REVIEW

Mutations in gene regulatory elements linked to human limb malformations

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ABSTRACT

Most of the human genome has a regulatory function in gene expression. The technological progress made in recent years permitted the revision of old and discovery of new mutations outside of the protein-coding regions that do affect human limb morphology. Steadily increasing discovery rate of such mutations suggests that until now the largely neglected part of the genome rises to its well-deserved prominence. In this review, we describe the recent technological advances permitting this unprecedented advance in identifying non-coding mutations. We especially focus on the mutations in *cis*-regulatory elements such as enhancers, and *trans*-regulatory elements such as miRNA and long non-coding RNA, linked to hereditary or inborn limb defects. We also discuss the role of chromatin organisation and enhancer–promoter interactions in the aetiology of limb malformations.

INTRODUCTION

Congenital limb malformations are a group of developmental disorders with an estimated prevalence of 1 in 500 of live births.^{1,2} They may occur in isolation or be associated with additional defects.^{3,4} Major risk factors leading to limb abnormalities include exposure to teratogens or genome alterations.^{2,5} Studies of congenital limb malformation have allowed to better understand the causative role of genes in limb development. Intriguingly, different mutations within one gene may lead to distinct skeletal phenotypes, whereas mutations in various genes of the same developmental pathway may result in similar or identical limb abnormalities (reviewed in ref 6). Importantly, an increasing number of mutations identified outside of the protein-coding areas support the notion that alterations in non-coding regulatory regions may lead to similar skeletal defects as mutations in their target genes. For instance, mutations in enhancers involved in regulation of the short stature homeobox-containing gene (*SHOX*) result in its downregulation, which leads to similar or identical skeletal phenotype as caused by loss-of-function mutations in one allele of the *SHOX*.⁷ In both cases, the level of *SHOX* mRNA is significantly reduced, which affects limb development.⁸ Several mutations in *SHOX* enhancers have been characterised as molecular factors involved in the aetiology of *SHOX*-related disorders, which is described in detail in the ‘Mutations in enhancers linked to human limb malformations’ section.

A vertebrate limb emerges from the lateral plate mesoderm and develops under the coordinated

action of three signalling centres. The zone of polarising activity, expressing sonic hedgehog (*Shh*), regulates the anterior-posterior axis of limb formation. The apical ectodermal ridge engages FGF (Fibroblast Growth Factor) signalling pathways, controlling the proximal-distal axis. Finally, the wingless-type MMTV integration site family, member 7A (*WNT7A*) signal from the dorsal ectoderm is responsible for the coordination of the dorsoventral limb axis.⁹ The crosstalk and coordination among these signalling centres are thus crucial for the correct limb patterning. During the evolution, the differences in the timing and intensity of the expression of genes involved in these signalling pathways are partly responsible for the distinct limb morphology among species.¹⁰ Within one species, however, even discrete changes to the spatiotemporal regulation of gene expression caused by mutation in regulatory elements frequently result in limb malformations. For instance, alterations of the zone of polarising activity regulatory sequence (ZRS) involved in regulation of *SHH* result in a range of skeletal abnormalities caused by ectopic expression of *SHH*.^{11–13} Thus, it becomes apparent that not only the protein-coding regions but also the regulatory apparatus is critical for the limb shape diversity.

In this review, we provide an update on the recent technological developments and the results of studies on the role of mutations in *cis*-regulatory and *trans*-regulatory elements affecting limb development.

ENHANCERS AND ENHANCER–PROMOTER INTERACTIONS IN REGULATION OF GENE EXPRESSION

Enhancers are regulatory elements that coordinate the spatiotemporal gene expression by interaction with gene promoters.¹⁴ They are located genome-wide, within introns, intergenic regions, gene desert areas or even gene exons.¹⁵ Enhancers can control the expression of target genes at very variable distance, in an orientation-independent manner, and sometimes across the chromosomes.¹⁶ Therefore, they may act in *cis* (within the same chromosome) or in *trans* (between chromosomes). Enhancers provide a structural scaffold for transcription factors (TFs) and cofactors, bridging them to promoters.^{17,18}

Promoters are regulatory elements located upstream from transcription start sites and are involved in the initiation of gene transcription by recruitment of TFs and RNA polymerase. The function of an enhancer is thus to augment the level of expression of its target gene.¹⁴ Consequently,

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mutations in enhancers and reorganisation of chromatin three-dimensional (3D) architecture that affect the enhancer–promoter interaction have been correlated with developmental disorders, including limb malformations.^{5 12}

Development of next-generation sequencing (NGS) techniques has resulted in the identification of many novel enhancers and helped in mapping of their interaction with target promoters. Several strategies have been developed to interrogate the enhancer regulatory regions and enhancer–promoter connections. The first one examines DNA accessibility using two techniques—DNase I hypersensitive sites sequencing or assay for transposase-accessible chromatin using sequencing—to investigate the ‘open state’ of chromatin structure which is correlated with the active enhancers and promoters.¹⁹ The second one is focused on the analysis of interactions between chromatin regions, among others allowing to map the physical enhancer–promoter connections. It applies the chromatin interaction analysis by paired-end tag sequencing as well as the chromatin conformation capture (3C) or its several variants, including 4C, 5C, T2C and Hi-C.²⁰ The third strategy interrogates the promoter-specific and enhancer-specific epigenetic marks using chromatin immunoprecipitation followed by sequencing. This technique examines histone modifications (eg, H3K27ac or H3K4me1 specific for enhancers and H3K4me3 characteristic for promoters), as well as cofactors and TFs interacting with either enhancer regions or gene promoters (eg, p300 specific for enhancers; CCCTC-binding factor (CTCF) for insulators and so on).^{21 22} The abovementioned techniques are frequently used in parallel to better map regulatory elements and enhancer–promoter connections.

