Chapter 4

Molecular Defects and Cellular Dysfunctions in Restricted Growth Conditions

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Additional information is available at the end of the chapter

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Abstract

Restricted growth (RG) or dwarfism is a varied phenotype ascribable to many different causes, most of which are genetic. Conditions associated with disproportionate short stature (DSS) are usually caused by *de novo* dominant mutations in genes coding for proteins involved in cartilage/bone development. Rarer conditions, which may occur in inbred families, show an autosomal recessive inheritance. Causative mutations, consequent to cellular dysfunctions, genotype-to-phenotype correlations in RG conditions such as achondroplasia, hypochondroplasia, thanatophoric dysplasia, severe achondroplasia with delay in development and acanthosis nigricans, pseudoachondroplasia, multiple epiphyseal dysplasia, diastrophic dysplasia, achondrogenesis, and osteogenesis imperfecta, are discussed in this chapter.

Keywords: dwarfism, cartilage, bone, chondrocyte, osteoblast

1. Introduction

Human height is a genetically complex phenotype. In recent years, several genome-wide association studies (GWAS) have collectively identified hundreds of common variants with a putative effect on determining adult height [1]. The variability between individuals has a normal distribution; extremes in height are often caused by monogenic mutations in genes involved in growth control. Gain in height in children is determined by the rate of endochondral ossification, i.e. the rate of proliferation of chondrocytes at the growth plate, a thin layer of cartilage that is found in most bones, other than skull and facial bones. Newly generated cartilage tissue is remodeled into bone tissue; as new bone is progressively created at the growth plate, bones grow longer and children grow taller. At puberty increasing levels of

estrogen, in both females and males, lead to increased apoptosis of chondrocytes in the growth plate; growth slows down and later stops when the entire cartilage has become replaced by bone, leaving only a thin epiphyseal scar. Systemic factors such as growth and thyroid hormones provide important signals for the regulation of cartilage/bone growth by modulating expression of locally produced factors, such as tissue-specific transcription factors (e.g. short stature homeobox-containing factor, SHOX), multiple fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), secreted signaling factors such as Wnts, and many others. Cartilage extracellular matrix components, secreted by chondrocytes, also play a crucial role in regulating growth plate activity. Dysfunctions in any of the multiple players in this complex process may cause genetic growth disorders. Gene mutations affecting various stages of the bone formation process, e.g. osteoblast differentiation, bone extracellular matrix deposition and mineralization, may as well result in substantial growth deficiency, a hallmark feature of osteogenesis imperfecta, a molecularly heterogeneous group of connective tissue disorders.

In this chapter, we will describe some paradigmatic conditions of restricted growth from the cell biologist's perspective. We will first consider various *cartilage disorders* and then some *bone disorders*. Our approach will start from the description of gene defects, the cellular dysfunctions they cause, their consequences on the extracellular matrix, and finally we will describe briefly the associated phenotypes, trying to compare, whenever possible, similar conditions.

2. Cartilage disorders associated with impaired height

Chondrodysplasias causing dwarfism comprise a group of skeletal disorders associated with improper regulation of cartilage growth during endochondral ossification (see **Figure 1** for a

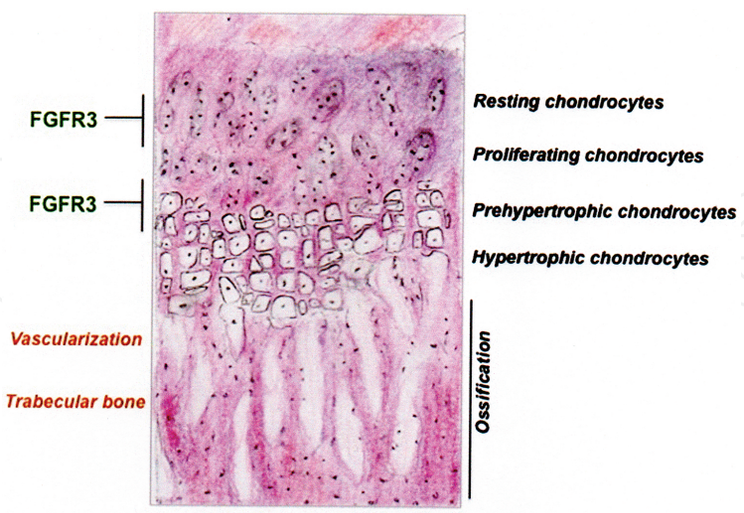

Figure 1. Endochondral ossification. Bone elongation is a tightly regulated process leading to the formation of a cartilage template subsequently replaced by bone. During the process, resting chondrocytes of the growth plates mature into proliferating chondrocytes, which in turn differentiate into prehypertrophic and then hypertrophic chondrocytes. The latter die by apoptosis and are replaced by trabecular bone, as a result of the following vascularization and ossification processes. FGFR3 plays a key role as negative regulator of chondrocytes proliferation and differentiation.

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simplified sketch of the process). Although this notion had been widely accepted for a long time, the idea that morphological assessment of the growth plate could be used to distinguish among the different disorders was successfully proposed only in the 1970s [2]. This brought to the concept of "chondrodysplasia families" formulated in the 1980s and the hypothesis that chondrodysplasias that look similar could be pathogenetically related. But it was only in the 1990s, with the advent of molecular genetics identifying the mutated genes associated with different chondrodysplasias, that in many instances chondrodysplasia family disorders turned out to be caused by mutations within the same gene, indeed [3].

2.1. FGFR signaling defects

Dwarf-associated chondrodysplasias are caused by genetic alterations in the *Fibroblast Growth Factor* (*FGF*) *Receptor 3* (*FGFR3*) gene and include achondroplasia (ACH), hypochondroplasia (HCH), thanatophoric dysplasia types I and II (TDI and TDII), and SADDAN [4]. A summary of the most recurrent mutations is shown in **Figure 2**. FGFR3 is a tyrosine kinase highly expressed in the resting and proliferating chondrocyte zones, where it plays key roles in controlling chondrocyte proliferation and/or subsequent cell cycle exit leading to differentiation into prehypertrophic chondrocytes. The transient pool of prehypertrophic chondrocytes will progress into hypertrophy generating an expansive strength required for bone elongation. Hypertrophic chondrocytes will eventually die by apoptosis or differentiate into trabecular osteoblasts allowing bone formation. Several signaling effectors fine-tune the transition from resting to hypertrophic chondrocytes, among which are FGFs. FGF family comprises secreted

