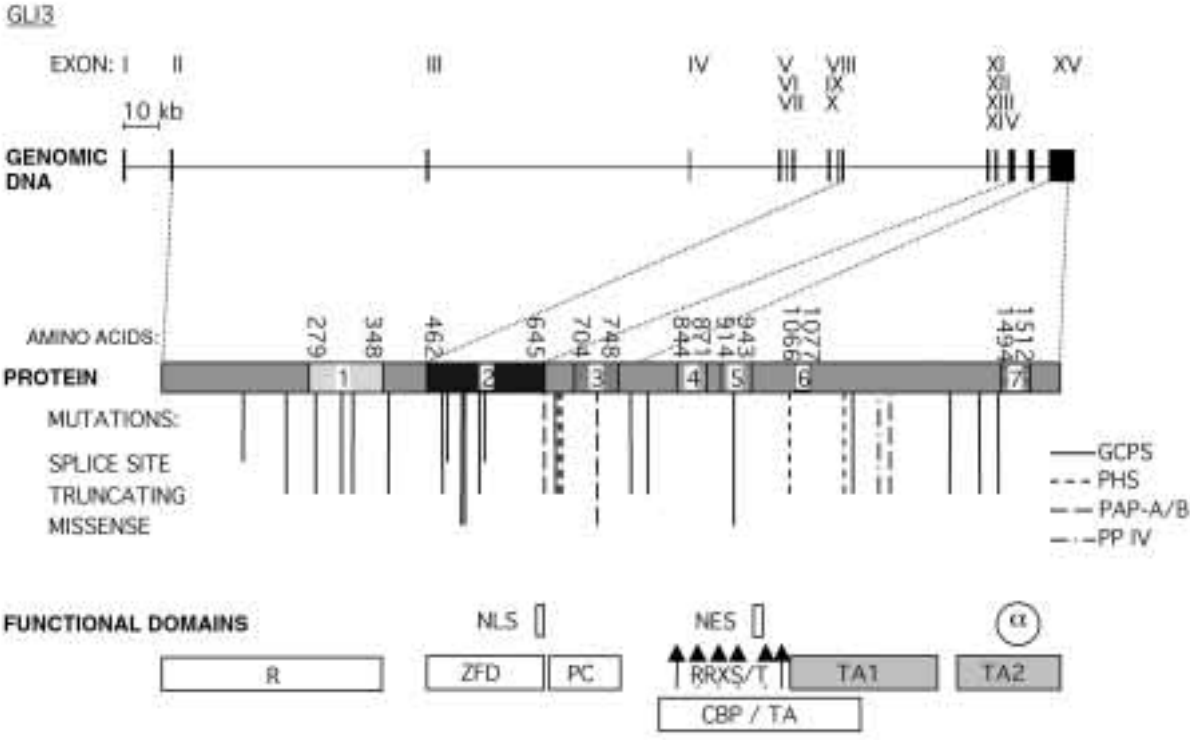

Fig. 2. Diagram indicating the type of point mutations in the *GLI3* gene observed in four different polydactyly-syndromes GCPS, PHS, PAPA/B, PPIV at the position in which the protein is affected. *In the bar representing the protein, regions of sequence homology in the GLI family (1-7) are shown. To indicate the lack of obvious genotype-phenotype correlation, putative and experimentally proven functionally important sites of the protein are shown below (R, repression domain; ZFD, DNA binding zinc finger domain; PC, protein cleavage site; RRXS/T, phosphorylation sites; CBP/TA, CBP-binding/transactivating domain; TA1 and 2, transactivating domains; NLS/NES, nuclear localization/export signals; α, α–helical domain. The diagram of the genomic structure of the* GLI3 *gene (above) shows that about half of the protein is encoded in a single exon XV.*

MIM146510) characterized by central polydactyly (Kang *et al.,* 1997), and the preaxial polydactyly, type IV (PPIV, MIM174700) (Radhakrishna *et al.,* 1999). Based on the role of Ci in the wing embryonal disk of *Drosophila* and the sites of the first *GLI3* point mutations described to cause GCPS, PHS, or PAPA/B, Biesecker (1997) proposed that complete loss of function of one copy of *GLI3* might cause GCPS, that a GLI3 molecule truncated C-terminal of the zinc finger region (ZFD), retaining the potential to bind to specific DNA-sequences as well as the putative N-terminal repressor region, might cause PHS, whereas a truncation leaving intact a more C-terminal domain potentially binding GLI3 to the microtubuli could cause polydactyly type A.