Large-scale epigenetic analysis of human and mouse genomes has identified three different states of enhancers: primed, active or poised. Each of the states is characterised by a specific profile of histone modifications, for instance, primed enhancers are enriched in H3K4me1.²³ They are able to bind pioneer TFs, proteins that play a role in blocking DNA methylation, recruiting chromatin modifiers and repositioning the nucleosomes. Thus, the pioneer TFs increase the accessibility of the regions for other TFs.²⁴ Interestingly, primed enhancers are associated with inactive genes, ‘waiting’ for a specific trigger prior to transcription.²³ Active enhancers are enriched in H3K27ac and H3K4me1 and also bind pioneer TFs and coactivators. One of the coactivators interacting with active enhancers is histone acetyltransferase p300, an enzyme involved in the regulation of chromatin structure by promoting chromatin decompaction.²¹ Analysis of DNase I hypersensitive sites showed that they are located within the ‘open structure’ regions of chromatin, suggesting that this state is involved in the regulation of gene expression.²⁵

The signature of poised enhancers has been characterised by enrichment of H3K4me1 and low level of H3K27ac. It is also marked by the interaction with polycomb repressive complex 2 (PRC2) and the presence of H3K27me3. The poised enhancers have been associated with early development and pluripotent cells, such as human and mouse embryonic stem cells.²³ Nevertheless, the role of enhancers at the poised state is poorly understood. They interact with gene promoters but they are unable to initiate their expression. The inactive state of poised enhancers is lost during the differentiation, and is correlated with loss of H3K27me3 and increase of H3K27ac, suggesting that PRC2 may be involved in specific regulation of this type of enhancers.^{23 26}

Currently, enhancers are considered as transcriptional units producing unique enhancer RNAs (eRNAs).²⁷ The eRNAs differ from other functional non-coding RNAs, such as long non-coding RNAs (lncRNAs) or miRNAs. They are typically shorter

than 2 kb, highly unstable, not polyadenylated RNAs without additional post-transcriptional modifications.²⁸ In addition, eRNAs are typically bidirectionally produced and are characterised by a low-level expression, which can be measured by CAGE (cap analysis of gene expression) or PRO-cap (a precision nuclear run-on sequencing variant). eRNA transcription is a mark of active enhancers and is used to help in identifying functional enhancers.²⁸ Despite the unstable nature of eRNAs, they have been shown to be biologically relevant. For example, the recruitment of the RNAP II (RNA Polymerase II) to the promoter of myogenic differentiation 1 (*MYOD1*) was shown to be facilitated by eRNAs.²⁹

Mutations in enhancers linked to human limb malformations

Brachydactylies (BDs) are one of the examples linking limb malformation to mutations in enhancers. Several types of BDs have been classified from A to E. They are characterised by shortening of selected fingers and/or toes.³⁰ Most of BDs were correlated with mutations in protein-coding regions. For instance, BD type E (DBE; MIM: 113300) was genetically linked to mutations in the open reading frame of gene encoding homeobox D13 (*HOXD13*).⁴ An interesting exception is a 17-member family with DBE where no mutations in *HOXD13* protein-coding sequence were identified, suggesting that mutations in the non-coding regions may be responsible for this phenotype. High-resolution array comparative genome hybridisation analysis has identified a novel ~440 kb microdeletion within the so-called regulatory archipelago of HOXD cluster (table 1).³¹ This regulatory region is located centromeric to HOXD cluster and contains several limb-specific enhancers.³² Generation of mice harbouring a similar mutation resulted in shortening of metacarpals, recapitulating the human phenotype. Deletion of the enhancers located in the regulatory archipelago in the mouse led to low expression of *Hoxd13* (down to ~10%), which is most likely responsible for the phenotype.^{31 32}

The next skeletal disorder associated with enhancer mutations is split hand/foot malformation 1 (SHFM1; MIM: 183600). It is a rare, inherited disorder characterised by aplasia/hypoplasia of phalanges, metacarpals and metatarsals, lack of selected digits, and cleft of hands and feet. SHFM1 is also correlated with deafness, intellectual disability and craniofacial malformations.^{33 34} Seven independent genetic loci have been linked to this malformation so far, including the 7q21.3 region.³⁴ Reporter studies in zebrafish and mice have revealed that this region encompasses two conserved limb-specific enhancers.³⁵ They are located in exons 15 and 17 of dynein cytoplasmic 1 intermediate chain 1, *DYNC1I1* gene. Analysis of 134 unrelated family cohort revealed that alteration in these enhancers led to limb malformation (table 1).³⁶ The mechanism of action is most likely based on the misregulation of their target genes: distal-less homeobox 5 (*DLX5*) and distal-less homeobox 6 (*DLX6*). Importantly, both the *DLX5* and *DLX6* have been previously linked to SHFM type I.³⁵