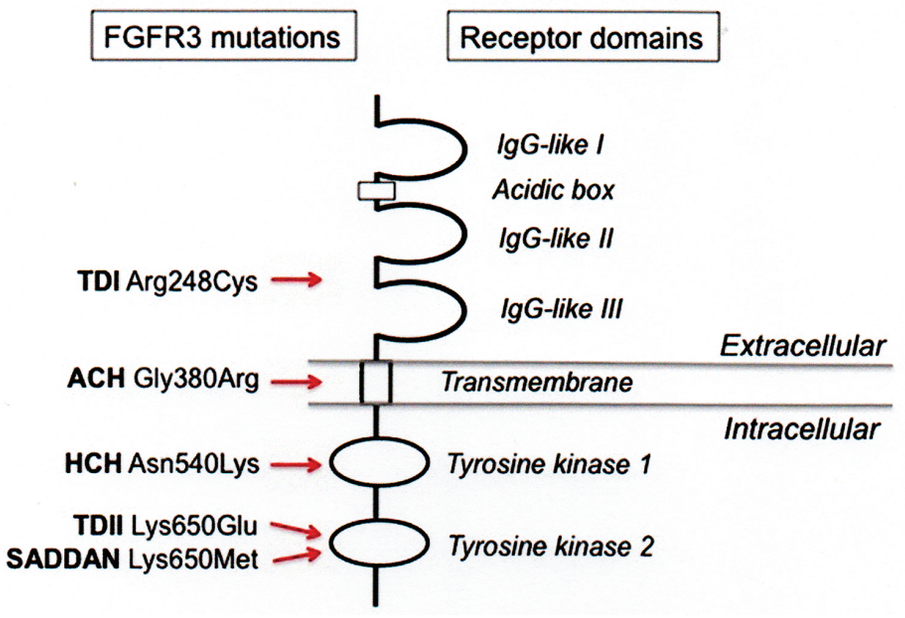

Figure 2. Recurrent FGFR3 mutations in chondrodysplasia. FGFR3 is a tyrosine kinase receptor composed by three IgG-like domains and an acidic box in the extracellular space, a transmembrane domain and two intracellular tyrosine kinase domains. TDI, ACH, HCH, TDII, and SADDAN diseases are caused by point mutations affecting different protein domains. Red arrows indicate where the most recurrent mutations associated with each different chondrodysplasia fall in FGFR3 molecule.

proteins that bind and activate FGFR3 as specific ligands and key roles in endochondral ossification have been attributed to FGF9 and FGF18 [5]. In the presence of heparin or heparan sulfates, FGFs bind to FGFR3 inducing receptor dimerization and subsequent autophosphorylation, which represent the activated state of FGFR3. Once stimulated, FGFR3 molecules trigger the activation of the RAS-MAPK and PLCγ intracellular signaling pathway or—depending on the cell type—of the PI3K or STAT pathways [3]. The mentioned signaling pathways have been shown to regulate several processes including cell proliferation and differentiation and can be activated by other members of the FGFR family (FGFR1, FGFR2, and FGFR4). However, unlike FGFR1, -2, and -4, FGFR3 uses those pathways to negatively regulate bone elongation, as proved by gene ablation in a mouse model resulting in extra-long bones [6]. Further supporting this notion is the recent identification of a missense impairing mutation (Arg621His) in FGFR3 that causes CATSHL (Online Mendelian Inheritance in Man, OMIM #610474) syndrome in humans, also characterized by extra-long bones [7]. A critical step in FGFR function is represented by receptor activity attenuation, a process required to avoid excessive signaling duration. Dwarfism-associated chondrodysplasias are all characterized by gain-of-function mutations that render FGFR3 constitutively active, but with graded levels of signaling potential [4].

Achondroplasia (*ACH*, OMIM #100800) is the most common among human chondrodysplasias and occurs with an incidence of 1:10–30,000 live births. Average adult male height is 131 cm, while average adult female height is 123 cm. Affected newborn infants present with disproportionate shortening of the limbs, a long and narrow trunk, a large skull with frontal bossing, a hypoplastic midface and exaggerated lumbar lordosis, as major clinical features [8]. Skeletal X-rays reveal characteristic abnormalities in the long bones of the limbs, which appear short. This form of dwarfism is caused by point mutations in FGFR3 characterized by autosomal dominant inheritance, as first discovered in the 1990s when heterozygous mutations were mapped to FGFR3 locus on chromosome 4. Almost all patients with typical achondroplasia features bear a glycine-to-arginine substitution at position 380 (Gly380Arg) in FGFR3, resulting from a spontaneous mutation to non achondroplastic parents (primarily fathers), in more than 80% of cases. This conversion shows the highest rate of occurrence among the known genetic germline substitutions, and a correlation with paternal age had been made [9]. FGFR3 protein is composed by an extracellular ligand-binding domain, a transmembrane domain, and an intracellular split tyrosine kinase domain (**Figure 2**). The Gly380Arg mutation falls in the transmembrane region causing a gain in receptor function, which reduces growth plate activity. Transgenic mice models for ACH (see Section Methods) allowed the characterization of FGFR3 function during skeletal development and postnatal growth, via analysis of the consequences derived from the mutation. Cartilage overexpression of FGFR3 bearing the achondroplasia mutation produced small mice with short bones resembling those seen in human achondroplasia. Studies coming from these mice led to postulate a defect in chondrocyte proliferation and/or differentiation, histologically giving rise to a disorganized growth plate [10].

FGFR3 is highly expressed in both proliferating and prehypertrophic chondrocytes where it normally limits their growth rate (**Figure 1**). At the molecular level, the FGFR3-Gly380Arg

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mutant showed ligand-independent activation and a specific defect in receptor down-regulation resulting in prolonged signaling activity [11]. Interestingly, cartilage targeted overexpression of a ligand (FGF9) that activates FGFR3 also generated a dwarf mouse [12]. These evidences established that FGFR3 signaling negatively regulates bone growth. Among the signaling effectors downstream to FGFR3 activation, STAT and MAPK signals have been the most studied in relation to skeletal development (a scheme of major FGFR3 signaling pathways is presented in **Figure 3**). FGFR3 is thought to inhibit chondrocyte proliferation through the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1), where the latter controls chondrocyte proliferation and terminal differentiation through the recruitment of p38 and ERK effectors [8, 13]. Biochemically, replacement of glycine 380 with arginine causes ligand-independent activation of FGFR3, which increases the constitutive level of phosphotyrosine on FGFR3 [14]. The consequent unregulated signal transduction through FGFR3 impacts growth plate function and therefore long bone development. Several approaches to reduce FGFR3 signaling by blocking receptor activation or inhibiting downstream signals have been proposed. The most promising utilizes an analog of C-type natriuretic peptide (CNP), which antagonizes the mitogen-activated protein (MAP) kinase pathway downstream of the FGFR3 receptor [15].

