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Chapter 9

The Mechanism of Action of Novel Antimyeloma Agents and Newer Therapeutic Strategies

Yossi Cohen*
Institute of Hematology, Asaf Harofhe University Hospital, Zerifin, Israel
Sackler, Faculty of Medicine, Tel Aviv University, Israel

Abstract

Several decades of "stagnation" in the introduction of new antimyeloma agents beyond the traditional corticosteroids and alkylating agents have been occupied from the late nineties by an exciting new era characterized by rapid or even 'explosive' development of a variety of new agents starting from the dramatic comeback of thalidomide, this time as a novel agent against multiple myeloma (MM) followed by the appearance of completely new classes of agents directed against specific molecular targets. The revolution in drug development since then has been manifested by the growing number of clinical trials assessing numerous combinations of older and new first and second generation agents and more recently also by modified routs of drug administrations. Despite this, the progress in elucidation of the basic mechanism of action of many of the novel agents in use has been lagging behind the clinical data acquired and this knowledge gap is becoming an obstacle for the ability to improve the available treatments against resistant disease or to design newer molecules which target the desired pathways more directly. This chapter will summarize the current knowledge on the mechanism of action of some of the mostly used classes of novel agents and those under advanced stage of evaluation. It is noteworthy that in many instances modern research failed to support the general idea standing behind the mechanism offered originally and new theories came instead. Thalidomide is one of the most fascinating examples in all times, for which the obscure binding site and the unknown molecular pathway mediating its diversified physiological effects turned much of the attention towards the clinical manifestations seen and the biological changes observed under experimental conditions,

* Corresponding author: Tel: +972 8 9778223, Fax: +972 8 9779772, Email: yosefc@asaf.health.gov.il.

whether of clinical relevance or just another speculation as also happened with the second generation IMiDs. The proteasome inhibitors in contrast have their binding site recognized already from the first introduction to clinic but even there the means by which the proteasome blockade translates into tumor cell killing remains largely unknown considering the failure to confirm the historical attribution of the cytotoxicity to NFkB inhibition. Another therapeutic modality that has gained a lot of success in the treatment of cancer but in myeloma is still seeking for its place is targeted therapy using monoclonal antibodies directed against specific tumor associated antigens or extracellular factors. The limited benefit of the antibodies studied so far in myeloma is an indication that our targets are not optimally selected and we have to improve our knowledge on myeloma initiating cells and their unique immunophenotype. All these issues will be addressed comprehensively and new insights will be introduced, which will be many times inconsistent with the established literature dogmas.

The IMiDS

More than a half of all pregnant women tend to experience morning sickness. With this statistic in mind, a German company Grunenthal developed a drug for expecting mothers in the late 1950s called thalidomide. Several years after its introduction, more than 10,000 babies were born with severe birth defects. These malformations included stunted or missing limbs, deformed ears and valvular abnormalities. More than 40% of births were still-born or the infants died shortly after birth. This devastation created a huge scare and sparked decades of research in order to figure out what caused these severe birth defects and how to prevent this from occurring again. However, after years of interrogation, in 2006, thalidomide was approved by the FDA for the treatment of multiple myeloma (MM), in addition to its off-label use since the seventies for the treatment of miscellaneous inflammatory conditions.

Structurally, thalidomide contains a phtalimide ring linked to a glutarimide ring via an asymmetric carbon atom. Several factors joined to make the thalidomide binding site and its mode of action so difficult to decipher and one of the most devastating medical mysteries ever: 1. Its peculiar spectrum of biological activities that involve many organs, including the central nervous system (sedation, hypnosis), the peripheral nervous system (neuropathy), the immune system (immunomodulation and reduction of TNFα levels), the cardiovascular system (arrhythmias), the coagulation system (thrombosis), the hematopoietic system (e.g., lenalidomide efficacy in the 5q- dysplasia) and of course the teratogenicity, which as a group are lacking any apparent common denominator. 2. The lack of activation of any known proapoptotic pathway and the few measurable changes observed in treated organisms, excluding the variable decrease in TNFα levels, which remains the best recognized biomarker. 3. The large number of circulating metabolites, exceeding 100 byproducts, which contributed to the complexity in working with this compound. 4. The species specificity that characterized the thalidomide embryopathy, which later turned to be one of the ultimate criteria required to be fulfilled in any explanation for the teratological effects of the drug. The last character limited animal modeling to pregnant rabbits, chickens and monkey until recently, but also implied that the teratogenicity is not necessarily exerted by the parent drug itself but is possibly mediated by one of its active metabolites, generated after processing by microsomal enzymes in a species specific manner. Over the years, the withdrawn from the markets and the difficulties mentioned minimized the global effort invested on the subject,

and fade the motivation to solve the thalidomide mystery ever, even though the continues progress in the developmental research and occasional breakthroughs in relevant fields generated new insights, from time to time, leading to new interpretations of the findings combined with "fresh" speculations, suited for the contemporary knowledge, which eventually matured into acceptable models based on discoveries on limb patterning, oxygen-stress related apoptosis, neurotoxic and vasculotoxic effects of thalidomide, which will be discussed in detail below. However, it seems that the most exciting discovery, as far as can be appreciate today, occurred in the year 2010, when a protein called cereblon (CRBN), previously implicated in mental retardation was unexpectedly found to be the thalidomide binding protein. Moreover, knockdown of the zebrafish paralogue of CRBN, i.e., zCRBN induced analogous teratologic malformations to the human embryopathy, including retardation of pectoral fin and otic vesicle (ear) formation, and inhibition of Fgf8 expression in fin buds. This discovery was more recently followed by the demonstration that lenalidomide-bound cereblon acquires the ability to target the proteasomal degradation of two specific B cell transcription factors, Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3). In addition it was found that in myeloma cell lines, the loss of IKZF1 and IKZF3 is both necessary and sufficient for lenalidomide's therapeutic effect, suggesting that the antitumor and teratogenic activities of thalidomide-like drugs are dissociable. Therefore, it is becoming more and more realistic that we are standing now very close to the end of the mystery. But, are we really there? The documented history of the drug thought us that the thalidomide mystery was "deciphered" too many times over the last decades and it is also too early to propose possible connections between the initial molecular events mentioned, starting with the drug binding and the variety of clinical manifestations observed. For the latter purpose however, it might be constructive to reorganize the numerous pieces of data acquired over the past decades (which are accessible from more than 8,200 PubMed titles on the value "thalidomide") and try to extract relevant information, which will aid integrating old and new findings under a common evidence based frame.

Here I summarized the data, which was looking the most pertinent to our goal, separated into several different categories, which will be discussed below. Although it could sound more natural for the aim of this chapter to start directly from the antiangiogenic subject, which has been already accepted as the leading explanation for the antimyeloma activity of the IMiDEs, I preferred keeping the teratological issue always a step forwards and from there spreading elsewhere, due to the explicit molecular findings discovered during investigation of the thalidomide embryopathy and the ability to connect some of them with the non teratological effects of the IMiDEs. Moreover, the wired collection of non-antitumoral effects of the IMiDs, which include the mysterious propensity of thalidomide to the developing limbs, its efficacy against drug reaction following dapsone administration in erythema nodosum leprosum (ENL), the unique hypnotic properties of this agent characterized by increase in the REM sleep duration and its usefulness against congenital and acquired vascular malformations togetether with the impressive antitumoral properties of the IMiDEs, which eradicate myeloma cells specifically (~15% complete response to lenalidomide + dexamethasone combination in relapsed/refractory patients), and induce hematological and cytogenetic response in the 5q- dysplasia; all of the above effects might be coexistent of course secondary to drug or metabolite activities on completely different molecular pathways, but on the other hand such a selective collection of target sites could also imply for the existence of as yet unrecognized common thread (e.g., cereblon targets), responsible for many

of the drug activities mentioned. Our journey will start with lessons from the thalidomide embryopathy, subcategorized into vascular, neurological, oxygen-stress, adhesion molecules and developmental findings, which will be extended if possible and compared to corresponding findings in myeloma in order to find possible molecular targets which are modified in a stereotypical manner by the IMiDEs.

Lessons from the Thalidomide Embryopathy

By far, the most characteristic features of the thalidomide embryopathy were the unique pattern of distribution of embryological defects and the short sensitive period; embryotoxicity with limb defects noted from the 27th to 40th gestational day of exposure; all cases of phocomelia occurred from the 27th to 42nd gestational day. Unfortunately, the last character was also one of the great contributors to the disaster extent by giving the impression that the malformation is sporadic and thereby delaying its linkage with the drug. For the researchers however, the time interval mentioned made easier to delineate the embryonic structures involved and to determine their developmental stage during the incident, so even before the modern molecular biology era a remarkable progress was made, which occassionally yielded fascinating discoveries. Another distinctive feature was the strict species specificity, which will gain special attention latter owing to its great discriminative value. Of more than 30 theories elaborated in attempt to explain the thalidomide mystery in terms of molecular or pathophysiological mechanisms I preferred to concentrate on five and ignore those which were not supported by sufficient evidence. As already mentioned, the five leading hypotheses could be classified, in the most simplistic manner, into vascular, neuronal, oxygen-stress, adhesion molecule and patterning defects on the basis of the suggested etiologies. Each of these hypotheses will be introduced first in its pure teratological context and then if supported by evidence it will be evaluated in the context of myeloma.