Inspection of a larger series of *GLI3*-mutations in different GLI3-morphopathies (Kalff-Suske *et al.,* 1999, and Fig. 2), indeed, does not reveal mutations in patients with PHS N-terminal of the zinc finger region (ZFD). However, mutations truncating the open reading frame in GCPS, PAPA/B, and PPIV, a preaxial polydactyly associated with *GLI3* mutations, are located throughout the coding sequence. GCPS, in particular, is associated with truncating mutations not only in the N-terminal half but also on the C-terminal side of the zinc fingers and even close to the C-terminus. Unless mRNA decay prevents translation of the mutated transcripts exclusively in GCPS, the derived truncated proteins should retain an intact zinc finger region as well as the predicted repressor domain at the N-terminus.

When an obvious connection between the loss of specific functional domains and distinct phenotypes can not be drawn, often, modifier genes or stochastic events are invoked. However, both explanations are not easily compatible with the observation that the phenotypes are consistent within individual families, i. e. only one type of GLI3 morphopathy each, with preaxial or postaxial polydactyly, is observed in different pedigrees in which mutations in neighbouring positions segregate (Radhakrishna *et al.,* 1997; Radhakrishna *et al.,* 1999).

Asymmetry of Limb Malformations

The wealth of individual reports of limb deformities in humans allows conclusions about mechanisms governing asymmetry in the phenotypic expression between the right and the left side of an individual.

The "invention" of the neural crest leading to the true vertebrates among the chordates is considered the deciding event that imposed permanent lateral asymmetry on the visceral situs, leaving paraxial structures such as the locomotory appendages in a relatively undisturbed, metameric, bilaterally symmetrical arrangement. This inherent symmetry, frequently, is disrupted in malformations: As a rule, skeletal dysplasias, in which there is a generalized abnormality in bone or cartilage, have far more symmetrical pattern of involvement than the dysostoses, in which the skeletal involvement is predominantly manifested in abnormalities of individual bones or in a group of bones. All dysostoses, especially limb malformations, must be regarded as asymmetrical until proven otherwise (Opitz and Utkus, 2001).

As an example, only of 1/4 of the cases of sporadic fibular a/hypoplasia are bilateral, in 62% of the unilateral cases the right side is affected, in 38% the left (Lewin and Opitz, 1986).

Observing unilateral involvement in tibial deficiency, frequently associated with a high degree of preaxial polydactyly, even in dominantly inherited cases, Wiedemann and Opitz (1983) invoked a mechanism of "developmental resistance". This concept applies to many discrepancies in normally symmetrical traits, identified in humans by careful, quantitative morphometric studies (fluctuating asymmetry) (Clarke, 1992). Discrepancies are thought to appear when the "developmental resistance" responsible for "developmental homeostasis", the stability inherent in complex developmental processes, is overrun by genetic and/or environmental disturbances (Livshits and Kobyliansky, 1991; Parsons, 1992; Clarke, 1993).

Limbs can be regarded monozygotic twins paralleling the frequent non-concordance in monozygotic twins with limb anomalies (Richieri-Costa and Opitz, 1986; Utkus *et al.,* 2001). Epigenetic as well as non-genetic factors can be invoked.

By breeding congenic lines of guinea pigs for over 30 years and carefully documenting phenotypes and potentially important parameters S. Wright (1984) could determine the contribution of non genetic factors in a model of multifactorial segregation resulting in the highly heterogeneous phenotype of atavistic restoration of a little toe in pure-bred animal lines: 4 classes contributed roughly equally to variance: 1) factors common to all siblings (permanent environmentally induced differences among the mothers), 2) fac-