One of the most prominent examples of non-coding mutations linked to skeletal disorders is located in ZRS, a highly conserved, limb-specific intragenic enhancer, located around 1 Mb upstream of gene *SHH*.¹¹ Mutations within this region lead to ectopic expression of *SHH*, which results in alteration of the anterior-posterior axis and digits patterning. Interestingly, distinct size mutations in ZRS region may lead to different phenotypes (table 1). For instance, two microduplications (~255 kb and ~179 kb) have been linked to Hass-type polysyndactyly (MIM: 186200), characterised by supernumerary fingers

Table 1 Non-coding variants linked to human limb malformations

Type of regulatory or topological element	Name of element/region	Target gene	Size and type of variant	Variant localisation	Phenotype	Reference
Enhancer	ZRS	<i>SHH</i>	255 kb dup	Chr7:156,437,229–156,692,706 dup (hg19)	Hass-type polysyndactyly (MIM: 186200)	12
			179 kb dup	Chr7:156,491,887–156,671,016 dup (hg19)		
			75 kb dup	Chr7:156,570,780–156,646,750 dup (hg19)	Laurin-Sandrow syndrome (MIM: 135750)	
			47 kb dup	Chr7:156,563,856–156,610,632 dup (hg19)		
			16 kb dup	Chr7:156,578,108–156,594,751 dup (hg19)		
		SNVs		LMBR1:c.423+5252A>G	Tibial hemimelia-polydactyly-triphalangeal thumb syndrome (MIM: 188740)	ClinVar variation IDs: 4902, 4903, 126371, 4900, 4906 ¹³
				LMBR1:c.423+5134C>G		
				LMBR1:c.423+4915C>T		
				LMBR1:c.423+4842T>C		
				LMBR1:c.423+4808T>C		
		SNVs		LMBR1:c.423+5252A>G	Preaxial polydactyly (MIM: 174500)	ClinVar variation IDs: 4902, 515097, 4903, 4898, 126371, 4907, 30497, 4900, 4899, 30496, 4906, 4897, 426635 ⁹⁵
				LMBR1:c.423+5176A>G		
				LMBR1:c.423+5134C>G		
				LMBR1:c.423+4917G>A		
				LMBR1:c.423+4915C>T		
	eExons of DYNC111	<i>DLX5, DLX6</i>	167 kb del	Chr7:95,615,187–95,783,313 (hg19)	Split hand/foot malformation (MIM: 183600)	36
			510 kb del	Chr7:95,624,825–96,135,521 (hg19)		
			205 kb del	Chr7: 95,667,046–95,872,044 (hg19)		
			169 kb del	Chr7:95,693,341–95,862,369 (hg19)		
		<i>HOXD13</i>	~440 kb del	Chr2:176,065,894–176,504,173 (hg19)*	Brachydactyly type E (MIM: 113300)	31
		<i>SHOX</i>	~423 dup	ChrX:8 09 125–1 232 802 (hg19)*	Leri-Weill dyschondrosteosis (MIM: 127300)	8
			~290 kb dup	ChrX:6 75 000–9 64 000 (hg19)*		
			~103 kb dup	ChrX:4 40 364–5 43 359 (hg19)*		
			~47.5 kb del	Xp22.33 chrX:7 00 549–7 48 093, (GRCh37)		
		CNE3, CNE5 CNE3, CNE5 CNE9	~286 kb del	ChrY:1 24 349–4 09 949 (GRCh36)*	Idiopathic short stature (MIM: 300582)	38 8
			~287 kb dup	ChrX:2 19 634–5 06 573 (hg19)*		
			~113 dup	ChrX:8 28 453–9 41 084*		
	CTCF	Boundary site of PAX3 TAD and upstream TAD	~1.75–1.9 Mb dup	2q35-36†	Brachydactyly	5
TAD	17q24.3 region	<i>KCNJ2</i>	~2 Mb dup	17q24.3†	Cooks syndrome (MIM: 106995)	3
	2q35 region	<i>IHH</i>	~900 kb dup	2q35†	Polysyndactyly	5
	2q36 region	<i>WNT6</i>	1.1 Mb inv	2q36†	Feingold syndrome (MIM: 164280)	
	2q36 region		1.5M dup	2q36†		
miRNA	miR-17~92		165 kb del	13q31.3†	Feingold syndrome (MIM: 164280)	69
	miR199 and miR214	<i>SP7, SMAD1</i>	~2.4 .Mb del	Chr1:168,007,914–170 448 198 (hg18)*	1q24q25 deletion syndrome	72
	RMRP-S1 and RMRP-S2	Multiple targets including <i>PTCH2</i> and <i>BMP2</i>	70A>G	9p13†	Cartilage-hair hypoplasia (MIM: 250250)	77
			Compound heterozygosity for a ~25–5 dup and a 146G>A			
lncRNA	HOXC-AS2 and HOXC-AS3	<i>HOXD</i> genes	175 kb del	Chr12:54,165,001–54,335,668 (hg19)	Congenital vertical talus	93

*Minimal range of mutation based on aCGH.