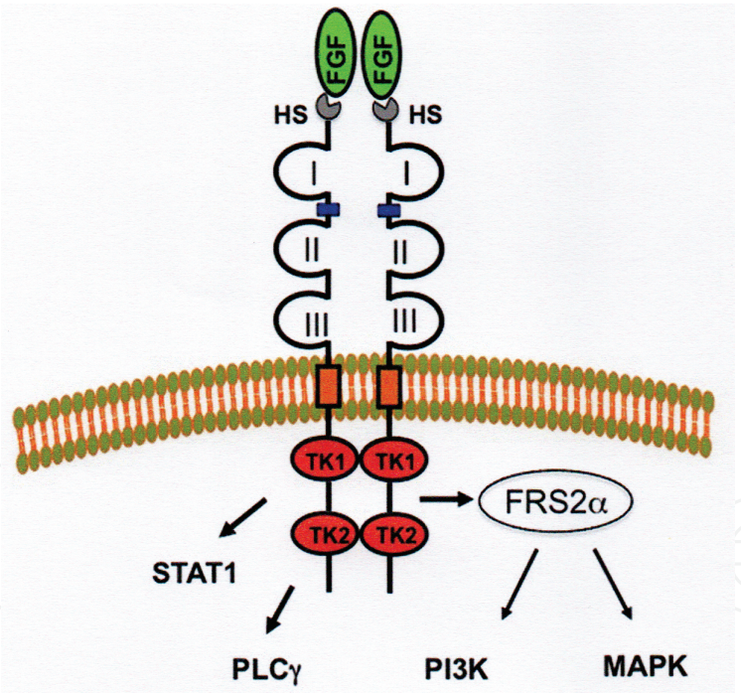

Figure 3. Signaling pathways downstream to FGFR3. Upon binding to specific FGF ligands and heparan sulfates (HS), FGFR3 undergoes dimerization, which causes conformational changes leading to tyrosine autophosphorylation. In this state, the receptor is active and engenders intracellular signal transduction by recruiting specific downstream effectors. STATs and MAPK signaling pathways play key roles in regulating the growth plate function.

Hypochondroplasia (*HCH*, OMIM #146000) shows skeletal features similar to but milder than those seen in ACH, whose differences can be distinguished on clinical and radiographic grounds. Key features are short-limbed dwarfism, lumbar lordosis, stocky build, short and

broad bones, and macrocephaly. Adult height ranges between 128 and 165 cm; 2.3 SD below the mean in children, but in some cases stature appears normal. The incidence of HCH is not precisely known, but it is believed to be about as common as ACH. Based on the clinical report of a peculiar case, allelism of HCH and ACH genes was suggested [16]. Subsequent genetic analyses indicated the Asn540Lys substitution in FGFR3, as the most recurrent mutation in HCH patients. The Asn540Lys mutation is inherited in an autosomal dominant manner and accounts for about 60–65% of cases, according to the clinical heterogeneity observed for this disease [17]. Although the amino acid change is semi-conservative, it occurs in a very conserved region of the tyrosine kinase 1 domain. The Asn540Lys mutation favors FGFR3 dimerization determining a gain of function associated with a mild but constitutive autophosphorylation. The lower degree of tyrosine phosphorylation of FGFR3-HCH compared to FGFR3-ACH was correlated with a milder clinical phenotype.

Thanatophoric dysplasia (*TDI*, OMIM #187600) and TDII (OMIM #187601). TDI is a severe autosomal dominant skeletal disorder that is lethal in the neonatal period of life. Two clinically defined TD subtypes have been classified: type I (TDI), characterized by micromelia with bowed femurs and, occasionally, by the presence of cloverleaf skull deformity of varying severity, and type II (TDII) characterized by micromelia with straight femurs and a moderate-to-severe cloverleaf skull deformity. TDI, which is more common, originates from several amino acid substitutions in extracellular and intracellular domains of FGFR3 protein, such as Arg248Cys, Tyr373Cys, and diverse substitutions of the natural stop codon in sense codons, such as X807Gly, X807Arg, and X807Cys, which result in the elongation of FGFR3 protein at the C-terminus by 141 amino acids. Conversely, only the Lys650Glu mutation located in activation loop of the kinase domain of FGFR3 has been associated with TDII [18]. The estimated birth incidence is approximately 1/20,000–1/50,000 being more frequent for TDI than for TDII. Most individuals with TD die within the first few hours or days of life by respiratory insufficiency secondary to reduced thoracic capacity or compression of the brainstem. Several mutations causing TDI lead to constitutive dimerization of FGFR3 due to the introduction of novel cysteines as it is the case for the Arg248Cys mutation, one of the most recurrent ones. Differently, TDII-associated Lys650Glu mutation induces a strong and constitutive ligand-independent tyrosine phosphorylation of FGFR3. This causes the recruitment and activation of several downstream key signaling effectors, among which are members of the STAT family, as shown in cell culture systems, human fetuses, and in a mouse model [19]. Moreover, the constitutive tyrosine phosphorylation acquired by the FGFR3-TDII receptor causes its accumulation within the endoplasmic reticulum (ER)/Golgi compartments impairing receptor trafficking toward the plasma membrane [20].

Severe achondroplasia with delay in development and acanthosis nigricans (*SADDAN*, OMIM #616482). SADDAN is a very rare skeletal disease: only few cases have been described. It clinically resembles TDI, but most of the SADDAN patients survive the perinatal period. This syndrome is additionally characterized by severe neurologic impairments, especially in long survivors and by the development of extensive areas of acanthosis nigricans [21]. SADDAN is caused by a heterozygous mutation in FGFR3 changing lysine 650 in methionine (Lys650Met). Of interest is the observation that the same lysine, when mutated into glutamic acid, gives rise