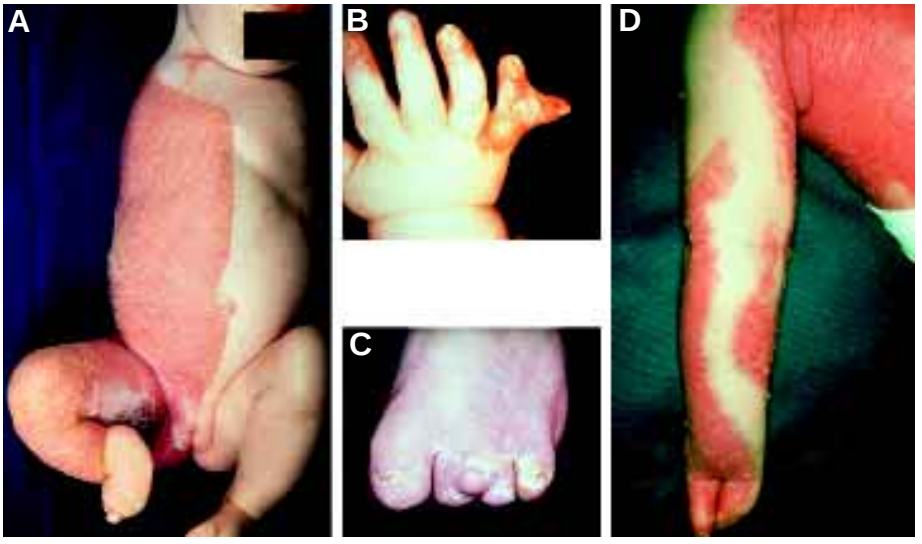

Fig. 3. CHILD syndrome (Congenital Hemidysplasia with Ichthyosiform nevus and Limb Defects) affects one half of the body. *Depending on the distribution of clones in which the mutated X-chromosome is the active one, different limb malformations result, such as lateralized aplasia of the upper limb and dysplasia of the lower limb* **(A)**, *postaxial polydactyly* **(B)**, *brachydactyly/syndactyly* **(C)**, *split hand* **(D)**. *Courtesy of Drs. Happle and Koenig, Marburg, and Dr. Yaguchi, Tokyo.*

tors common only to the littermates (temporary environmentally induced differences among the mothers), 3) factors peculiar to individuals (such as delay in implantation), and 4) developmental accidents relating separately to each foot (Wright, 1984).

Among the epigenetic causes underlying asymmetries are lyonization, somatic/clonal mosaicism, mosaic aneuploidy/polyploidy, and chimaerism.

Mosaic asymmetries are most easily observed and demonstrated in heterozygotes of X-linked conditions as a consequence of Lyonization (Opitz and Utkus, 2001).

One particularly instructive example is CHILD syndrome (Congenital Hemidysplasia with Ichthyosiform nevus and Limb Defects), a male lethal X-linked condition, which shows predominant unilateral involvement (Happle *et al.,* 1980; Happle *et al.,* 1995) (Fig. 3). CHILD syndrome (MIM308050) is caused by mutations in *NSDHL* (NAD(P)H steroid dehydrogenase-like protein) located at Xq28 encoding a 3ß-hydroxysteroid dehydrogenase. (Happle *et al.,* 2000; Koenig *et al.,* 2000). The exceptional occurrence of CHILD syndrome in a boy with a normal chromosome constitution 46, XY (Happle *et al.,* 1996) can be explained either by somatic mosaicism resulting from an early postzygotic mutation or a gametic half-chromatid mutation (Lenz, 1975; Happle, 1993).

Two X-linked dominant male-lethal traits, bare patches (Bpa) (Phillips *et al.,* 1973) and striated (Str) (Phillips, 1963) had previously been associated with mutations in *Nsdhl* (Liu *et al.,* 1999). The human CHILD phenotype is characterized by an inflammatory nevus with striking lateralization and strict midline demarcation, as well as ipsilateral hypoplasia of the body (Happle *et al.,* 1980; Happle *et al.,* 1995). The lateralization is not evident in the mouse mutants (Phillips, 1963; Phillips *et al.,* 1973; Liu *et al.,* 1999). NSDHL functions in the distal Kandutsch-Russell pathway for cholesterol biosynthesis upstream of EBP (emopamil binding protein) mapping at Xp11.22-p11.23. This protein acts as a Δ8-Δ7 sterol isomerase and is defective in CDPX2 (chondrodysplasia punctata 2, X-linked dominant) another syndrome with limb defects. A third developmental defect which also involves the limbs, Smith-Lemli-Opitz syndrome (SLOS), is associated with mutations affecting the last step in this pathway, a 7-dehydrocholesterol reductase.

These three syndromes and several more rare developmental disorders suspected to be associated with other defects in cholesterol biosynthesis identify a novel group of «metabolic» malformation syndromes. Cholesterol is found as an essential component in cell and mitochondrial membranes and in the layers that make up the myelin sheaths in the central and peripheral nervous system (Clayton, 1998; Farese and Herz, 1998). Its singular hydroxyl group permits the formation of ester bonds employed for lipid transport and the storage of cholesterol. Recently, it has been discovered that cholesterol is important in the control of embryonic development for Hedgehog signalling, both as a partner for the activation of the signalling molecule itself and for the transduction of the signal (Cooper *et al.,* 1998; Incardona and Eaton, 2000; Mann and Beachy, 2000; Ingham and McMahon, 2001, c. f. Fig. 1).

This led to the notion that the severe developmental malformations in CHILD syndrome as well as in CDPX2 and SLOS may originate from impaired signalling by human hedgehog paralogues, in particular Sonic Hedgehog (SHH) and Indian Hedgehog (IHH) (Herman, 2000; Koenig *et al.,* 2000; Mann and Beachy, 2000).