†Location based on aCGH. The authors did not provide exact breaking points.

aCGH, array comparative genome hybridisation; CNE, non-coding DNA element; CTCF, CCCTC-binding factor; del, deletion; dup, duplication; inv, inversion; lncRNA, long non-coding RNA; SNV, single nucleotide variant; TAD, topologically associating domain; ZRS, zone of polarising activity regulatory sequence.

with cup-shaped syndactyly of hands.¹² Another group of micro-duplications (~75 kb, ~47 kb, ~16 kb) has been associated with the Laurin-Sandrow syndrome (MIM: 135750), characterised by polysyndactyly of hands, overlapping with Hass-type polysyndactyly, but also additional skeletal defects such as mirror image polysyndactyly of feet, duplication of fibula with lack of tibia and nasal defects.¹² Finally, point mutations within ZRS have been associated with tibial hemimelia-polydactyly-triphalangeal thumb syndrome (MIM: 188740), characterised by short lower limbs with lack of tibias, polydactyly and digitalisation of the thumbs.¹³ Also patients with preaxial polydactyly, postaxial polydactyly, syndactyly and triphalangeal thumb have been linked to point mutation in ZRS (MIM: 174500).¹¹ Correlation between the size of mutations and diverse skeletal phenotypes may suggest that not only alteration in ZRS but also disruption of local chromatin architecture may play a key role in the aetiology of limb malformations. However, in contrast to predictions, the genetic manipulations of the local chromatin structure of the ZRS-SHH promoter region have shown limited or no change of gene regulation during limb development and no limb malformations in animals with rearranged chromatin architecture.³⁷

SHOX-related congenital limb disorders have also been linked to enhancer mutations. Alterations in enhancers located within *SHOX* genomic region have been reported in Leri-Weill dyschondrosteosis (LWD; MIM: 127300) and idiopathic short stature (ISS; MIM: 300582).⁷ The phenotypic spectrum of these disorders includes short stature, mesomelia and Madelung deformity of the wrist.^{7,8} Further comparative genomic and functional studies of the *SHOX* cis-regulatory landscape have identified eight downstream and three upstream conserved non-coding DNA elements (CNEs) with enhancer activity. Four of these produce eRNA (CNE4, CNE5, CNE7 (ECR1) and CNE9 (ECS4)).⁸ Importantly, mutations in the CNEs affect the formation and growth of limb bones. They most likely lead to defects in *SHOX* expression, resulting in skeletal phenotypes (table 1). For instance, a ~47.5 kb downstream deletion identified in 30 patients with LWD, or ISS, encompasses the CNE7 regulatory element, leading to misregulation of *SHOX*.⁷ In another case, a ~286 kb deletion of two upstream enhancers has been associated with the ISS phenotype of two probands. Both patients have been reported with haploinsufficiency of the *SHOX*.³⁸ Moreover, duplication of CNEs may also lead to a reduction of the *SHOX* mRNA level and cause the skeletal phenotype. For example, duplication encompassing upstream cis-regulatory elements CNE3 and CNE5 has been reported in one family with ISS.⁸ Another duplication including CNE9 has been associated with the phenotype of four patients with ISS.⁸ Moreover, a ~290 kb duplication affecting all of the identified upstream CNEs was reported in one family with LWD.⁸ Other cases of LWD were associated with duplications of downstream CNE8, CNE9 and X:970 000 putative element.⁸ The mechanism of pathogenic effects is most likely based on the disruption of local chromatin architecture, which affects the long-range interaction between cis-regulatory elements and *SHOX* promoter.

CHROMATIN ORGANISATION AND TOPOLOGICALLY ASSOCIATING DOMAINS

Studies of 3D chromatin structure have provided new insights into the role of chromatin architecture in the gene regulation and enhancer specificity. Chromatin is organised in the 3D nuclear space in a specific fashion. Each chromosome is located in a definite nucleus location, termed chromosome territory.³⁹ Additionally, analysis of Hi-C contact maps has shown