a. Angiogenesis and the IMiDs

Based on the negative impact of thalidomide or its derivatives on angiogenesis under certain experimental conditions, the antiangiogenic properties of the IMiDs turned to be one of the most acceptable explanations for both the teratogenic insults as well as for the antimyeloma activities of the IMiDS. The earliest description of vascular injuries induced by application thalidomide was documented in 1966. In this forgotten article, Arthur Jurand from the Institute of Animal Genetics, Edinburgh incubated fowl eggs until Hamburger-Hamilton (HH) stage 17 or 18. The egg's shells were then slightly opened and thalidomide in suspension (0.2 ml of thalidomide 10 or 20 mg/ml) was introduced into the egg-white in close proximity to the embryo or injected into the yolk sac and then sealed with Parafilm (1). Thalidomide introduced into chicken eggs preincubated for 64-68 h causes dilation of the axial limb-bud artery in 24 h after treatment i.e., at stages 21-25. The injured endothelial cells became extremely thinned and formed vesicular projections into the lumen. In some cases there was necrosis in the mesoblast outside the dilated artery. In the most extreme degree of injury the mesoblast was almost completely destroyed. At the ultrastructural level the mitochondria in the endothelial cells of the dilated artery became swollen and vacuolated. These results suggested that the primary cause of thalidomide abnormalities is an injury of the endothelial lining of the axial limb artery. However, it took almost 30 years to rediscover the

negative influence of thalidomide on vascular vessels and the seminal work on this topic was done by Robert D'Amato et al. from the Harvard Medical School and reported in 1994. The authors examined the effect of thalidomide on growing vasculature in the chicken chorioallantoic membrane and in the rabbit cornea and they were able to show that orally administered thalidomide can inhibit the angiogenesis induced by basic fibroblast growth factor (b-FGF) in the rabbit cornea [2]. Specifically, chicken chorioallantoic membrane assay was performed by implantation of 0.5% carboxymethylcellulose pellet containing 650 ng of the potent angiogenic protein b-FGF bound to sucralfate on the chick embryo chorioallantoic membrane through a hole cut in the egg shell. After 48 h incubation, neither thalidomide nor EM-12, a related teratogenic analog, exhibited any inhibitory activity on blood vessel growth and this observation was consistent with the known species specificity. Corneal neovascularization was induced by implantation of a similar pellet into corneal micropockets of New Zealand White rabbit, 2 mm from the limbus. Histological examination demonstrated progressive blood vessel growth into the cornea toward the pellet with only rare inflammatory cells evident. This angiogenic response was not altered by severe immune suppression with total body irradiation, and pellets with sucralfate alone did not induce angiogenesis. Oral treatment with a teratogenic dose of thalidomide (200 mg/kg) resulted in an inhibition of the area of vascularized cornea that ranged from 30 to 51% in three experiments with a median inhibition of 36%. The teratogenic analog EM-12 was also inhibitory, with a median inhibition of 42%, while nonteratogenic analogs of thalidomide exhibited no activity. Electron microscopic examination of the corneal neovascularization of thalidomide-treated rabbits revealed specific ultrastructural changes similar to those seen in the deformed limb bud vasculature of thalidomide-treated embryos. During the following years, similar results were reported by other groups, using alternative angiogenic stimulators and/or different thalidomide derivatives and in 1998, a German group showed comparable results using vascular endothelial growth factor (VEGF) (500 or 750 ng) instead of b-FGF in a corneal neovascularization model in rabbits. In that study, animals received two daily feedings of thalidomide (200 mg/kg) and after the 5th day of treatment a significant inhibition of corneal angiogenesis could be recognized, which persisted for more than 16 days [3]. Later they repeated the investigation using thalidomide and two of its derivatives, supidimide and EM12 and applied either b-FGF or VEGF for initiation of the neovascular response [4]. It was demonstrated that all of the three compounds examined inhibit neovascularisation induced by either b-FGF or VEGF containing pellet. EM12, the most teratogenic derivative was also the most potent inhibitor of angiogenesis in this model. In 1997, the Harvard's group reutilized their original corneal neovascularization model but now in mice and they were able to show that in this species, intraperitoneal but not oral administration of thalidomide can inhibit b-FGF or VEGF-induced corneal neovascularization [5]. Notice that this finding negates the species specificity criterion, which will be discussed in detail latter, albeit route of administration was different in this particular instance. The species specificity criterion was also missing in a guinea pig model of foreign body granuloma in which oral administration of thalidomide reduced vascular density [6]. In some more recent studies, an attempt has been made to delineate also the molecular mechanisms underlying the effects of the IMiDs on vascular vessels. A North American group used for this purpose the thalidomide analogue cc-1069, which inhibits endothelial cell proliferation more efficiently than thalidomide. This inhibition was associated with a marked decrease in the activity of the nuclear factor SP1 and a moderate inhibition of NFkB activation in nuclear extracts of endothelial cells [7]. The

same compounds however did not impair the viability of the endothelial cells and there was no effect of thalidomide or the thalidomide analogue on the proliferation of a glioma cell line (U251) in vitro. A German group examined the effects of thalidomide on the development of capillary structures in embryoid bodies differentiated from murine embryonic stem cells. At a concentration of 100 μg/ml thalidomide significantly inhibited blood vessel development, when administered during the period of vasculogenesis and angiogenesis, known to occur between day 4 and 8 of embryoid body development [8]. However, 10 μg/ml of the compound did not exert significant effects on the formation of capillary structures. Examination of the intracellular REDOX state indicated that thalidomide exerts its anti-angiogenic properties via the generation of reactive oxygen species (ROS). An Italian group demonstrated in 2003 that thalidomide can inhibit the proliferation of endothelial cells induced by b-FGF and VEGF in-vitro, more so if the cells are grown on vitronectin [9]. At the molecular level, exposure to thalidomide reduced the release of the angiogenic factors MMP-2 and IL-8 by endothelial cells, suggesting a further pathway for the antiangiogenic activity of drug. A Japanese group studied the antiangiogeneic properties of thalidomide in zebrafish [10]. At a concentration of 800 μM for 40 hours, thalidomide caused shortness in length and loss of blood flow in 93% of the zebrafish embryos. The vascular defect was accompanied by additional complications such as severe pericardial edema and heart failure, and subsequently the embryos died by 5 days of development. Except for reduced body length, no deleterious effects were observed on the general morphology of embryonic structures. Vascular defects in the region corresponding to the dorsal artery and vein could be visualized both morphologically and immunohistochemically by the loss of expression of VEGF receptors and these changes appeared only after transient increase in the ceramide content through activation of neutral sphingomyelinase (nSMase). Moreover, the synthetic cell-permeable ceramide, N-acetylsphingosine (C2-ceramide) inhibited the embryonic angiogenesis in a similar fashion as thalidomide. Finally, the blockade of ceramide generation by antisense morpholino oligonucleotides for nSMase prevented the thalidomide-induced ceramide generation and the vascular defect. In contrast, sphingosine-1-phosphate (S1P) inhibited nSMase-dependent ceramide generation and restored the thalidomide-induced embryonic vascular defect with an increase of expression of VEGF receptors. Similar results were observed in human umbilical vein endothelial cells (HUVECs), on which thalidomide induced inhibition of cell growth, generation of ceramide through nSMase, and depletion of VEGF receptors, that could restored to the control levels by pretreatment with S1P. These results implied therefore that the antiangiogenic activity of thalidomide is regulated by the balance between ceramide and S1P signal.

In the year 2009, more than 40-years after Jurand reported his vascular findings in chick embryos, Christina Therapontos et al. from Harvard, returned to this embryonic model and showed that thalidomide derivatives can induce limb defects by preventing angiogenic outgrowth during early chick limb formation [11]. Therapontos et al. applied thalidomide and various thalidomide metabolites over the upper body and amniotic fluid of HH stage 17–19 embryos, at a similar or lower concentration relative to the therapeutic dose of thalidomide used in humans (chick = 160–1,280 μg/kg; human = 700–2,000 μg/kg). This method of drug application preferentially treats the right limb, allowing using the left limb as a control. Anti-inflammatory and thalidomide hydrolysis byproducts (5,6-OH thalidomide; 5′OH-thalidomide; PG Acid (N-phthaloylglutamic acid) had no effect on embryonic or limb development at any concentration tested. However, CPS49, a tetrafluorinated analogue of

thalidomide that is chemically and structurally related to thalidomide breakdown products, induced severe limb defects similar to those seen in thalidomide victims. Specifically, CPS49 (100 μg/mL; 160 μg/kg), which has potent antiangiogenic and antitumor effects in vitro, induced defects in 82% (n = 250/306) of forelimbs and 10% (n = 32/306) of hind limbs. No defects were observed in any other regions of the embryo. To determine the effects of CPS49 on the development of the forming limb vasculature, the vascular network was visualized with Indian ink. Within 2 h of treatment, before obvious changes in limb morphology were observed, CPS49 consistently induced a 20% reduction in vessel density relative to controls. Vessel density continued to decrease and was reduced by 34% and 45% by 3 h and 6 h post treatment, respectively, whereas limb area was unaltered or only mildly reduced. By 24 h, vessel density was decreased by 64% and the limb bud itself was severely truncated. Remarkably, other blood vessels and the structure of other tissues in the embryo were unaffected. Embryos that were viable at day 10 were stained to investigate limb cartilage patterns. CPS49 treatment resulted in 4 main truncation phenotypes that resemble the range of limb defects seen in thalidomide-affected children: complete forelimb loss (amelia; 21%), a short humerus-like structure (36%), a shortened humerus with short ulna (21%), or a severely truncated limb with 2 shortened cartilage elements articulating with each other (21%). The expression patterns of genes essential for limb outgrowth like Fgf8 in the apical ectodermal ridge and Fgf10 in the underlying mesenchyme, were decreased significantly, but only after the changes apparent in blood vessels. Expression of Tbx5, required for forelimb initiation, was detectable at all-time points. In contrast, expression of sonic hedgehog, involved in digit patterning, and Msx1, a marker of the progress zone, was normal until 6 h, but reduced or undetectable by 24 h. In summary, this model was consistent with the concept that vascular insult is underlying the thalidomide embryopathy from the aspects of tissue specificity and the sequence of detectable changes, which were evident in blood vessels prior to the pathological and molecular manifestations which appeared later in the primordial tissue. This model however, was limited to thalidomide analogue, which leaves a room for skepticism concerning the implication to humans, as will be discussed in more detail latter.