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to TDII. A mouse model for the SADDAN disease (see the methods section) highlighted milder long bone abnormalities than in the TDII mouse model, and overgrowth of the cartilaginous tissues was observed in the rib cartilage, trachea, and nasal septum. The presence of the Lys650Met mutation causes the highest constitutive tyrosine phosphorylation among the ligand-independently activated mutant FGFR3 associated with chondrodysplasia. Analogously to FGFR3-TDII, in cell culture models, the SADDAN mutant triggers the activation of several signal transduction effectors from the ER/Golgi, where it is kept due to its premature tyrosine kinase activation [22]. Although all FGFR3-related skeletal dyplasias manifest with profound shortening of the long bones, the phenotypic severity ranges from relatively mild HCH to neonatal lethal TD, where the degree of severity correlates with the degree of activated FGFR3. The different mutations activate FGFR3 and correspondingly inhibit chondrocyte proliferation to different levels when compared to wild-type (wt) FGFR3, with a relative strength being [4]:

$$\text{wt} < \text{Arg540Lys}(\text{HCH}) < \text{Gly380Arg}\ (\text{ACH}) \ll \text{Arg248Cys}(\text{TDI}) = \text{Tyr373Cys}\ \leq \text{Lys650Glu}\ (\text{TDII}).$$

2.2. COMP defects

Thrombospondin-5, better known as cartilage oligomeric protein (*COMP*), is a pentameric extracellular matrix (ECM) protein primarily expressed in chondrocytes and musculoskeletal tissues [23]. Each monomer comprises four domains (**Figure 4**). COMP interacts with several ECM proteins, including collagen II, collagen IX, matrilins, proteoglycans, and others. Through these interactions, COMP plays an important role in matrix assembly. Gene mutations affecting the structure of COMP pentamers cause two different skeletal dysplasias with autosomal dominant inheritance: pseudoachondroplasia (*PSACH*) and multiple epiphyseal dysplasia type 1 (*EDM1*)

PSACH (OMIM #177170) is a disproportionate dwarfing condition with involvement of the long bones, spine, and joints (incidence=1:20.000). Unlike ACH, it is not recognizable at birth: PSACH newborns are normal. Growth retardation is seldom recognized until the second year of life or later, at which time the body proportions resemble those of persons with achondroplasia, but the head circumference and facies are normal. Average adult male height is 120 cm; average adult female height is 116 cm. Radiographic findings of PSACH are distinctive, and another distinctive finding consists of significantly low COMP plasma levels. Scoliosis and lumbar lordosis are common spine abnormalities; osteoarthritis develops by the second/third decade of life affecting all joints. *Molecular pathology—In vitro* studies on human cells and analysis of mouse PSACH models demonstrated that pseudoachondroplasia is an endoplasmic reticulum storage disease, caused by improper folding of mutant COMP. Many causative mutations have been described: they produce either single amino acid substitutions or deletions and cluster in the highly conserved type 3 (Calcium binding) repeat domain TSP 3 (depicted in color pink in the diagram; **Figure 4**). A recurrent mutation in which aspartic acid 469 is deleted (D469 del) accounts for 30% of PSACH cases. All PSACH mutations exert a dominant-negative effect: pentamers composed of both structurally abnormal and normal subunits are retained within the endoplasmic reticulum (ER), promoting an excessive ER stress

response and ultimately premature chondrocyte death [24]. The growth failure of patients with PSACH may be explained by an increased death rate of growth-plate chondrocytes. The observation that COMP-deficient mice are not dwarfed and show a normal skeletal phenotype suggested that loss of COMP in the cartilage ECM *per se* is not the primary defect in PSACH [25].

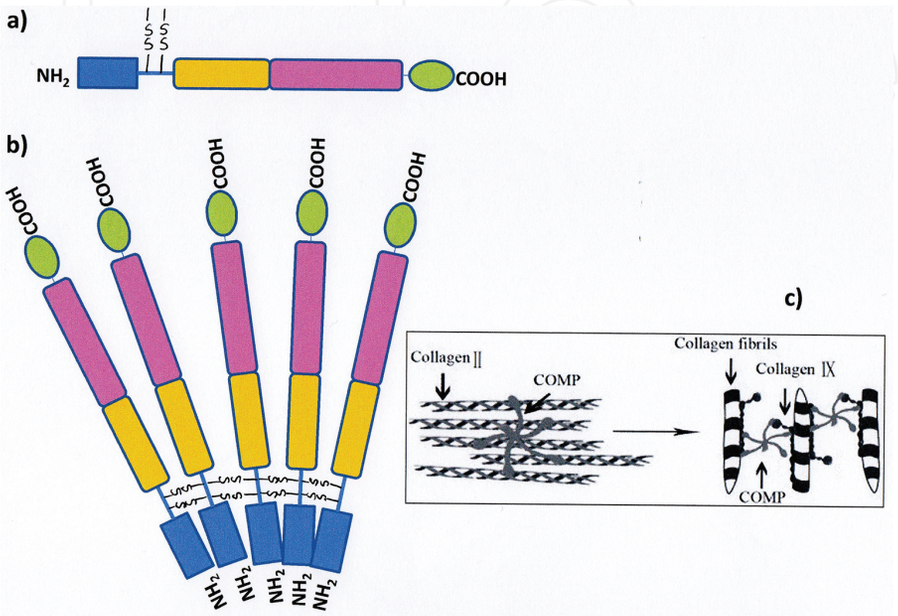

Figure 4. COMP structure and function in the ECM. (a) Schematic representation of COMP monomer, which comprises four domains: An N-terminal pentamerization domain (blue), an EGF-like domain (orange), a highly conserved TSP3 (calcium binding) domain (pink), a C-terminal globular domain (green). (b) COMP oligomer made of five identical disulfide-linked monomers. (c) Schematic representation of COMP interactions with collagens II and IX in cartilage ECM (Open-i nlm.nih.gov).

Epiphyseal dysplasia multiple 1 (*EDM1*, OMIM #132400) is a skeletal dysplasia characterized by mildly impaired height and early onset osteoarthrosis. Due to genetic heterogeneity (defects in other genes may cause similar phenotypes), only 38% of multiple epiphyseal dysplasia (*MED*) spectrum can be ascribed to COMP mutations. MED patients with COMP mutations can be recognized because of significantly low COMP plasma levels. Causative mutations are amino acid substitutions clustered in TSP3 domain; therefore, a phenotypic overlap between PSACH and EDM1 can be observed. However, missense mutations resulting in MED or mild PSACH phenotypes have been described also in the C-terminal domain of COMP (depicted in color green in the diagram; **Figure 4**).

2.3. Sulfate transporter defects

A group of chondrodysplasias showing moderate to lethal phenotypes have been associated with mutations in SLC26A2 gene, which codes for a sulfate-chloride transmembrane exchanger, DTSTD. This protein is predominantly present in chondrocytes, and it ensures proper sulfation of proteoglycans, essential components of the cartilage extracellular matrix. The

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highly organized structure of cartilage ECM is of crucial importance for the endochondral ossification process. Furthermore, sulfated proteoglycans are important for transmission of FGF signaling [26]. In humans, as well as in animal models, impaired sulfation of proteoglycans due to DTDST gene defects causes a continuous phenotypic spectrum of skeletal dysplasia. The clinical phenotype is modulated by the degree of residual protein activity, as shown in **Figure 5**. All conditions are recessively inherited; heterozygous carriers appear to be asymptomatic [27].