that mammalian genomes are organised into different-sized topologically associating domains (TADs), delineated by the boundaries enriched in CTCF binding sites. The size of TADs may be hundreds of kilobases up to several millions bases, with average length of ~880 kb.⁴⁰ CTCF is an insulator protein which inhibits the interaction between enhancers and gene promoters (figure 1). Thus, binding of CTCF proteins defines the TAD boundaries and is responsible for a specific pattern of enhancer-promoter interaction, which when mutated may lead to limb malformations.^{5,41} Polymer simulations and chromatin architecture studies have discovered that intra-TAD interactions are dynamic,⁴² whereas the TAD organisation within the genome is highly stable and conserved across cell types, tissues and related species.^{5,43} Based on the loop extrusion model, TADs are formed due to the cohesin-CTCF-dependent chromatin loop formation.^{39,44} The process begins with attaching a cohesin complex to the chromatin. The ring structure of cohesin complex allows interacting with two chromatin regions at the same time leading to formation and extrusion of a chromatin loop until the cohesin reaches the CTCF boundary sites.^{40–42} Therefore, the interactions between cis-regulatory elements and gene promoters are coordinated by both the presence of cohesin complex and CTCF binding sites. Importantly, the deletion of CTCF binding sites causes loss of the ‘insulation effect’, resulting in TAD rearrangements and gene misregulation, correlated with the aetiology of some limb malformations.^{5,45,46} However, it is important to mention that despite overwhelming evidence about the role of TAD boundaries in gene regulation, the genome-wide depletion of CTCF binding has a limited effect on global gene transcription (described in ref 47). Additionally, the deletion of CTCF binding sites located at the TAD boundaries around *Sox9* and *Kcnj2* loci has caused an increase in inter-TAD interaction without TADs’ fusion. The deletions of all major intra-TAD CTCF binding sites resulted in TAD fusion, suggesting that CTCF sites located at the boundaries as well as intra-CTCF sites play a role in TAD formation.⁴⁷ Surprisingly, it was shown that fusion of *Sox9-Kcnj2* TADs caused by deletion of CTCF binding sites has not caused strong changes in gene expression of the genes, suggesting that in this case TAD boundaries were non-essential for gene regulation. However, inversions and/or insertions of *Sox9-Kcnj2* TADs’ boundaries resulted in gene misexpression.⁴⁷ Therefore, it suggests that CTCF located at TAD boundaries may not be always essential for gene regulation; however, when redirected, they may result in gene misexpression.⁴⁷

The chromatin architecture also has an impact on gene expression by controlling the accessibility of proteins to transcriptionally active or inactive genomic regions. Interestingly, while the transcriptionally active chromatin tends to localise away from the nuclear envelope, transcriptionally silent chromatin locates within the nuclear space close to the membrane and interact with the nuclear lamina (NL).³⁹ The chromatin localised near the NL is termed lamina-associated domains and is marked by the features typical for heterochromatin, such as absence or low transcription level, and H3K27me3 histone modification.⁴⁸ Collectively, the chromatin compaction together with its architecture and nuclear localisation play a crucial role in gene regulation, which when disrupted may be responsible for the molecular aetiology of limb abnormalities.

Disruption of TADs leads to deregulation of genes and hereditary diseases

Studies of three unrelated patient families with a novel type of BD have revealed that deletion of CTCF binding site led to local

Figure 1 Chromatin organisation in gene regulation. (A) At the nuclear level, chromatin is organised in chromosomes, which occupy the defined territories. At the chromosomal level, chromatin is organised in TADs. The TAD organisation is stable and conserved among tissues and species, while intra-TAD interactions are highly dynamic. The cohesin complex and CTCF are the main factors which play a role in the formation of TADs and intra-TAD interactions. (B) TADs have been originally discovered by Hi-C technique. They act as regulatory units within which *cis*-regulatory elements can interact with gene promotes. The interaction between regulatory elements from neighbour TADs is inhibited by boundary sides separating TADs, which are enriched in CTCF binding regions. The ChIP-Seq together with ATAC-seq and functional assay data have helped to characterise the content of regulatory elements within the TADs. Several epigenetic marks may be used to identify active enhancers, such as histone H3 Lys27 acetylation (H3K27ac) or histone H3 Lys4 monomethylation (H3K4me1). Other marks may be used to interrogate promoter regions, such as histone H3 Lys4 trimethylation (H3K4me3). ATAC-seq, assay for transposase-accessible chromatin using sequencing; ChIP-seq, chromatin immunoprecipitation followed by sequencing; CTCF, CCCTC-binding factor; TAD, topologically associating domains.

gene deregulation. Specifically, the 4C-seq data have shown that absence of CTCF boundary site separating paired box 3 (*PAX3*) from the next TAD put *PAX3* under the control of normally not accessible enhancer, therefore rewiring the enhancers–*PAX3* promoter interaction (table 1).⁵ Such interaction is termed ‘enhancer adoption’ and may be caused either by a fusion of two TADs or formation of a new TAD with the novel regulatory landscape. Importantly, the generation of mice harbouring a similar deletion resulted in upregulation of *Pax3* and BD-like phenotype, indicating that deregulation of *PAX3*, caused by rewiring enhancer–promoter interactions, plays a crucial role in the skeletal phenotype.⁵

Cooks syndrome (MIM: 106995) is another disorder linking reorganisation of TADs with the aetiology of limb malformations.³ It is an autosomal dominant ectodermal dysplasia characterised by brachydactyly, lack of phalanges, absence of the nails and abnormal length of phalanges.⁴⁹ Genetic analysis of seven affected individuals revealed that ~2Mb duplications in the 17q24.3 region led to the disruption of local chromatin architecture and formation of a new TAD (table 1). This mutation changed the regulatory landscape of the potassium voltage-gated channel subfamily J member 2 (*KCNJ2*), leading to its misexpression.³ Functional studies of inwardly rectifying K⁺ channel *Irk2* (Irk2), a *KCNJ2* *Drosophila* homologue, have shown that *Irk2* is involved in Dpp/BMP (Decapentaplegic/Bone Morphogenetic Proteins) signalling.⁵⁰ Since several mutations in the components of the BMP pathway play a role in the aetiology of skeletal disorders, it is tempting to suggest that defect in the expression of *KCNJ2* may be an important factor in this limb malformation.