Not far from the discovery of the antiangiogenic properties of thalidomide [2], the therapeutic implications of this finding were sought in the malignancy setup. In the experimental level, an Israeli group injected B16-F10 melanoma and CT-26 colon carcinoma cells to mice subcutaneously, intravenously and intraperitoneally, combined with daily gavage of 0.3-1.0 mg thalidomide from day two or ten after tumor cell injection [12]. However, in this model, no inhibition of tumor growth or metastasis was noted in thalidomide treated mice as compared to control mice. In addition, both the control and the treated mice developed an intact network of new blood vessels. Nevertheless, a few years later the antitumoral activities of thalidomide were also examined in the clinical setup. In one study, oral thalidomide 800 mg/d given to 39 adults with previously irradiated, recurrent high-grade gliomas resulted in two objective radiographic partial responses (6%), two minor responses (6%), and 12 patients with stable disease (33%). Eight patients were alive more than 1 year after starting thalidomide, although almost all with tumor progression [13]. In another phase II trial, which included 18 patients with recurrent glioma, who had failed radiotherapy and chemotherapy regimens, only 6% of the patients achieved any clinical response and the median survival from the start of thalidomide treatment was 2.5 months only [14]. In contrast, the IMiDEs demonstrated extreme therapeutic efficacy in MM, and this discovery marked the beginning of a new era for patients and provided researchers an opportunity to address the

antiangiogenic properties of the drug in situ, which led to interesting conclusions that will be discussed below.

Angiogenesis and the IMiDS in MM

The assumption that the activity of thalidomide on its target sites is mediated via its antiangiogenic properties became assessable in the malignancy setup owing to the availability of authentic specimens from thalidomide treated patients. Two methods have been adopted for this purpose in MM, the first was measurement of the cytokine levels and the second utilized BM biopsies to answer this question directly. Baseline cytokine levels were associated in some of the studies with the tumor load and with the outcome. For example, in a Spanish study that included 38 patients with relapsed/refractory MM, baseline serum levels of VEGF were significantly higher and levels of hepatocyte growth factor (HGF) were significantly lower in thalidomide responders, whereas serum levels of b-FGF and IL-6 did not correlate with the response to treatment [15]. In an Australian phase II trial that included 75 patients with relapsed/refractory myeloma, elevated VEGF levels were also predictive for a superior response to thalidomide and in responders VEGF levels decreased significantly from a median of 65.8 pg/mL (range: 9.2–562.4) to 43.3 pg/mL (range: 0–208.1) at best response [16]. Because serum cytokine levels is derived from secretion by both the normal and the tumor cells, the influence of the IMiDEs on cytokine release from the tumor cells proper can be estimated only by direct assessment of this question in a pure population of tumor cells. To this end, a French group analyzed the modulation of the expression pattern by lenalidomide (a second generation IMiDE),using real-time quantitative PCR, in a collection of 23 MM cell lines (17). Surprisingly, although lenalidomide inhibited the proliferation of two-thirds of the myeloma cell-lines examined, as measured by ^{3}H-thymidine incorporation, it paradoxically increased the expressions of TNFα, insulin-like growth factor-1 (IGF-1) and IL-8 (an angiogenic factor) in both lenalidomide sensitive and lenalidomide resistant MM cells. The last observation doubted therefore the assumption that the IMiDEs work in myeloma through inhibition of angiogenesis. However, relying upon cytokine levels might be misleading because stimulation of angiogenesis might be mediated via direct cell-cell contact between tumor and endothelial cells or alternatively could be exerted by unmeasured cytokines. For this discrimination we will need also the micro vessel density (MVD) data.

In contrast to the cytokine levels, the MVD provides a direct estimation of the vascular density in the tissue and it can be also repeated during the course of treatment. To this end, Yaccoby et al. utilized their severe combined immunodeficiency-human (SCID-hu) host system and examined the effect of thalidomide on MVD in a mice model of myeloma [18]. In this model, tumor cells interact with the human microenvironment to produce typical myeloma manifestations in the hosts, including stimulation of neoangiogenesis. To "humanize" the metabolism of thalidomide, SCID-hu mice received implants of fetal human liver fragments under the renal capsule in addition to subcutaneous implants of a fetal human bone. It was found that daily thalidomide given by peritoneal injection significantly inhibited myeloma growth in 7 of 8 experiments, each with myeloma cells from a different patient, in hosts implanted with human liver. In contrast, thalidomide exerted an antimyeloma effect in only 1 of 10 mice without liver implants thus confirming that human-like metabolism is required for the antimyeloma effects of thalidomide, a reminiscence of the species specificity

in thalidomide teratogenicity. In addition, human bone MVD in the untreated controls was higher than in thalidomide-responsive hosts but not different from nonresponsive ones. Also the expression of VEGF by MM cells and by other cells in the human bone was not affected by thalidomide treatment in any experiment. In conclusion, although the response to thalidomide was correlated with MVD reduction, there was definitely no indication that the decrease in MVD was the cause of the tumor reduction rather than being secondary to reduced tumor burden. In agreement with the findings described, serial MVD measurements in BM samples from MM patients before and after starting thalidomide treatment led virtually to the same conclusion. For example, a Japanese group evaluated BM biopsies from 51 patients with untreated myeloma or monoclonal gammopathy of undetermined significance (MGUS) and found as expected that BM MVD is higher in MM and is correlated with the tumor load. After starting thalidomide the monoclonal component decreased by at least 30% in seven of 11 evaluable patients whereas MVD decreased in only three of the responders (19). A Spanish study from 2008, examined the same issue in a group of 44 patients (14 newly diagnosed, 30 refractory/relapsed), who were treated with novel agents (thalidomide, lenalidomide, bortezomib) combined with dexamethasone. In 19 of the patients, a pretreatment BM biopsy was available and MVD was examined using anti-CD34 antibody. In addition, serum levels of various angiogenic factors (VEGF, b-FGF, and HGF) and cytokines (IL-6 and TNFα) were also measured. Despite the correlation found between MVD and the tumor load, MVD was not significantly correlated with either disease stage or response to therapy. Also baseline serum levels of the cytokines measured were not correlated with the response to treatment and no significant correlation was found between BM MVD and the serum levels of angiogenic cytokines. In fact, b-FGF serum levels even increased after starting therapy. The final conclusion therefore, was that no correlation exists between MVD and the serum levels of angiogenic cytokines, neither between each of them and the response to therapy [20]. Similar conclusions could be drawn from the Australian phase II trial mentioned, which included overall 75 patients with relapsed/refractory myeloma [16]. For example, before treatment some of the patients had high MVD levels without having a large marrow infiltrate and vice versa. After treatment, the MVD fell significantly in responders from a median baseline count of 21 (range: 7–71) to 10 (range: 4–20) at best response; and increased in patients who progressed during the study from 10 at baseline or best response (range: 1–130) to 20 at first progression (range: 1–113). However, the MVD did not decrease in all thalidomide responders and six of the 19 responders who had serial MVD measurements showed no change or even some increase in their MVD despite the clinical response. In addition, in the majority of responders in whom MVD did decrease, this was not follow a strict linear pattern but after an initial decrease the MVD often reached a plateau or even increased despite an ongoing response to therapy.

In conclusion, the lack of reduction of MVD levels in the myelomatous BM prior to the cytoreduction induced by the IMiDEs undermines the prevailing concept that the antimyeloma activity of the IMiDs is mediated via inhibition of angiogenesis. Also the cytokine levels measured were inconsistent with primary suppression of angiogenic stimulation as the explanation for the antimyeloma activity of the IMiDEs. Therefore, outside the context of research, MVD or cytokine measurements are lacking any beneficial value. An exception to the last statement is concerned for the subset of patient with the Polyneuropathy, Organomegaly, Endocrinopathy, M-protein and Skin changes (POEMS) syndrome, a rare

variant of plasma cell dyscrasia; for which patient's group, serum VEGF levels are strongly correlated with the response to thalidomide [21, 22].

The failure to link the antitumoral effects of the IMiDEs to inhibition of angiogenesis should not let us forget that the vascular meshwork is not just a transporter but it also participates in many physiological and pathological processes in the marrow such as homing, migration, proliferation and tumor invasion and recent data even placed BM vessels in the center of a provisional "vascular niche" entity, at least for the hematopoietic stem cell [23]. Therefore, it is still possible that the IMiDEs are doing part of their antitumural work via modification of the vascular wall endothelium. Although this possibility has not been examined as yet, it is noteworthy that the IMiDEs modify gene expression [24] and enhance endothelial cells maturation status. The significance of this differentiation in its broader sense will be elaborated at the end of this section but some of the clinical applications of the cell maturating property of thealidomide will be presented here. For example, in hereditary hemorrhagic telangiectasia (HHT) thalidomide reduced the severity and frequency of nosebleeds (epistaxis) in the majority of treated subjects [25]. The blood hemoglobin levels increased due to reduced hemorrhage and enhanced vessel wall stabilization. In mice heterozygous for a null mutation in the Eng gene (encoding endoglin), used as an experimental model of HHT, thalidomide treatment stimulated mural cell coverage and through this rescued vessel wall defects. Thalidomide also increased platelet-derived growth factor-B (PDGF-B) expression in endothelial cells and stimulated mural cell activation. The effects of thalidomide were partially reversed by pharmacological or genetic interference with PDGF signaling from endothelial cells to pericytes. Biopsies of nasal epithelium from individuals with HHT showed that similar mechanisms can explain the protective effects of thalidomide on humans. Thalidomide showed protective effects also in non-hereditary vascular malformations, as demonstrated in an open-label, randomized controlled trial [26]. On the molecular levels, stabilization of vessel wall was associated in one study with downregulation of Hif-1α, Ang2, Notch1 and Dll4, which are all tending to become overexpressed under hypoxic conditions [27]. Finally, thalidomide was protective for cultured endothelial cells, which were injured by preincubation with doxorubicin. In this model, doxorubicin induced a dose- and incubation time-dependent and caspase-3-mediated apoptosis of endothelial cells [28]. Thalidomide alone caused no changes in intact endothelial cells in terms of morphology, cell viability or activation of caspase-3. In contrast, when thalidomide was added to doxorubicin-injured endothelial cells, there was protection from cell death, increase in viability of endothelial cells, induction of differentiation and formation of neotubules.