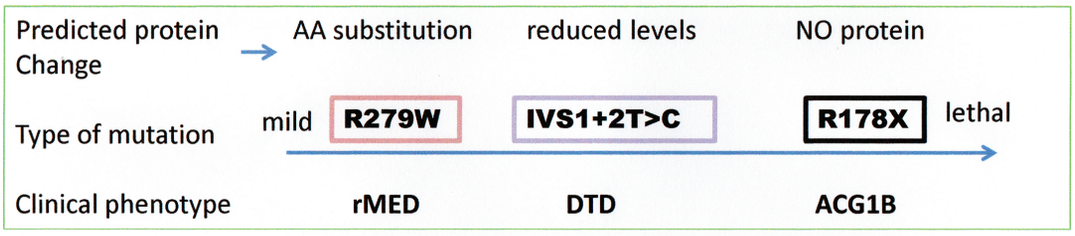

Figure 5. Schematic representation of genotype-to-phenotype correlations along the spectrum of SLC26A2 gene mutations.

Missense mutations such as R279W cause an amino acid substitution (arginine to tryptophan), which alters but does not abolish the sulfate transporter activity. This is a recurrent mutation found in the Finnish population as well as in other Europeans; at the homozygous state, it results in *recessive multiple epiphyseal dysplasia* (*rMED*, OMIM #226900) with mildly shortened or normal stature. Exact data about its prevalence are not available. An example of "intermediate" mutation is IVS1+2T>C, a splicing mutation which in homozygosity leads to reduced levels of mRNA and related product. It is common in the Finnish population, probably because of a founder effect, but it has been found in Central Europe too. Homozygous individuals have an intermediate clinical phenotype, *diastrophic dysplasia* (*DTD*, OMIM #222600), associated with short stature (adult height 135–150 cm for males; 100–120 cm for females), joint contractures, and other characteristic clinical signs. Finally, mutations that produce null alleles, such as the above-cited R178X, lead to no detectable DTDST protein activity within chondrocytes, and in homozygous individuals result in an extremely severe skeletal dysplasia, achondrogenesis type 1B (*ACG1B*, OMIM #600972). This condition is characterized by extremely short limbs, severe hypodysplasia of the spine and the rib cage, and is invariably lethal [28]. No data on the prevalence of ACG1B are available.

3. Bone disorders featuring short stature: osteogenesis imperfecta

Osteogenesis imperfecta (*OI*) is a chondro-osseous dysplasia characterized by fragile, deformed bones, short stature, and low bone mass (incidence: 1:15–20,000 births). Traditionally, it has been considered a bone disorder due to defects in type I collagen, the most abundant protein of bone, skin, and tendon extracellular matrices. In fact, 85–90% of OI cases are caused by dominant mutations in either of two genes, COL1A1 and COL1A2, causing both quantitative

and structural defects in collagen [29]. Within the past decade, the discovery of new disease genes has exceeded the idea of OI as a collagen-related disorder. Recessively inherited forms of OI with lethal to moderately severe phenotypes may be caused by defects in genes whose products are involved in post-translational modifications and/or folding of type I collagen [30–32]. Finally, recent molecular findings of causative mutations for both dominant and recessive forms of OI in non-collagenous genes have enlightened new perspectives [33]. OI at present appears as a molecularly and phenotypically heterogenous disorder characterized by defective bone mineralization; moreover, since the recessive types of OI are caused by deficiency of proteins found in both cartilage and bone, a new concept of OI as chondro-osseous dysplasia is arising. The classification has evolved with the new genetic discoveries. The original classification of Sillence *et al*. (1979), divided OI in four types, from mild to lethal, on the basis of clinical and radiographic features [34]. **Table 1** summarizes an updated classification of different OI forms along with their causative genes and their effect on growth deficiency. The disease genes list will almost certainly become longer with time, thanks to the whole exome new sequencing approaches. From the biologist's perspective, a logical and "user-friendly" classification pools the genetic types on the basis of altered intracellular or extracellular metabolic pathways.

3.1. Defects in collagen

Type I collagen is a heterotrimer made of two α1(I) and one α2(I) chains. It is synthesized as a procollagen molecule, with N-terminal and C-terminal globular domains flanking the helical domain. N-terminal and C-terminal propeptides are removed after secretion by specific proteases in the extracellular matrix. After processing, the collagen helices are capable of spontaneous assembly into fibrils, to be further stabilized by crosslinks. The helical domain is characterized by uninterrupted G-X-Y triplets since just the small glycine side chain fits the internal helical space. The most common genetic defects in dominant OI are missense mutations causing glycine substitutions within the helical domain and consequently structural defects in collagen heterotrimers. Gly substitutions delay helical folding and, in this way, promote post-translational overmodifications. Misfolded chains disturb intracellular metabolism, delay collagen secretion, and affect extracellular matrix deposition and mineralization. Phenotypic consequences vary depending on the nature of substituting amino acid, helical position, and chain type. In the α1(I) chain, substitutions with charged or branched side chains disrupt helix stability and are mostly lethal. In the α2(I) chain, substitutions are mainly non-lethal. Heterozygous COL1A1 loss-of-function mutations result in synthesis of reduced amount (about 50%) of structurally normal collagen and cause the mildest form of *OI* (*type I*). Heterozygous COL1A2 loss-of-function mutations in COL1A2 do not cause any apparent OI phenotype. Examples of COL1A1 and COL1A2 mutations and corresponding phenotypes are listed in **Table 2**.

3.2. Defects in collagen post-translational modifications

Procollagen undergoes several post-translational modifications, most of which occur in the endoplasmic reticulum. Such modifications are required for its correct folding, secretion, and

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extracellular matrix assembly. A complex of three proteins in a 1:1:1 ratio (CRTAP, P3H1, CyPB) called the 3-hydroxylation complex post-translationally modifies selected prolines in type I collagen chains in osteoblasts and type II collagen chains in chondrocytes. Deficiency of any of the three partners of the 3-hydroxylation complex, caused by loss-of-function mutations in both alleles of the corresponding gene, results in clinically distinct forms of moderate to lethal recessive OI (types VII, VIII, and IX, respectively, see **Table 1**). Common features are very low BMD, rhizomelia, bone fragility, and moderate to very severe growth deficiency. These recessive forms of OI are much rarer than the dominant forms (they account for 2–5% of OI cases detected in North America and Europe) and occur prevalently in inbred families.