Feingold syndrome (F-syndrome; MIM: 164280) is a limb disorder which has also been linked to alteration in the chromatin architecture of TADs.⁵ Additionally, F-syndrome has been correlated with mutation in *MYCN* proto-oncogene (*MYCN*) and microdeletion encompassing miR-17~92 region (detailed explanation in the ‘Disruption of miRNA genes leads to limb abnormalities’ section). Thus this is an example of a condition when the same phenotype is induced by different types of mutations. It is an autosomal dominant disorder described by short stature, thumb hypoplasia, brachymesophalangy of the second and fifth fingers, microcephaly, and toe syndactyly. Additionally, F-syndrome has been correlated with learning disability, heart and kidney anomalies, hearing loss and asplenia.^{51 52} The 4C data analysis of two unrelated families has shown that 1.1 Mb inversion or 1.5 Mb duplication led to alterations in chromatin architecture, affecting TAD organisation (table 1). Thus, rearrangement of chromatin structure led to disruption of the Wnt family member 6 (*WNT6*) *cis*-regulatory landscape, causing its deregulation by rewiring enhancer–promoter connections.⁵ The *WNT6* is involved in chondrogenesis and bone formation, and its ectopic expression has been associated with limb malformation in chicken.⁵³ Finally, reconstruction of the 1.1 Mb inversion in mice resulted in enhancer adoption and ectopic expression of *Wnt6* in distal limb autopod mesenchyme.⁵ Altogether, it strongly suggests the causative role of *WNT6* misexpression in the aetiology of F-syndrome.

Studies of two probands with a polysyndactyly have correlated rearrangements within TADs with limb malformation. Polysyndactyly is a common skeletal defect characterised by an additional

Figure 2 Role of miRNA in post-transcriptional regulation of gene expression. (A) After transcription and maturation, the miRNAs interact with several proteins (eg, AGO and TNRC6) leading to the assembly of RNA-induced silencing complex (RISC). (B) The miRNA is involved in the coordination of mRNA level via initiation of targeted deadenylation followed by mRNA degradation. (C) The miRNAs regulate gene expression of target genes due to a negative influence on the proper formation of initiation complex, leading to translational repression. (D) A small fraction of 'free' miRNA in the cytoplasm is caused by lncRNA 'sponging', which affects the dynamics of miRNA–mRNA interaction events and regulates gene expression either negatively or positively depending on the target mRNA. AGO, argonaute; lncRNA, long non-coding RNAs; RISC, RNA-induced silencing complex.

number of fingers and/or toes that are connected by interdigital webbing.⁵⁴ The 4C data analysis of two human samples carrying a ~900 kb duplication encompassing Indian hedgehog (*IHH*) locus (2q35) has revealed that this mutation led to shift of *IHH* gene to *EPHA4*-containing TAD (table 1). Thereby, transposition of *IHH* to a different TAD led to enhancer adoption and *IHH* misexpression,⁵ and patients carrying this mutation have been also diagnosed with craniofacial abnormalities. The ~600 kb deletion region encompassing *Ihh* locus (2q35) in mice also resulted in *Ihh* enhancer adoption, causing severe polydactyly and craniofacial dysmorphism,⁵⁵ altogether supporting the role of chromatin rearrangements in the aetiology of polysyndactyly.

MIRNAS: POST-TRANSCRIPTIONAL GENE REGULATORS

miRNAs are small, ~22 nucleotides long, non-coding RNAs.⁵⁶ Depending on the origin, miRNAs are synthesised by canonical or non-canonical pathways (reviewed in ref 57). They act as *trans*-regulatory elements, coordinating gene expression through two main mechanisms: translational repression, or mRNA deadenylation followed by decay (figure 2).^{52 58}

The miRNAs cooperate with several proteins leading to the assembly of the RNA-induced silencing complex (RISC). Once miRNA attach to argonaute (AGO) protein, the RISC is able to locate cognate mRNA and usually binds to its 3'UTR region. The target site is recognised by the complementarity with 2-7nt/2-8nt miRNA 'seed sequence'.^{59 60} Another molecule interacting with AGO is TNRC6/GW182. It recruits the CCR4–NOT and PAN2–PAN3 deadenylase complexes responsible for the shortening of the 3'-poly(A) tail of mRNA.^{58 59} Moreover, the TNRC6/GW182 and AGO interact with proteins involved in removing the mRNA 5'–7methylguanosine cap initiating targeted degradation of mRNA.^{61 62} The RISC is also involved in the inhibition of the formation of translation initiation complex, crucial for proper interaction of mRNA with the ribosome.^{52 63–65}

Disruption of miRNA genes leads to limb abnormalities

Added level of regulatory complexity is caused by the fact that a single miRNA may target several genes, whereas one gene can be regulated by different miRNAs.⁶⁶ In those cases, miRNAs compete for target mRNA with other class of non-coding RNAs, such as lncRNAs (reviewed in ref 67). The crosstalk between miRNAs, lncRNAs and mRNAs creates a complex gene regulatory network, which when disrupted by mutation or misexpression of miRNA may lead to disorders, including limb malformations.