The phenotypic differences among these disorders as well as from their mouse homologues indicate that the deficiencies might not simply be due to reduction in cholesterol levels but rather to the inability of (aberrant) sterol precursors to perform the multiple tasks of cholesterol properly (Incardona and Eaton, 2000; Incardona *et al.,* 2000).

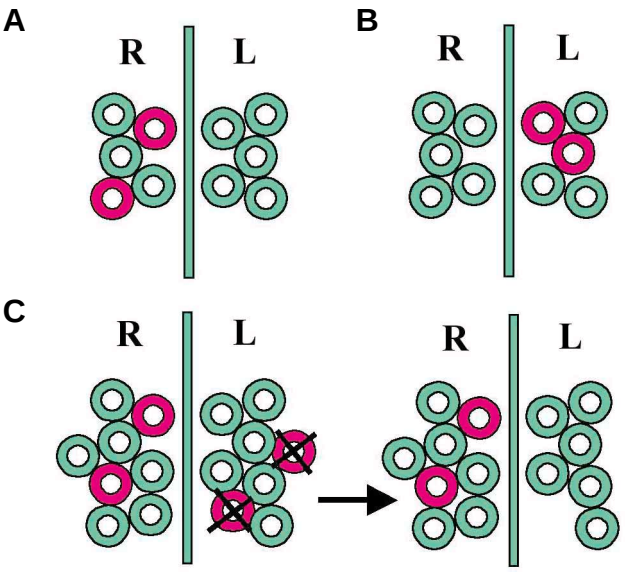

Fig. 4. Model for lateralized developmental defects. *Cells close to the node on either side of the primitive streak are symbolized. R, right; L, left.* **(A,B)**. *Random X-inactivation: critical role of very few enzyme-deficient organizer cells distributed by chance preferentially to one or the other side?* **(C)** *Random X-inactivation: selection against active mutated X-chromosome, if cells on one side need hedgehog signals for development?*

This notion is corroborated by observations that compounds resembling cholesterol in structure, such as the steroidal alkaloid cyclopamine and its related compound jervine act as teratogens, which block SHH signalling at the level of signal transduction resulting in phenocopies of Hedgehog signalling defects (Mann and Beachy, 2000; Gofflot *et al.,* 2001).

In CHILD syndrome the enzymatic block affects NSDHL, one factor of a complex of 3 separate enzyme activities demethylating in the pathway from lanosterol to lathosterol a 4.4-dimethyl-compound in two sequential reactions. There, the 3ß-hydroxysteroid dehydrogenase activity encoded by NSDHL, potentially, might be responsible for the replacement of an intermediate carboxyl group by a 3-keto group, which, in the next step, would be reduced to a ß-hydroxysterol, thus generating the only reactive hydroxyl moiety for ester formation in the cholesterol molecule (Baudry *et al.,* 2001). A cholesterol precursor without the potential to form esters might impair signalling through Hedgehog proteins and thus explain the dysplasias observed in CHILD syndrome.

Even though related, the clinical phenotypes resulting from mutations in *NSDHL* or *EBP* are distinctly different (Koenig *et al.,* 2000). The most striking dissimilarity is the lateralization of the defects observed in CHILD syndrome, contrasting with the classical bilateral Lyonization pattern found in CDPX2 (Happle, 1979; Happle *et al.,* 1980).

Organizer cells with either a wild type or a mutated *NSDHL* on the active X-chromosome might determine a large developmental field including the skin, the bones, the brain, the kidney, and other organs on one side of the body (Fig. 4 A,B). However, if both cell

types, *NSDHL*$^{+}$ or $^{-}$ are a priori equally distributed throughout the early embryo, a strictly unilateral expression of the dysplasia in female patients and the exceptional male might be explained by selection against cells deficient in SHH signalling in the unaffected half of the body, preferentially the left side, allowing the wild type cells to proliferate (Fig. 4C). Indeed, the requirement for SHH in signals originating from the node is restricted to the left of the midline (Capdevila *et al.,* 2000; Mercola and Levin, 2001).

This example demonstrates that to understand the mechanisms causing asymmetry of limb malformations, perhaps not only in CHILD syndrome, the interactions of signals determining left-right pattern formation with patterns emerging from random X-chromosome inactivation have to be determined.

Electronic Supplementary Material for this paper, entitled *"Mapped human loci associated with limb defects"* is available at the following address:
http://www.ijdb.ehu.es/abstract.0207/esm1grzeschik.htm

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