In summary, although the antimyeloma effects of the IMiDs could not be explained in terms of quantitative vascular changes, the impact of the IMiDs on endothelial cell maturation may imply that MVD is not the real player here but qualitative changes on vascular endothelium may represent one of the mechanisms behind the antimyeloma activity of this class of agents. This possibility may be supported by the marked induction of vascular modulating genes like IL8, PTGS2 (encoding for COX-2) and AREG (encoding for amphiregulin) in primary MM cells after their removal from the BM niche in contrast to low and stable expression of the strong vasculogenic genes like VEGF and b-FGF, which are known to induce angiogenesis quantitatively [29]. The induction pattern mentioned actually reflects the response of the tumor cells to the interruption of their interactions with integral niche structures and signals, which are missing in vitro. The lack of induction of strong

angiogenic factors such as VEGF and b-FGF in vitro, also suggests that in niche the angiogenesis is not a limiting factor but on the other hand the tumor cells have to stimulate their vessels to function according to their needs in terms of niche signaling in analogy to the vascular niche model.

b. Oxidative Stress and the IMiDs

The growing interest on oxidative stress during the last decades did not skip the thalidomide mystery and the seminal work on this topic was published in 1999 by Parman et al. In that study, Parman et al. showed in rabbits that thalidomide stimulates embryonic DNA oxidation and teratogenicity, both of which were abolished by pre-treatment with the free radical trapping agent α-phenyl-N-t-butylnitrone (PBN). In contrast, in mice, a species resistant to thalidomide teratogenicity, thalidomide did not enhance DNA oxidation, even at a dose 300% higher than that used in rabbits [30]. Therefore, the authors concluded that these results constitute direct evidence that the teratogenicity of thalidomide may involve free radical-mediated oxidative damage to embryonic cellular macromolecules. In 2002, Hansen et al. located the oxidative damage to the limb bud itself [31]. They treated thalidomide-resistant Sprague-Dawley rat embryos (gestation day 13) with thalidomide in utero (300 mg/kg/day) and found no changes in intracellular glutathione (GSH) distribution in the limb bud tissue whereas thalidomide-sensitive New Zealand White rabbit embryos (gestation day 12) showed selective intracellular glutathione (GSH) depletion in the limb bud progress zone after treatment with thalidomide (300 mg/kg/day). This finding was accompanied by preferential decrease in NFkB expression in rabbit limb bud cells but not in rat limb bud cells. In addition, PBN rescued NFkB expression in thalidomide-treated cultures of limb bud cells compared with cultures that received thalidomide only. In situ hybridization showed a preferential decrease in expression of the limb patterning molecules Twist, Fgf8, and Fgf10 after thalidomide treatment (400 mg/kg per day) in rabbit embryos while the expression in rat embryos was not affected. Intravenous co-administration of thalidomide and PBN restored normal patterns and localization of these molecules in rabbits. These findings were considered as indicating that NFkB binding is diminished due to selective thalidomide-induced REDOX changes in the rabbit, resulting in a significant attenuation of the expression of genes necessary for limb outgrowth. Later, in 2004, the same group reported that the failure of the progress zone mesenchyme to express Fgf10 and Twist resulted from the failure of NFkB to bind to its DNA promoter [32]. In contrast, Knobloch et al. suggested in 2007 that the oxidative damage is mediated by bone morphogenetic proteins (Bmps). They injected thalidomide (50 μg) into the extraembryonic blood vessels of HH 19 chick embryos (750 μg/kg egg weight) and of total 309 treated embryos, the forty-seven (15%) which survived until day 7 showed uni or bilateral limb truncations including amelia
[33]. Of note, both proximal and distal structures were affected in all cases and uni or bilateral microphthalmia was seen in 21% of the embryos but as expected, phthalimide did not cause limb or eye defects. On the molecular level, they decided to check the expression of Dkk1 and found a remarkably higher expression of this gene in the limb buds from thalidomide treated HH 23/24 chick embryos compared to controls as well as in human but not in mouse embryonic fibroblasts. Since Dkk1 is controlled by Bmp signaling during early limb development, they continued to check the upstream regulation and showed that indeed there was induction of Bmp4, Bmp5 and Bmp7 following injection of thalidomide into the extraembryonic blood vessels of HH 19 chick embryos. In addition, in primary embryonic

cells isolated from the mesenchyme of chicken limb buds as well as in primary embryonic chicken fibroblasts thalidomide (38.7 μM = 10 μg/ml) increased the apoptotic rate x6 compared to controls but it did not increase apoptosis in primary mouse embryonic fibroblasts. As in whole limb buds, thalidomide induced the expression of both Bmp4 and Dkk1 in progress zone limb bud cells and in human embryonic fibroblasts but not in mouse embryonic fibroblasts whereas recombinant Noggin and anti-Dkk1 antibody completely neutralized thalidomide-induced cell death as also did Gsk3β inhibitors. In-vivo, Dkk1 and Gsk3β inhibitors reduced the truncation and microphthalmia proportions induced by thalidomide. Altogether, the results of this study suggested that limb bud cell apoptosis induced by thalidomide is initiated by ROS and mediated by blocking of Wnt/β-catenin signaling. Later, in 2008, the same group revealed that PTEN/Akt pathway participates in the apoptotic signaling cascade induced by thalidomide and that PTEN stabilization contributes to the limb anomalies via Akt suppression and caspase activation [34]. Finally, the discovery of Tbx5 and Sall4 gene abnormalities in congenital limb defects (Holt-Oram and Okihiro syndrome, respectively), prompted Knobloch et al. to investigate these genes also in the context of thalidomide limb defects and indeed RT-PCRs done on RNA extracted from wing buds of HH 23/24 chick embryos as well as from human embryonic fibroblasts revealed reduced transcription of Tbx5 and Sall4 in response to thalidomide [35]. When considering the vascular and oxidative insults together, it makes sense that both phenomena are related or in other words that the vascular insult was secondary to the oxidative stress, as demonstrated in embryoid bodies [8]. It is also noteworthy that the oxidative-stress hypothesis has not been extended to myeloma so far.

c. Adhesion Molecule and the IMiDs

The earliest referral to thalidomide in the context of adhesion was documented in 1984 and the finding was that the thalidomide metabolite EM12 inhibits the attachment of tumor cells to concanavalin-A coated surface [36]. Since1994, more data have been acquired, especially from German studies. Initially, it was found in human volunteers that thalidomide modulates the expression of many adhesion molecules such as CD18 (the common beta-chain of the beta 2-integrins), CD54 (ICAM-1), CD49b (VLA alpha 2) and CD49d (VLA alpha 4) [37]. In 1996, the same group demonstrated that EM12, triggers dramatic downregulation of several surface adhesion molecules (e.g., CD11a/CD18, CD49d/CD29, CD61, etc.) on early limb bud cells and on cells from some other primordia during early organogenesis of a primate (marmoset, Callithrix jacchus) [38], whereas oral administration of two daily doses of EM12 (2 x 50 mg/kg body weight) to Wistar rats from day 7 to 10 of pregnancy did not downregulatethe same adhesion molecules in limb bud cells from that species [39]. The species specificity demonstrated was considered by the authors as representing causal relationships between the downregulation of adhesion molecules and the teratogenic action of thalidomide. Based on the observation that thalidomide can bind at the N-terminal domain of N-cadherin by mimicking a tryptophan residue another German group suggested in the year 2000, that thalidomide might disturb cellular recognition and migration during morphogenesis by interaction with the N-terminal domain of N-cadherin, which is critical for the homodimerization of the adhesion molecule [40]. When considering the relationships found between the thalidomide embryopathy and adhesion molecule modulation it is readily seen that species specificity was the main association in favor of the suggested mechanism and

even that was in conflict with the tissue specificity criterion required to be fulfilled in any explanation for the thalidomide embryopathy, as will be elaborated in detail latter.