One of the examples linking limb abnormalities to mutations in miRNAs is the aforementioned F-syndrome (MIM: 164280). As previously described, this disorder was correlated with mutations in the MYCN and disruption of *WNT6* *cis*-regulatory landscape caused by chromatin rearrangement.^{5 51 68} Nevertheless, the analysis of high-resolution comparative genomic hybridisation data from 10 patients with F-syndrome has identified that microdeletion affecting miR-17~92 region also plays a crucial role in the molecular aetiology of this disorder.⁶⁹ Reconstruction of the mutation in the mouse model led to brachymesophalangy of the second and fifth digits, similar to the patient's phenotype. The biallelic mutation of miR-17~92 was lethal. Mouse embryos (miR-17~92Δ/Δ) had hypoplasia of the mesophalanx of the second digit and lack of the mesophalanx of the fifth digit.⁶⁹ Since the patients with F-syndrome skeletal defects have been characterised to carry mutations either in MYCN or miR-17~92, or to have altered *WNT6* expression caused by TAD reorganisation, it is tempting to speculate that these three components may be involved in the same developmental pathway. Indeed, the MYCN may directly bind to the region coding miR-17~92 leading to its transcriptional activation.⁶⁹ Furthermore, the studies of neuroblastoma cell lines with or without expression of MYCN suggest that MYCN is involved in the regulation of *WNT6*.⁷⁰ Altogether, these data strongly imply

that mutations in all abovementioned regions are involved in the molecular aetiology of F-syndrome through the modulation of WNT6-dependent pathway.

Another example of limb malformation linked to a mutation in miRNAs is 1q24q25 deletion syndrome. It is a rare inherited disorder characterised by short stature, small hands and feet, brachydactyly, clinodactyly, microcephaly, and severe cognitive disability.⁷¹ Currently, only a few patients with skeletal abnormalities and lack of cognitive disability have been reported, suggesting that microdeletion narrowed to the dynamin 3 (*DNM3*) region could play a vital role in the skeletal phenotype (table 1).⁷² Yet the expression of *DNM3* mRNA was detected at high levels in the brain and low levels in the skeleton, excluding the *DNM3* gene as a potential candidate for the syndrome.⁷¹ Interestingly, two miRNAs (miR199 and miR214) are encoded within intron 14 of *DNM3*. The miR214 is involved in the inhibition of osteogenic differentiation through regulation of osteoblast-specific transcription factor SP7, whereas miR199 affects BMP signalling pathway by targeting SMAD1.^{73 74} Moreover, alteration of the miR199-miR214 cluster in a mouse model led to short stature and cranial deformity, partially recapitulating patients' phenotype and strongly suggesting the role of the miR199-miR214 cluster in the aetiology of limb malformation of 1q24q25 deletion syndrome.⁷⁵

Cartilage-hair hypoplasia (CHH; MIM: 250250) is another example of limb disorder associated with the misexpression of miRNA (table 1). It is an autosomal recessive disorder characterised by severe short-limbed dwarfism, metaphyseal dysplasia, immunodeficiency, predisposition to cancers and severe anaemia. The phenotypes are linked to mutations in *RMRP*, a gene encoding one of the units of RNase MRP ribonucleoprotein complex.^{76 77} Interestingly, expression analysis of fibroblast and B cells from patients with CHH has revealed that alteration in *RMRP* expression led to a significant reduction of two miRNAs, the *RMRP*-S1 and *RMRP*-S2. Both miRNAs are involved in the regulation of more than 900 genes including downregulation of

patched 2 (*PTCH2*), which results in induction of SHH signalling and downregulation of morphogenetic protein receptor type 2 (*BMPR2*) decreasing the effective BMP signalling in limb development.⁷⁷ Collectively, it suggests that miRNAs may be important factors in the skeletal phenotype of CHH.