Mode of Inheritance	OI type/ OMIM #	Defective gene	Defective protein	Cellular disturbance	Short stature*
Autosomal dominant (85–90% of OI cases)	I/#166200	COL1A1	Collagen I	Collagen quantitative defect	No
	II/#166210	COL1A1 or COL1A2	Collagen I	Collagen qualitative/ structural defect	Lethal
	III/#259420	COL1A1 or COL1A2	Collagen I	Qualitative/structural defect	+++
	IV/#166220	COL1A1 or COL1A2	Collagen I	Qualitative/structural defect	+
	V/#610967	IFITM5	BRIL	Bone matrix mineralization	+
Autosomal recessive (10–15% of OI cases)	VI/#613982	SERPINF1	PEDF	Bone matrix mineralization	++
	VII/#610682	CRTAP	CRTAP	Collagen hydroxylation	++
	VIII/#610915	LEPRE1	P3H1	Collagen hydroxylation	+++
	IX/#259440	PPIB	CyPB	Collagen hydroxylation	+/++
	X/#613848	SERPINH1	HSP47	Collagen chaperoning	+++
	XI/#610968	FKBP10	FKBP65	Collagen chaperoning	+
AR—very rare	XII/#613849	SP7/OX	OSTERIX	Osteoblast differentiation	+++
	XIII/#614856	BMP1	PICP endopeptidase	Abnormal procollagen I C-terminal propeptide processing	+++
	XIV/#615066	TMEM38B	TRIC-B	Intracellular [Ca] modulation	+
	XV/#615220	WNT1	WNT1	Wnt signaling pathway (bone formation)	++
	XVI/#616229	CREB3L1	OASIS	Bone formation	+++

*+ = ~−1 SD; ++ = ~−2 SD; +++ = >−2 SD.

Table 1. OI types and related gene/protein defects.

OI type	Gene	Mutation	Phenotypic defect	Reference
I AD	COL1A1	c.757 C>T p. R253 stop	Haploinsufficiency (decreased amount of structurally normal collagen)	[35]
II AD	COL1A2	c.1874 G>A p.G625 D	Structurally abnormal collagen chains	[35]
III AD	COL1A1	c.2461 G>A p.G821 S	Structurally abnormal collagen chains	[35]
IV AD	COL1A2	c.577 G>A p.G193 S	Structurally abnormal collagen chains	[35]
V AD	IFITM5	c.-14 C>T p. +MALQP	Functionally abnormal IFITM5 protein (gain of function)	[36]
VI AR	SERPINF1	c.423delG + c.423delG p.I142Sfs*9	Lack of PEDF protein	[37]
VII AR	CRTAP	(c.118_133del16insTACCC)+ (c. 118_133del16insTACCC) p.Q40Yfs*117	Severe impairment of prolyl 3-hydroxylation complex activity (collagen post translational modification)	[38]
XI AR	FKBP10	c.1399+1G>A + c.1399+1G>A aberrant splicing	Lack of FKBP65 protein	[32]

Table 2. Examples of causative mutations in different types of OI found at the Verona molecular diagnostic center.

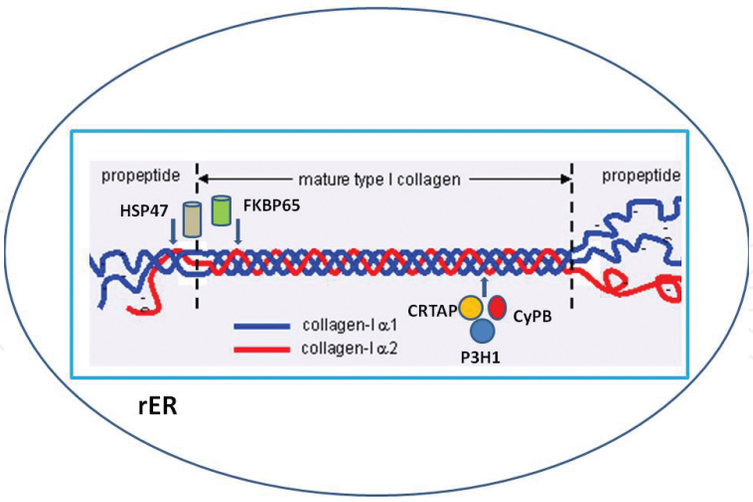

Figure 6. Type I collagen chains synthesis, post-translational modification and folding. Schematic representation of rough endoplasmic reticulum (rER) within an osteoblast. Several rER resident enzymes perform post-translational modifications on alpha 1 (blue) and alpha 2 (red) chains, before and during their folding. In particular, the prolyl 3-hydroxylation complex (P3H1 + CyPB + CRTAP) is shown. FKBP65 and HSP47 proteins are involved in subsequent maturation steps, such as folding and cross-linking, before secretion occurs. Defects, due to mutations in the corresponding genes, in any of the proteins shown in the figure, are responsible for several types of osteogenesis imperfecta (see text for details).

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3.3. Defects in collagen folding and secretion

Folding of post-translationally modified α chains is assisted by ER-resident collagen-specific chaperones. Absence or dysfunction of two collagen chaperones, HSP47 and FKBP65, due to mutations in both alleles of the corresponding genes (SERPINH1 and FKBP10, respectively) have been reported to cause very rare recessive OI. A single patient has been reported so far with HSP47 deficiency and a severe *OI* phenotype (*type X*) with considerable growth deficiency [39]. Several patients with a milder form of *OI* (*type XI*) due to FKBP65 dysfunction have been reported, showing long bone fractures, short stature, and ligamentous laxity [40]. Common cellular features in these two clinically distinct forms are intracellular aggregation and delayed secretion of collagen, normally post-translationally modified, but unstable (increased sensitivity to protease digestion was demonstrated *in vitro*). These observations corroborate the idea that specific chaperones are necessary to monitor collagen helix folding and stabilization during transit through the secretory pathway. **Figure 6** schematically illustrates the role of gene products mentioned in Steps 3.1, 3.2, and 3.3.