Nevertheless, when speaking of myeloma, adhesion molecule modulation becomes much more meaningful and therefore this issue will be summarized briefly. The major proteins involved in the interactions between MM cells and the extracellular matrix include CD44, very late antigen 4 (VLA-4), VLA-5, leukocyte function-associated antigen 1 (LFA-1, CD11a), neural cell adhesion molecule (NCAM, CD56), intercellular adhesion molecule 1 (ICAM-1, CD54), vascular cell adhesion molecule 1 (VCAM-1, CD106), syndecan-1 (CD138), and monocyte chemoattractant protein (41). The initial homing of MM cells to the BM milieu is mediated by binding of the stromal-derived growth factor (SDF-1α) in the BM to its C-X-C chemokine receptor (CXCR-4) located on MM cells. The interaction between MM cells and BM stromal cells leads to increased production of IL-6, a myeloma cell growth and survival factor, as well as additional growth factors. In addition, SDF-1α also modulates the expression of VLA-4, LFA-1, VCAM-1, and ICAM-1 that mediate the adhesion between MM cells and BM stromal cells. Furthermore, adhesion of MM cells to BM stromal cells enhances NFκB activity that further upregulates IL-6 and VEGF. Also TNFα, which is secreted by MM cells, enhances the expression and secretion of IL-6 from BM stromal cells [42]. TNFα also activates NFκB and induces the expression of LFA1, ICAM-1, VCAM-1, VLA-4, and MUC-1 on MM cell lines as well as VCAM-1 and ICAM-1 on BM stromal cells. The IMiDs diminish IL-2, interferon-γ, and IL-6 regulator suppressor of cytokine signaling (SOCS) expression in immune (CD4T, CD8T, natural-killer T, natural-killer) cells from both BM and peripheral blood (PB) of MM patients [43]. In pre-clinical models, inhibition of adhesion molecules by statins restored drug sensitivity to cultured MM cells by reducing cell adhesion mediated drug resistance (CAM-DR), however the effects of the IMiDEs on CAM-DR has not been examined as yet.

d. The Nervous System and the IMiDs

The mechanisms discussed so far were purely hypothetical based and relied upon experimental models to support the feasibility of the suggested insults in terms of species and organ specificity. The last two hypotheses are much more complex and apart from species and tissue specificity they are trying to face with the unique distribution of deformities based on diagnostic findings like x-rays from thalidomide victims. The first of the two hypotheses linked the teratogenesis with the neuropathy which is frequent in thalidomide users.

The earliest attribution of the thalidomide embryopathy to a neurological insult was documented in 1966. Gavin Gordon from Cumberland, UK was impressed from the fact that the thalidomide neuropathy is predominated by sensory symptoms while weakness is usually mild and involves proximal rather than distal muscles [44]. Gordon explained: "The wide variety of deformities in neonates following the use of thalidomide as a sedative by the mother can only be the result of a toxic factor acting on the initial nerve impulses to muscles in the organogenetic phase of development. This explanation is developed to cover all the deformities seen in 'thalidomide babies' and is correlated with the pathogenic picture in adults following prolonged dosage". In 1968, Fullerton et al. described the clinical and laboratory findings in 22 patients who suffered from thalidomide neuropathy, during follow up periods between four to six years after stopping the drug. Symptoms and signs were unchanged in approximately 50% of cases; have improved in a quarter, and the remainder have recovered. Sensory nerve action potentials were recorded and found to be closely related

to the clinical state. Sural nerve biopsies from six patients, performed two to six years after stopping thalidomide, showed selective loss of large diameter fibers. There was no segmental demyelination. In two of the nerves a marked increase in number of small fibers was taken to indicate regeneration. In 1973, Janet McCredie and William McBride revived Gordon's hypothesis (probably unaware of it) and created the basis for the axonal substraction theory [46]. Their assumption was based on detailed radiological review of five cases, supported by a summary of 50 similar children in Britain, which revealed that the bones and joints of thalidomide victims are absent, hypoplastic, tapered, or fused, and the surface area of skin is reduced. These signs were considered analogous to the trophic changes in adult sensory peripheral neuropathy. The authors put forward the hypothesis that the teratogenic action of thalidomide is exerted on the neural crest and its derivatives, and that the reduction deformities of the limbs represent embryonic peripheral neuropathy. During the next years, McBride developed an experimental model which supported the neurological etiology of dysmelia [47]. Thalidomide was administered to pregnant rabbits in dosages of 150-250 mg/kg/day on days 8-12 of gestation. These females produced 40 offspring, 21 of which were deformed. Four control females produced 34 offspring, none of which was deformed. Degenerative changes were found in the neurons and axons of dorsal root ganglia in day-13 experimental embryos, i.e., at least 16 hours before the earliest signs of thalidomide dysmelia have been reported in rabbits. Since the dorsal root ganglia form in rabbits on days 11 and 12, the changes evident on day 13 indicated that degeneration of neurons and axons may be a pathogenetic factor in thalidomide-induced peripheral deformities. McBride and Stokes also proposed that the size of nerve injury is the factor which determines the extent of deformity: damage to peripheral sensory nerves results in preaxial abnormalities while a greater damage results in amelia [48]. Further support to the toxic neuropathy explanation for the embryopathy came from the study by McCredie et al. which was published in 1984 [49]. Pregnant rabbits were given oral thalidomide (150 mg/kg/day) on days 7-11 of gestation and seven fetuses with partial or total absence of the tibia, five treated fetuses without deformities, and four untreated controls were examined. The sciatic nerves were dissected and transverse sections were taken from an identical site. In all treated animals there was a significant reduction in total fascicular area and in the number of large diameter fibers. However, in deformed fetuses a significant depletion of total fiber numbers was evident as compared with the controls. It was concluded therefore that dysmelic deformities of the limbs are secondary to toxic embryonic neuropathy occurring when damage to the nerves reduces the transverse fascicular area below a critical threshold. The toxic neuropathy demonstrated experimentally, encouraged McCredie and Willert to go a step forwards and try to explain also the deformity distribution pattern and in 1999 they reported their findings, based on comparisons between the limb defects as appeared in x-rays from thalidomide victims and the sclerotome map [50]. The sclerotomes are areas of segmental sensory innervation of the limb skeleton defined by the radiation of referred pain. According to the sclerotome subtraction theory, injury to the sixth cervical nerve in the embryo will affect the sixth cervical sclerotome, resulting in defects in parts of the thumb, radius, humerus and scapula. Likewise, damage at the level of the fourth lumbar nerve will reduce or subtract the fourth lumbar sclerotome, sparing the foot but reducing the tibia, femur and pelvis. As expected, there was a high overlap (73.5%) between the skeletal deformities seen and the sclerotome map and these results were considered as confirming the principles of skeletal reduction according to the sclerotomes with involvement of the sensory nervous system in the process of limb morphogenesis and

teratogenesis. Further support to the sclerotome subtraction theory came from the investigation of limb development by Stephen et al. The author placed tantalum foil barriers either lateral to the mesonephric duct or parallel to the long axis of the chick embryo in the wing field of stage-12 to -15 chick embryos [51]. These barriers blocked the somatopleure's communication with more medial tissues at specific somite levels. The results of these experiments indicated that the limb is not induced from one specific point, and portions of the humerus appear to be induced segmentally along the entire limb field. It was proposed then, that the humerus is originated from several separate components which fuse to form the definitive bone. The "segmentation" of limb development in this study could fit the sclerotome subtraction theory, however several findings contradicted this theory. First, a German publication from 1982, showed that removal of axolotl nerve 4 (the largest brachial nerve, estimated to contribute 50-60% of the forelimb axons) by repeated resection resulted in pronounced reduction deformities with impaired regeneration of skeletal limb elements [52]. However, removal of nerve 3 or 5 gave less pronounced phenotype and although the dependency of skeletal element formation upon nerves was verified it did not follow a strict segmental subtraction, probably due to overlapped innervation of nerves 3, 4, and 5 to all four axolotl digits. More strikingly, Streker and Stephens placed a foil barrier into early chick embryos lateral to the neural tube in order to block brachial plexus-level neural crest cells from reaching the limb [53]. However, despite the interrupted innervation to the limb, the limb skeleton developed normally. Thus, the proposals that the segmental pattern of the limb is determined by level-specific nerves and that diminished levels of innervation will result in skeletal malformations were both refuted.

It is now well agreed that mesenchymal rather than ectodermal insult stood behind the thalidomide limb deformity, as first suggested by William McBride. It is also clear that the contribution of the neurological findings described to the understanding of the mechanism of action of the IMiDEs is small because both the physiological findings and the histological data acquired from affected nervous specimens of thalidomide users were not specific and molecular investigation was not done.

The situation is slightly different in respect to the CNS, since thalidomide has unique influence on the REM (rapid eye movement) sleep pattern, which its control is largely established. It is also important to recognize the general influence of thalidomide on the CNS, as described in 1960: "Thalidomide is a new sedative hypnotic drug which produces no toxic effects when administered orally to animals in massive doses. The drug has a quieting effect on the CNS, reducing the voluntary activity of laboratory animals and promoting sleep. Unlike the barbiturate drugs it does not cause an initial excitation in mice, incoordination or narcosis. It potentiates the actions of other CNS depressants, in particular the barbiturates. Its sedative effects are counteracted by CNS stimulants. Thalidomide proved to be virtually non-toxic. Mice tolerated the maximum oral dose (5 g/kg) that could be administered, without ill effects. Sleeping mice could be easily aroused, and then they resumed normal but slow movements and showed little interest in their surroundings. In contrast, both phenobarbitone and glutethimide caused initial excitation, marked ataxia, and incoordination of movement followed by narcosis and death. CNS stimulants rapidly counteracted the depressant action of thalidomide in mice. They quickly resumed normal motility and with increasing doses of methylamphetamine they became hyperactive and convulsed. The motility of mice made hyperactive with methylamphetamine (5 mg/kg, subcutaneously) was not depressed by an

oral dose of thalidomide (500 mg). The interaction of methylphenidate (Ritalin) with thalidomide was very similar to that of methylamphetamine [54].