LONG NON-CODING RNAs

lncRNAs are a class of functional RNAs longer than 200 bp and devoid of protein-coding capability. They are typically tissue-specific with characteristic spatiotemporal expression pattern.⁷⁸ A number of studies have shown that lncRNAs regulate various biological processes including chromatin remodelling, epigenetic modification of DNA, transcription, translation or mRNA splicing (figure 3).^{79–81} Importantly, they can affect gene expression not only by acting in *cis* but also in *trans* (reviewed in ref 78). Thus, lncRNAs most likely function as a structural scaffold, recruiting regulatory complexes or interacting with the gene promoter itself. For instance, ANRIL (antisense non-coding RNA in the *INK4* locus) lncRNA, by recruiting polycomb complex PRC2, leads to repression of the p15^{INK4B} gene,⁸² whereas the EZR-AS1 by interaction with histone-lysine N-methyltransferase SMYD3 causes enrichment of H3K4me3 in the Ezrin (*EZR*) promoter region, leading to higher expression of *EZR*.⁸³ Another lncRNA, derived from the region upstream of gene dihydrofolate reductase (*DHFR*), acts by the formation of a stable complex with *DHFR* major promoter, repressing its expression.⁸⁴ Additionally, lncRNAs through bridging TFs to gene regulatory elements coordinate expression of multiple genes at the same time. For example, E2F1 TF due to interaction with lncRNA-HIT modulates the expression of its target genes, such as *FOXM1*, S-phase kinase associated protein 2 (*SKP2*), neural EGFL like 2 (*NELL2*) and docking protein 1 (*DOK1*).⁸⁵ Besides transcription control, lncRNAs are also involved in the regulation of gene expression at the post-transcriptional level. For instance, MALAT1 lncRNA coordinates alternative splicing of mRNA by interaction with

Figure 3 Role of lncRNA in the regulation of gene expression. (A) The lncRNAs indirectly regulate epigenetic silencing of gene expression by bringing silencing complexes (PRC2) to target genes. (B) The lncRNAs interact with TFs and transport them to gene promoters, modulating the transcription of specific genes. (C) The lncRNA via interaction with SR (Serine/Arginine-rich) splicing factors facilitates the alternative splicing. (D) Several lncRNAs interact with ribosome complexes, which suggests their regulatory roles in the translation process. lncRNA, long non-coding RNA; TF, transcription factor.

SR splicing factors.⁸¹ Another lncRNA 5S-OT affects splicing due to interplay with U2AF65 splicing factor.⁸⁶ Additionally, numerous lncRNAs participate in staufen (STAU1)-mediated mRNA decay. They are mainly involved in the formation of the binding site for STAU1 protein.^{79–87} lncRNAs play also a role in the crosstalk between competing endogenous RNAs, within the mRNA-miRNA-lncRNA regulation network.⁸⁸ For instance, they can function as a 'sponge' for miRNAs or compete with miRNAs for mRNA binding sites. For instance, MALAT1 and ANCR lncRNAs play a vital role in the modulation of osteogenic differentiation, due to 'sponging' of miRNA-143 and miRNA-758.^{89–90}

Intriguingly, the lncRNA synthesis process itself may regulate the gene expression. For example, the transcription of Airn lncRNA affects the expression of the insulin-like growth factor 2 receptor (*IGF2R*) gene and does not require Airn transcript. The mechanism of action is based on the transcriptional overlap of the promoter region of the target gene, which reduces the RNA polymerase II recruitment.⁹¹

Potential role of lncRNA genes in the development of limb abnormalities

Currently, more than 51 000 lncRNA genes have been annotated in the human genome.⁹² Taking under the consideration the functional role of lncRNAs in gene regulation, it opens a possibility that alteration in lncRNAs may play a role in limb malformations.

Genetic characterisation of patients with clubfoot and congenital vertical talus supports this hypothesis; both disorders are closely related and may occur in the same family or individual. The former is characterised by rigid flatfoot deformity, the latter is manifested by the inward position of the foot/feet.⁹³ Analysis of Affymetrix Genome-Wide Human SNP Array V6.0 and multiplexed direct genomic selection from 226 individuals with clubfoot and 27 with congenital vertical talus pointed out four deletions in the 5' of *HOXC* gene cluster, ranging from 13 kb to 175 kb (table 1).⁹³ Several lncRNAs are located within this region, such as HOTAIR, HOXC-AS2, HOXC-AS3 and HOXC13-AS. The HOTAIR plays a role in the repression of *HOXD10* gene by guiding PRC2 silencing complexes to its region, and mutation in *HOXD10* has been linked with congenital vertical talus.⁹⁴ The gene expression analysis in a patient with 175 kb deletion revealed a significant reduction of *HOXD10* and two lncRNAs, HOXC-AS2 and HOXC-AS3. Despite the fact that expression of HOTAIR was unaffected, it could be possible that other lncRNAs located in 5' of *HOXC* gene cluster might play a role in the regulation of *HOXD* genes.⁹³ Altogether, it is suggestive that alteration of *trans*-regulation of the *HOXD* genes due to misexpression or mutation in lncRNAs may be involved in the aetiology of clubfoot and congenital vertical talus.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recent technological advances in NGS-based methods resulted in the discovery of new layers of gene regulation. It also allowed for the identification of numerous mutations in gene regulatory elements linked to congenital limb disorders. Therefore, the examination of regulatory elements, or regulome, provides answers not only to the basic science questions but importantly gives clinically relevant information applicable to the diagnosis of patients. Better understanding of *cis/trans* regulatory elements may also lead to the development of potentially promising pharmacological solutions in limb malformations treatment. For instance, a small molecule targeting enhancers regions could be

applied for correction of gene misexpression, whereas targeting of miRNA and lncRNA could be used to specific regulation of genes.

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