3.4. Defects in bone mineralization

Autosomal-dominant *type V OI* and autosomal-recessive *type VI OI* were first described as distinctive forms of brittle bone disorder in 2000 and 2002, respectively, by Glorieux *et al*. on the basis of peculiar histological features revealing defects in the mineralization process [41, 42]. All patients with type V OI have distinctive mesh-like lamellation on bone histology; an osteoporotic phenotype associates paradoxically with exuberant bone formation in hypertrophic callus, affecting periosteal bone. As it was discovered in 2012, all

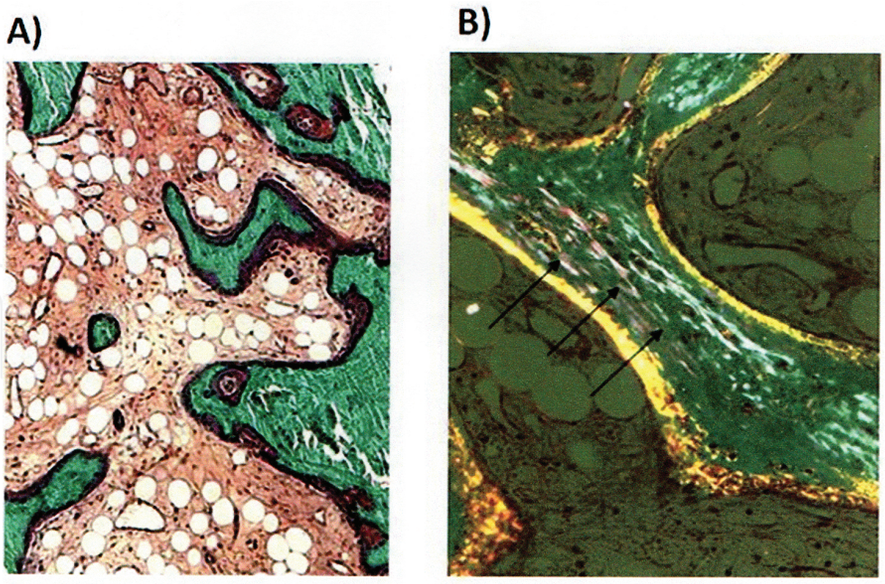

Figure 7. Defects in bone mineralization in OI type VI. (A) Goldner-stained iliac bone section of a OI type VI patient. Resorption lacunae and a large quantity of unmineralized osteoid (in red) are visible. (B) Bone section under polarized light. Arrows point to anomalies in the orientation of lamellae, reminiscent of a "fish-scale" pattern (magnification 200×). (Reproduced from J Bone Miner Res 2012; 50: 343–9 with permission of the American Society for Bone and Mineral Research).

patients with type V OI share the same heterozygous mutation (c.-14C>T) in the 5'UTR (untranslated transcribed region) of IFITM5 gene [43]. The mutation creates a novel start codon, thus adding five amino acids to the N-terminus of the protein, named BRIL, with a gain-of-function effect. BRIL is a transmembrane protein highly expressed in osteoblasts during mineralization. In the case of type VI OI, the causative gene is SERPINF1, which encodes for PEDF, a ubiquitously expressed multifunctional secreted protein. Patients affected by type VI OI, as well as the murine knockout model, do not produce PEDF because of different loss-of-function mutations in both SERPINF1 alleles [37, 44, 45]. Type VI OI children do not show skeletal abnormalities at birth, fractures do not occur until the age of 8–12 months, but a severe progressively deforming bone dysplasia with frequent long bone fractures develops thereafter. Various studies have demonstrated that PEDF, which is produced both by chondrocytes and osteoblasts, is necessary for osteoblast development, favoring the expression of osteogenic genes and mineral deposition. It stimulates the production of osteoprotegerin, thus inhibiting osteoclast maturation. The absence of PEDF is detrimental for bone homeostasis and osteoid mineralization. Type VI patients bone histology, in fact, reveals an increased amount of unmineralized osteoid and a peculiar fish-scale pattern under polarized light (**Figure 7**). Although mutations in IFITM5 and SERPINF1 seem to have opposite effects on mineralization-increased ectopic ossification in type V and reduced bone mineralization in type VI, further findings have demonstrated that the two gene products, BRIL and PEDF, do interplay in the process of bone mineralization [46].

3.5. Defects in osteoblast development

Mutations in two genes involved in osteoblast differentiation have been recently associated with recessive *OI* phenotypes: SP7 (*type XII*) and WNT1 (*type XV*). SP7 codes for a transcription regulator factor, OSTERIX, which plays an essential role in regulating the differentiation of preosteoblasts to osteoblasts. The unique type XII patient so far described [47], born to first-grade Egyptian cousins, harbors a homozygous frameshift mutation in SP7 gene which most likely leads to a dysfunctional OSTERIX protein. WNT1, a member of the Wnt family of secreted glycoproteins, is the activator of a complex intracellular signaling pathway. It plays an important role in bone formation and maintenance; the above-cited SP7 master gene and ALPL gene (which encode alkaline phosphatase, a key enzyme for bone mineralization) are among the downstream targets of Wnt signaling. Heterozygous WNT1 mutations may cause early-onset osteoporosis, while homozygous mutations impairing Wnt protein occur in patients with severe OI with short stature, frequent fractures, and vertebral compressions [48]. **Figure 8** illustrates schematically the role of gene products mentioned in Steps 3.4 and 3.5.

Schematic and simplified representation of the differentiation steps from bone marrow mesenchymal stem cell to mature osteoblast. The transcription factors with an inductive effect (RUNX2, OSTERIX) are indicated in red as well as the osteoblast-specific proteins PEDF and BRIL, as cited in the text. The WNT signaling pathway plays a crucial role in osteoblast differentiation, proliferation, and bone matrix formation/mineralization. Defects, due to

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mutations in the corresponding genes, in any of the proteins shown in the figure, are responsible for various types of osteogenesis imperfecta (see the text for details).