Almost forty years later, it was shown that thalidomide aggravates canine cataplexy, a pathological manifestation of REM sleep atonia seen in narcolepsy [55]. Specifically, after intravenous injections of thalidomide (0.25-8.0 mg/kg) to six narcoleptic and four control Dobermans from the Stanford Canine Narcolepsy Colony, long episodes (30 minutes) of complete muscle atonia appeared in all narcoleptic animals but not in control animals. Polysomnographic recordings in two narcoleptic animals demonstrated a de synchronized electroencephalogram (EEG) pattern with silent electromyogram (EMG), a typical polygraphic pattern of cataplexy. In contrast, intravenous diazepam, a commonly used hypnotic with GABAergic activity, had no effect on cataplexy in narcoleptic animals. In vitro receptor binding and enzymatic assays revealed that thalidomide does not bind to or enzymatically modulate the neurotransmitter systems reported to be involved in the regulation of cataplexy [56].

In order to find possible molecular targets of thalidomide in the CNS, based on its physiological activity, which will later serve us to define the connection between the various activities of the drug the control of REM-sleep will be briefly describe. The REM sleep consists of a dreaming state in which there is activation of the cortical and hippocampal EEG, rapid eye movements, and loss of muscle tone. In humans, thalidomide significantly increased the time spent in REM and stage 3-4 sleep as compared with placebo. On the other hand, thalidomide significantly decreased the time spent in stage 1, while the time spent in stage 2 was unchanged. The effect of thalidomide on REM and stage 3-4 sleep is unique as compared with other hypnotics [57]. A key molecule in the physiology of REM sleep is orexin (hypocretin). A series of studies have suggested that loss of hypothalamic neurons which produce orexin causes narcolepsy in humans while in other mammalian species orexin plays an extremely important role in the regulation of sleep/wakefulness states, especially in the maintenance of wakefulness [58]. The orexin neuropeptides produced by lateral hypothalamic neurons play a critical role in the maintenance of wakefulness by activating two distinct receptors, the orexin-1 (OX1R) and the orexin-2 (OX2R) receptor that are widely distributed throughout the brain. The orexin system is believed to stabilize the wake-sleep flip-flop switch in wake-active structures consisting of histaminergic, monoaminergic, and cholinergic neurons, and also to regulate the onset of REM sleep and associated muscular atonia in the brainstem. In accordance with the prominent function of orexins in sustaining wakefulness, pharmacological blockade of both OX1R and OX2R (OX1/2R) has been shown to promote sleep in various species, and the dual OX1/2R antagonists almorexant, SB-649868 and suvorexant have been clinically validated for the treatment of insomnia. Further investigations conducted in rodent models on the specific role of OX1R and OX2R in sleep modulation indicate that selective blockade of OX2R seems to be sufficient to initiate and prolong sleep [59].

The brainstem flip-flop switch is consisting of mutually inhibitory REM-off and REM-on areas in the mesopontine tegmentum. Each side contains GABA (gamma-aminobutyric acid)-ergic neurons that heavily innervate the other. The REM-on area also contains two populations of glutamatergic neurons. One set projects to the basal forebrain and regulates EEG components of REM sleep, whereas the other projects to the medulla and spinal cord and regulates atonia during REM sleep. The mutually inhibitory interactions of the REM-on and REM-off areas may form a flip-flop switch that sharpens state transitions and makes them

vulnerable to sudden, unwanted transitions-for example, in narcolepsy [60]. The principle nuclei mediating REM sleep and their interactions with other brainstem and forebrain nuclei were recently identified by elegant work in rat. The sublaterodorsal nucleus, which is equivalent to the subcoeruleus or perilocus coeruleus in the cat, is the major structure responsible for REM sleep [61]. More recent work has led to the concept of a putative on/off switch for control of REM sleep [60]. In this model, the ventrolateral part of the periaqueductal grey matter and the lateral pontine tegmentum represent the REM-off region; i.e., these nuclei turn off REM sleep. Lesions of these nuclei increase the amount of REM sleep. This region is regulated by multiple afferents, it is *inhibited* by GABAergic and galaninergic projections from the forebrain ventrolateral preoptic nucleus plus the cholinergic projections from the pedunculopontine nucleus/laterodorsal tegmental nucleus. The periaqueductal grey matter and lateral pontine tegmentum REM-off neurons are *activated* by projections from the noradrenergic locus coeruleus, serotonergic raphe nucleus and importantly, by hypocretinergic pathways from the lateral hypothalamus. Narcolepsy is characterized by inappropriate intrusions of REM sleep and severe reductions of brain hypocretin; this could be explained by loss of hypocretin projection, thereby tipping the balance in favor of REM-on firing [62]. In relation to thalidomide, it can be speculated on the basis of the flip-flop model that this compound modulates either the REM-off or REM-on signaling cascade. However, further evaluation of this issues will be presented in the next section.

e. Limb Patterning and the IMiDs

The last hypothesis to be discussed here seems to have very little in common with the known activities of the IMiDs in myeloma. Nevertheless, as will be demonstrated, limb development is controlled by a dynamic signaling sequence and therefore identification of the exact stage where limb development was interrupted will exhibit the signaling system which was active at that stage, which might be the specific target of thalidomide. It is now also the time to refer more comprehensively to the criteria adopted for the elimination of irrelevant information which masked the meaningful findings as outlined by Reinhard Neubert from Freie Universität, Berlin [63]: "Since there seems to be no end to speculations on the possible mode of the teratogenic action of thalidomide, it may be helpful to recall the criteria that should be fulfilled when seriously attempting to explain the teratogenicity of this type of compound. These criteria are essential to provide an explanation of the pronounced species and phase specificity, as well as the malformation pattern associated with the teratogenic action of thalidomide, and they imply that the investigations should be performed:

- In a thalidomide-sensitive species (primates or possibly rabbits)
- At the thalidomide-susceptible stage of embryonic development (a short but well defined period)
- In cells of thalidomide-susceptible primordia (e.g., of the limbs or the heart)
- Under conditions inducing a close to 100% teratogenic effect
- At doses not inducing embryo mortality or a pronounced growth retardation
- To demonstrate that the effect is confined to thalidomide and its teratogenic derivatives and not inducible with non-teratogenic derivatives with a very similar chemical structure (e.g., thalidomide isomers or enantiomers).

In considering each of the four theories described earlier to the spirit of Neubert's perspective, they all failed the dosage criterion owing to the use of non-adjusted doses of thalidomide in respect to the doses used by pregnant women. In such mega dose range, thalidomide generated toxic oxygen injuries within susceptible organs, including the microcirculation due to the oxidizing properties of thalidomide derivatives [64]. Also the species specificity demonstrated in those experiments was likely a reflection of differences among species in neutralizing the ROS generated by oxidizing thalidomide derivatives rather than being specific at the primordial level as have been demonstrated in monkey. For example, in green pregnant monkey, an oral administration of thalidomide in a single or 3-day treatment periods between days 28 and 33 resulted in defects of the limbs which resembled those observed in humans, macaques and baboons [65] and no internal abnormalities were noted in any of the thalidomide-treated fetuses [66, 67]. In baboon, a relatively large series of experiments involving single and multiple dose treatments from 4 to 24 mg/kg/day, defined the sensitive period between days 25 and 29 of gestation. Macaque species have also demonstrated a similar sensitive period to thalidomide. A cranio-caudal gradient of development was also evident, so treatment on day 25 most commonly affected the upper limbs, treatment on day 27 affected the lower limbs, and treatment on day 29 only affected the tail. Limb reduction defects, including amelia and phocomelia, were the most common observation in macaques as well as in baboons. Finally, it has to be emphasized that no clear dose–response was observed in those fetuses [68] and that the thalidomide analog CG 3033 (supimide) did not provoke any teratogenity in this species in a remarkable contrast to the observations in non-primate animals [69].

If we return to the thalidomide doses applied experimentally, recall that 0.25 mg/kg thalidomide was sufficient to aggravate canine cataplexy [55], while in humans, a single 100 mg dose (1-2 mg/kg) during the sensitive period was sufficient to produce malformations (and even 20 mg was documented) [70]. A 100 mg dose of thalidomide given to human volunteers resulted in a plasma concentration of 1 mg/L = 1 μg/ml, while 200-mg thalidomide led to a Cmax of 2.40 μg/ml; tmax, 2.40 h; AUC0-∞, 21.62 μg/h/ml and $t_{1/2}$ of 6.18 h [71]; as well as Cmax 2.00 μg/ml, tmax 3.2 h, AUC0-∞ 19.8 μg/h/ml and $t_{1/2}$ of 6.17 h according to data from another study [72]. In contrast, the thalidomide doses used experimentally were 400 mg/kg in the seminal work by Parman et al. [30], 200 mg/kg in the D'Amato [2] and Kruse [3] et al. studies; 100 μg/ml but not 10 μg/ml inhibited embryoid endothelial cells in the Sauer et al. study [8], while the thalidomide concentration used by the Japanese group in zebrafish was 800 μM = 206.7 μg/ml [10].

Just consider the 35% incidence of phocomelia but also 24% decrease in fetal body weight, 450% increase in the incidence of fetal resorptions and 780% increase in postpartum fetal lethality in the study by Parman et al. [30] as well as the severe systemic complications in zebrafish, which included severe pericardial edema with heart failure and subsequent embryonic death by 5 days of development [10] in comparison to the lack of any apparent abnormality in chick embryos when thalidomide was applied in the range of the human doses (chick = 160–1,280 μg/kg; human = 700–2,000 μg/kg) [11] and the lack of systemic morbidities in mothers of thalidomide babies and in MM patients. Thus, in considering the mega dose range of thalidomide used in the experimental models described earlier, it becomes possible to explain most of the findings, whether speaking of the vascular injuries, neuronal insults or the so called 'species specificity' as derived from the same toxic oxygen-stress generated by thalidomide metabolites in a dose dependent manner. So we are left now with

the last chance for getting a feasible explanation to the thalidomide embryopathy and with it to the mechanism of action of the IMiDEs in general. But before proceeding, a short introduction to limb development is needed.