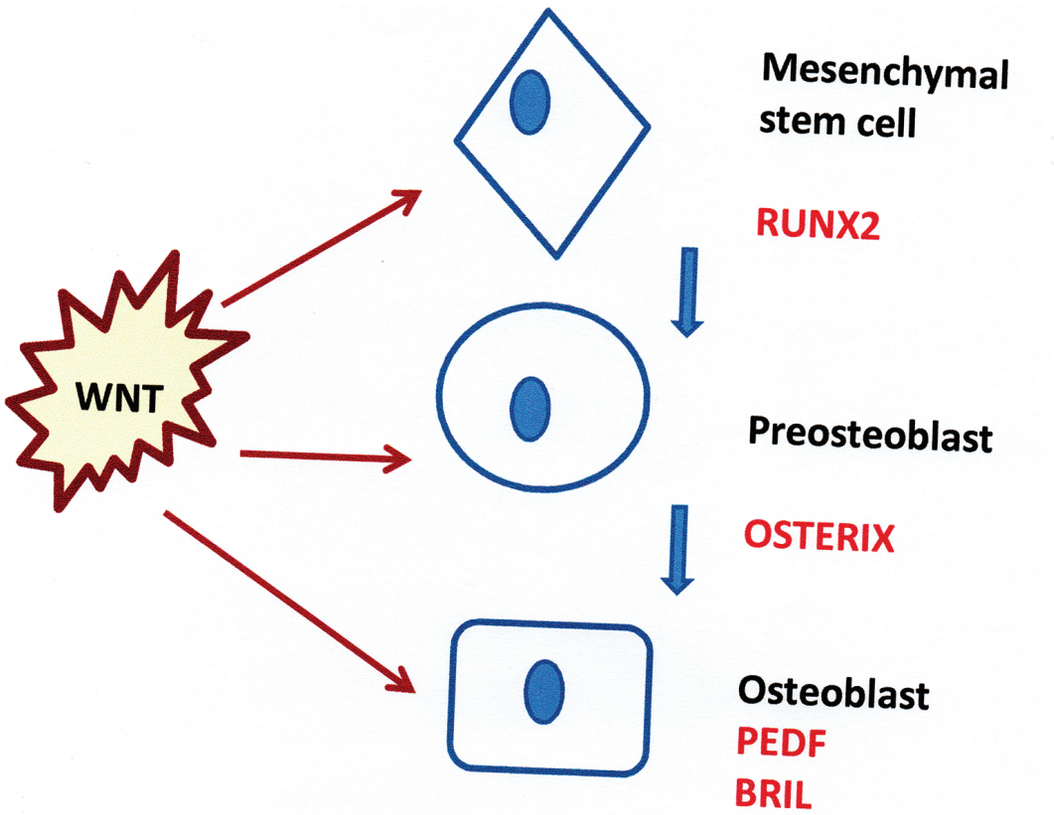

Figure 8. Osteoblast development.

3.6. Restricted growth in OI

Short stature is the most prevalent secondary feature of OI [49]. Only in the mildest form, OI type I, affected individuals have minimal bone deformities and normal stature. In all other types of OI, mild/moderate (+/++) to very severe (+++) growth deficiency is to be found (see **Table 1**). Short stature in OI is not caused by premature closure of growth plates; it can be the consequence of compromised extracellular matrix structure and mineralization, which impact on bone properties, leading to repeated long bones fractures, deformities, and bowing. Severely affected patients may be short because of vertebral compression fractures, severe scoliosis, lower limb deformities, and disruption of growth plates. However, growth can also be delayed in the absence of these abnormalities. The mean standing height of patients with OI is lower than that of their unaffected first-degree family members, regardless of severity. Truncal height is reduced, and head size increased in one-third of the patients with moderate or severe OI.

During childhood, there appears to be no difference between the standing heights of girls and boys, but women have lower height *z*-scores than men. The reduction in arm span *z*-score

generally follows the same pattern as for height: individuals with moderate or severe OI tend to have lower *z*-scores than individuals with mild OI. The arm span/height ratio appears to be increased in children with moderate or severe OI, but not in those with mild OI. Mean concentrations of insulin-like growth factor (IGF)-I and IGF-binding protein (IGFBP)-3 are generally normal, in the low range of age-specific reference values. Growth hormone (GH) deficiency is very rare in patients with OI [50]. The etiology of the growth restriction in children with moderate and severe OI is not entirely clear. It has been suggested that it could be viewed as a self-protective mechanism: a given mechanical load creates smaller stresses in a short bone than in a long bone; thus, a short bone will break less easily [51]. People with severe OI have a typical deformity of the growth cartilage, defined as "popcorn" appearance of the metaphysis. Microfractures of the growth cartilage may play a role in the growth problems experienced by these patients. There are no reports on the effects of puberty and hormonal changes on growth in children with OI.

3.7. Conclusions

The intent of this chapter was to give a molecular and cellular overview of selected conditions associated with impaired height, focusing on growth plate misregulation, cartilage extracellular matrix dysfunctions, osteoblast differentiation, and mineralization process impairments.

4. Methods

Most of the experimental data described in this chapter come from either *in vitro* studies performed on cultured cells or from *in vivo* studies performed on animal models.

4.1. *In vitro* cell cultures

Cell culture studies on mutated FGFR3 were mostly performed in chondrosarcoma RCS cells from rat, ATDC5 from mouse, or in heterologous cell lines as Hek293 or PC12. Single-codon substitutions were introduced into the cDNA encoding FGFR3 by site-directed mutagenesis, and the plasmids carrying different mutant molecules were transfected into cultured cells to allow protein expression. To address questions related to the biochemistry of mutant FGFR3 molecules, which is assessing the degree of receptor activation, FGFR3 proteins were isolated from cell lysates by immunoprecipitation techniques and analyzed by Western blot using specific antibodies directed to phosphorylated tyrosine. Intracellular receptor localization was visualized by immunofluorescence [22].

Studies in the field of O.I. are mostly based on cultivation of fibroblasts obtained (upon informed consent) from patients' skin biopsies. Fibroblasts are grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (penicillin and streptomycin) at standard concentration. Proteins, DNA, and mRNA are extracted and purified from cells for subsequent analyses. Detailed description of methods can be found in Refs [31, 38].

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4.2. Animal models

Transgenic mice described in the chapter were generated by targeting the specific genes of interest in murine embryonic stem cells with the homologous recombination technique, originally described by Thomas AND Capecchi [52]. Several mouse models orthologous to human skeletal dysplasia have been generated where gene expression was targeted to chondrocytes. The list includes ACH, TDI, TDII, and SADDAN [53, 54]). Histochemical analyses were performed on tissues isolated from proximal tibial growth plate tissue, generally prepared from 1-week-old mice.

4.3. Gene sequencing

The search of causative mutations described in the text was performed by sequencing exons and exon/intron boundaries of the candidate genes. Typically, single exons are amplified by PCR using appropriate primers and then subjected to automated sequencing according to standard protocols. When analysis of known established disease genes failed to identify the causative mutations, whole exome sequencing strategies were employed in order to identify novel loci [55, 56].

4.4. Growth plate histology

For analyses on human samples, tibial and/or femoral cartilage fragments were obtained from medically aborted fetuses upon informed parental consent. Pregnancies were legally terminated after ultrasonographic and X-ray detection of severe dwarfism.

4.5. Bone histology

Biopsies obtained from iliac crest (upon informed consent) are fixed in 70% ethanol and embedded undecalcified in methylmethacrylate resin. Bone sections are cut by microtome, stained by Goldner's stain, and mounted on microscope slides.

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