Limb development is proceeding from proximal (humerus) to distal (digits). The limb bud elongates by means of proliferation of the mesenchymal cells underneath the apical ectodermal ridge (AER), a thickening in the limb ectoderm that forms at the distal tip of the growing limb bud. This region of cell division is called the progress zone, and it extends about 200 μm in from the AER. The Fgf released from the AER keeps the progress zone mesenchymal cells dividing. The cellular matrix begins its differentiation by the condensation of cells that will form the cartilage templates of individual bones. As the limb grows outward, the proximal mesenchymal cells that are farthest from the AER and destined to become skeletal elements undergo condensation and initiate chondrogenesis. Thus, the condensations that give rise to proximal limb elements form first while the cells at the tip of the limb bud remain undifferentiated. As the bud continues to enlarge, more distal skeletal elements differentiate sequentially until the complete set of condensations is laid down. In summary, the condensations are proceeding from proximal (early) to distal (late) by the control of Fgfs released from the AER, which promotes proliferation and inhibits condensation and differentiation in mesenchymal cells nearest the apical ectodermal ridge. Experimentally, when AER is removed from an early limb bud, only the most proximal parts of the stylopod (humerus) are made. However, if an Fgf-containing bead is placed in the hole left by the removal of the AER, a normal limb will form [73]. From the findings described above, it becomes clear that the limb deformities seen in thalidomide babies could not result from interruption of AER signaling because these deformities frequently spared distal elements like hand and digits unless the entire limb was missing (Amelia).

In 1998, Cliford Tabin from the Harvard University proposed a new hypothesis based on the progress zone model of limb development [74]. Tabin's hypothesis consisted of two components ("double hit"), the first was familiar and consistent with the general thought that the primary event was an apoptotic insult to the limb bud mesenchyme (e.g., by impaired angiogenesis). The second "hit" tried to explain the sparing of distal-most elements (i.e., fingers) by introducing a secondary developmental defect. The idea was that the primary apoptotic "hit" required time until mesenchymal recovery and thus delayed limb elongation which in turn prolonged the time spent of proximal identity elements within a distalizing signal field (comprised of Fgf) originated from the AER (located in the tip of the limb bud) and consequently re-specified proximal identity cells to distal elements. The need for a secondary developmental "hit" was explained by Tabin: "Perhaps the most striking aspect of this syndrome is phocomelia (a severe shortening of the limbs). The long bones are shorter than normal and more proximal elements are lost, such that in extreme cases the hand or fingers are attached directly to the shoulder, a condition described as resembling the flipper of a sea. Here I show how these defects can be understood in the context of current models for limb patterning. Although the pharmacological basis for thalidomide's effect remains controversial, the net result of thalidomide exposure is a decrease in growth of the limb-bud mesenchyme. From the perspective of pattern formation, the challenge is to understand why the decrease in growth should lead to a specific loss of proximal limb elements. This effect could be viewed as paradoxical, as other perturbations that prevent limb bud growth, such as removal of the specialized AER at the tip of the limb bud, lead to the opposite patterning defect: deletions of distal structures. These truncations induced by AER removal are

explained by the 'progress zone' model of proximodistal patterning, and consideration in this context can also explain the distinct proximodistal defect seen with thalidomide. According to the progress-zone model, proximodistal structures are specified sequentially under the influence of a continuous signal, now believed to be a Fgf, produced by the AER. Initially, the entire limb mesenchyme has a proximal identity; left on its own, it would develop into proximal structures. However, the mesenchyme at the tip of the limb bud under the AER — the progress zone — is exposed to Fgf and becomes respecified to a slightly more distal fate. As limb development proceeds, the progress zone cells divide and, as a result of this growth, not all of these cells remain within range of the Fgf signal. Those too far from the AER maintain their already specified proximal fate, whereas those still in close proximity to the AER are once again respecified to a still more distal ate. This model explains the distal truncations seen following surgical removal of the AER: without the source of the distalizing factor Fgf, no further distal patterning occurs and only the proximal elements specified before the AER was removed are formed. The phocomelia observed following thalidomide treatment can also be understood from this developmental perspective. Thalidomide treatment blocks mesenchymal proliferation in the limb bud. I propose that this means that the progress zone does not increase in its number of cells, despite continued exposure to Fgfs from the AER. As a result, no progress-zone cells end up outside the range of the Fgfs, which would be required for them to remember proximally assigned fates. Instead, the entire progress zone is progressively distalized, ultimately programming the entire limb bud to form only the distal-most elements. Consistent with this view, an effect like that of thalidomide can be produced by irradiating the limb mesenchyme, limiting proliferation in the progress zone without affecting the distalizing activity of the AER. Based on these data, a similar mechanism was proposed as a general explanation for phocomelia. Loss of intermediate limb elements, the most common type of limb malformation seen in offspring of women who used thalidomide, is therefore a predicted consequence of altering growth in the progress zone while leaving intact the production of Ffgs by the AER. Many of the pharmacological mechanisms proposed to explain thalidomide's decreasing growth in the early limb bud, such as inhibition of the growth of blood vessels, could also contribute later to decreased growth of the skeletal elements, leading to shortening of the long bones. Although this is not strictly a patterning defect, it is commonly observed in thalidomide cases. Finally, after the period of exposure to thalidomide, the progress zone would recover, leading to the range of patterning defects occasionally seen, including Amelia (absence of limbs), phocomelia, and radial aplasia (lack of development of the radius). The limb patterning defects of thalidomide are therefore the understandable and expected result of decoupling distalization from outgrowth during limb patterning".

Despite being elegant, unique in attempt to explain the deformity distribution pattern, and introducing developmental considerations rather than sticking to toxic insult exclusively, Tabin's hypothesis did not peak too much interest and was even criticized by Reinhard Neubert et al.: "With regard to Tabin's hypothesis, we must consider the fact that the critical period for thalidomide-induced typical limb malformations (amelia and phocomelia) is very early. According to investigations in primates, developmental stages 11 to 14 are affected, reaching (for example in the marmoset *Callithrix jacchus*) a maximum at stages 11 to 12. Upper limb buds start to develop as early as stage 11. The AER, which is critical according to Tabin's hypothesis, does not occur until stage 14, too late to bring about amelia or phocomelia" [75].

In 2009, Tabin's hypothesis from 1999 was tested experimentally. Galloway and Tabin et al. induced apoptosis in limb bud mesenchyme using x-irradiation (first "hit"). However, despite the remarkable apoptosis in limb bud mesenchyme, the phenotype of the embryos was not as they expected: "Here, using a combination of molecular analysis and lineage tracing in chick, we show that x-irradiation-induced phocomelia is fundamentally not a patterning defect, but rather results from a time-dependent loss of skeletal progenitors. Because skeletal condensation proceeds from the shoulder to fingers (in a proximal to distal direction), the proximal elements are differentially affected in limb buds exposed to radiation at early stages. This conclusion changes the framework for considering the effect of thalidomide and other forms of phocomelia, suggesting the possibility that the aetiology lies not in a defect in the patterning process, but rather in progenitor cell survival and differentiation. Moreover, molecular evidence that proximodistal patterning is unaffected after x-irradiation does not support the predictions of the progress zone model" [76]. In other words, Tabin's et al. returned to the single "hit" apoptotic insult...

Now, after rejection of each of the five theories mentioned from one reason or another, we stay again with no a reasonable explanation to this saga. However, the last statement is not precise because limb patterning defect was excluded only in the context of a single speculative hypothesis whereas from almost any other consideration (e.g., the thalidomide doses used, its short half-life, the lack of general morbidities in mothers, MM patients and monkey), limb patterning defect remains the only possible explanation. However, the strongest argument in favor of limb patterning defect is derived from the spatiotemporal sequence of limb element loss (or sparing), which will be elaborated after description of the sequence by the words of Henkel and Willert from 1969: "In the upper limb mild manifestations of the deformity are restricted to the radial ray of the hand. Then follows, with increasing severity, involvement of the radius and only after the radius is either completely absent or its remnants have fused with the ulna is the humerus affected" [77], as well as the description by Smithells and Newman in 1992: "The bones of the upper limb are affected in a remarkably regular order, starting with the thumb, followed by the radius, the humerus, the ulna, and finally the fingers on the ulnar side of the hand (middle., ring, and little fingers) [78].

As readily noticed, the deformity sequence described was definitely non-linear, for the ulna was reduced only after the humerus and the ulnar digits were reduced even later, thereby making the whole sequence difficult to interpret from a developmental perspective. However, much of the confusion was probably caused by the intuitive tendency to relate to limb truncation as a proximodistal insult similar to the truncations produced in AER extirpation experiments, which gave rise to a linear sequence of bone element loss from distal-most to proximal. In contrast, in congenital limb defects for example, truncation deformities result many times from mutations in anteroposterior patterning genes, like Tbx5 in Holt-Oram syndrome, Sall4 in the Okihiro syndrome [79] as well as mutations in Shh, Wnt7a, Wnt5a and Wnt 3a, which are all associated with truncations and reduction deformities. Therefore, it is still possible that thalidomide interrupts the anteroposterior patterning sequence, provided that this sequence overlaps the limb defect sequence described in thalidomide babies.

Some evidence for sequential maturation of limb elements along the anteroposterior axis, came from the study by Koyama et al., which was published in 1966 [80]. Koyama et al. were concerned from the fact that the activities of the ZPA (zone of polarizing activity; essential for anteroposterior patterning) and AER dwindle early in embryogenesis and soon after

ceases, when in fact the proximal skeletal elements are still rudimentary in structure and the more distal ones are yet to become recognizable. One possibility was that the chondrocytes formed following mesenchymal condensation start themselves expressing properties similar to those of ZPA and/or AER and in so doing, may bring skeletal development to completion. To answer this question they isolated radius and ulna with attached presumptive periosteum from the stage 29 embryos, subdividing each element into one diaphysis and two epiphyses, and grafted the resulting tissue fragments to the anterior margin of host stage 19-20 wing buds. As negative control, they used fragments of most-anterior non-chondrogenic mesenchyme and overlying ectoderm from the same wings. The results confirmed their basic assumption and also revealed that the fragments of ulna had a much stronger polarizing activity than those from radius at this stage; ulna's diaphysis in fact displayed the strongest activity among all the fragments tested and induced supernumerary digits including digit 4 in about 25% of operated wings. Ulna's epiphyses and radius' diaphysis were weaker and induced digits 2, 3, or both but not digit 4. Tissue fragments of most-anterior non-chondrogenic mesenchyme had no appreciable activity, attesting to the fact that polarizing activity was topographically restricted to the cartilaginous structures. To further assess the specificity of the polarizing activity, they grafted fragments of stage 29 ulna's or radius' diaphysis to the center of host stage 20-21 wing buds rather than to the anterior margin. In that case, no induction of supernumerary digits was observed (0/7). Surprisingly, when they examined stage 31 embryos, the polarizing activity of ulna's diaphysis had decreased significantly compared to that observed at stage 29 while the activity of radius's diaphysis was now higher; indeed, radius' diaphysis induced digit 4 in about 25% of operated wings. The epiphyses of both radius and ulna had comparable moderate activity at this stage 31; however, with further development the activity decreased substantially while it was still significant in developmentally younger distal elements, such as metacarpals and first phalange at stage 34. Clearly, the presence of polarizing activity in developing cartilaginous elements occurs sequentially and transiently along the proximodistal and posterior-anterior axes. In summary, the acquisition of polarizing activity by the cartilaginous structures followed clear proximal-to-distal and posterior-to-anterior routes. Thus, [1] stage 25 cartilaginous humerus had polarizing activity while stage 25 prospective radius did not, [2] posteriorly-located stage 29 ulna had stronger activity than anteriorly-located stage 29 radius, and [3] ulna's diaphysis had stronger activity at stage 29 than 31 while radius's diaphysis was stronger at stage 31 than 29. Prior to inducing extra digit formation, the cartilaginous grafts induced Hoxd-12 and Hoxd-13 gene expression in adjacent competent mesenchymal tissue. Strikingly, the cartilaginous grafts activity also induced expression of Shh and polarizing activity in adjacent mesenchyme, which ZPA grafts cannot do; thus, the cartilaginous structures displayed activities "upstream" of those of the ZPA. The results thus supported the hypothesis that chondrocytes can themselves direct skeletal morphogenesis. In so doing and as a result of their inductive activities, the cells may also have an important role in the completion of limb patterning and morphogenesis.

The relevant conclusion from that study to our subject is that the differentiation of discrete bone elements during the post condensation stage (and probably also before that), is by no means progressing along the proximodistal axis, like the sequence of chondrocyte condensation but each element has its specific timing, likely depending on distance from the AER (which generates the Fgf gradient) and from the ZPA (responsible for the Shh gradient). In fact, the two morphogenic gradients mentioned (or the three if we include the Wnt

signaling along the dorsovental axis) might collaborate in the right limb location to create a permissive morphogenic constellation of signals for differentiation. If the maturation sequence described by Koyama et al. is indeed dependent on morphogenic gradients, than the earlier maturation of the ulna and ulnar digits (and therefore their sparing from thalidomide insults), could result from their closer proximity to the ZPA. Anyway, the differentiation sequence reported by Koyama's et al. partially overlapped the limb deformity sequence described in thalidomide victims wherein the radius was always reduced earlier than the ulna and the thumb before the ulnar digits. It was not a perfect match however, because the autopod (digits) differentiated lastly in the Koyama's et al. study in contrast to its most frequent sparing in thalidomide victims. Nevertheless, this instance provided firm evidence that the differentiation sequence of discrete bone elements after the chondrocyte condensation stage turns to be non-linear and therefore can overlap the cumulative sequence of the thalidomide limb defects. In order to find possible patterning factors which their interruption can mimic the phenotypic spectrum observed in thalidomide victims the relevant anteroposterior molecules will be described.

a. Hedgehog signaling: Shh is an example of a morphogen as defined by Lewis Wolpert's French flag model—a molecule that diffuses to form a concentration gradient and has different effects on the cells of the developing embryo depending on its concentration. Shh remains important in the adult. Of the hedgehog homologues, Shh has been found to have the most critical roles in development, acting as a morphogen involved in patterning many systems, including the limb and midline structures in the brain, spinal cord, the thalamus by the zona limitans intrathalamica and the teeth. Mutations in the human Shh, cause holoprosencephaly type 3 as a result of the loss of the ventral midline. Gli3 (the gene implicated in human Greig cephalopolysyndactyly syndrome) is proposed to negatively regulate Shh by restricting its expression and influence to the posterior mesoderm. Genetic analyses in mice showing that Shh and Gli3 are dispensable for formation of limb skeletal elements: Shh(-/-) Gli3(-/-) limbs are distally complete and polydactylous, but completely lack wild-type digit identities. Recently it was shown that the effects of Shh signaling on skeletal patterning and ridge maintenance are necessarily mediated through Gli3. It was propose that the function of Shh and Gli3 in limb skeletal patterning is limited to refining autopodial morphology, imposing pentadactyl constraint on the limb's polydactyl potential, and organizing digit identity specification, by regulating the relative balance of Gli3 transcriptional activator and repressor activities [81]. Ptch1 encodes a 12-pass transmembrane glycoprotein that functions as a negative regulator of the Shh signaling pathway. Binding of Shh ligand to the Ptch1 receptor liberates Smoothened activity, which ultimately leads to the release and nuclear translocation of activator and repressor forms of the Gli family of zinc finger transcription factors (Gli1, Gli2, and Gli3). Inactivation of Ptch1 via loss of function mutation leads to constitutive pathway activation due to unrestrained Smoothened activity. A diverse range of downstream targets have been identified in mammals, including members of other developmental pathways, such as Wnt, Bmp, and Notch, in addition to genes involved in cell cycle progression, apoptosis, and migration. Mice null for Ptch1 die during the early stages of embryonic development (at E9.5) due to severe developmental anomalies in embryonic patterning**.** Mutations in *PTCH1* cause Gorlin syndrome often with a midline cleft lip and mutations have also been found in holoprosencephaly patients [82, 83]. The family of Gli proteins (Gli 1-3) comprises the intracellular mediators of the hedgehog pathway, which regulates a myriad of developmental processes, one of which is limb development. Whereas

Gli 1 and Gli 2 seem to be dispensable during limb development, Gli 3 is especially crucial since all Gli 3-associated human congenital diseases comprise limb malformations. Furthermore, Gli3(-/-) mouse embryos exhibit pronounced polydactyly in conjunction with a loss of digit identities. Mutations in Gli3 cause several congenital diseases including Greig cephalopolysyndactyly syndrome and Pallister–Hall syndrome. Most of the clinical characteristics of Gli3 associated syndromes are distinct, but all share digit abnormalities. Only Gli3 seems to exert such an irreplaceable function in limb development since Gli1 and Gli2 deficient mouse embryos develop well patterned extremities. The effects of a loss of Gli3 function can be seen in *extra-toes* (*XtJ*) mice, which carry a *Gli3* null allele and represent a mouse model for GCPS. Heterozygous $Gli3^{+/-}$ mice display mild preaxial polydactyly (= supernumerary digits at the anterior side) while the limbs of homozygous $Gli3^{-/-}$ embryos exhibit severe polydactyly associated with a complete loss of digit identities. Consequently, Gli3 is believed to act in the regulation of both the number and the identity of digits [84].

b. Tbx5: The transcription factor Tbx5 is expressed in the developing heart, eyes and anterior appendages. Mutations in human Tbx5 cause Holt–Oram syndrome, a condition characterized by heart and upper limb malformations. Tbx5-knockout mouse embryos have severely impaired forelimb and heart morphogenesis from the earliest stages of their development. However, zebrafish embryos with compromised tbx5 function show a complete absence of pectoral fins, while heart development is disturbed at significantly later developmental stages. Tbx5b gene in zebrafish is co-expressed with its paralogue, tbx5a. Downregulation of tbx5a and/or tbx5b in zebrafish revealed that tbx5 genes have essential roles in the establishment of cardiac laterality, dorsoventral retina axis organization and pectoral fin development [85].

c. Bmps: are multifunctional growth factors that belong to the transforming growth factor beta (TGFβ) multigene family**.** In the vertebrate limb bud, the expression of several members of the Bmp gene family has been documented, namely Bmp2, Bmp4 and Bmp7. Current evidence indicates that Bmp2, Bmp4 and Bmp7 are the main source of Bmp signaling in the developing limb including AER formation, AER regression, cartilage and bone differentiation and interdigital webbing regression because of the early lethality of null mutants for Bmp2 and Bmp4, as well as their receptors, Bmpr1 and Bmpr2. In addition, these Bmps share functional similarities and may display functional redundancy. As a result, the role of one particular Bmp gene may be hidden by the activity of others. For example, although the mutation of Bmp7 by itself has little influence on limb development, it does enhance the Bmpr1b mutant phenotype [86].

d. Wnt7a pathways: Wnt7a is located in the dorsal ectoderm and it has two main pathways: one is via b-catenin (known as the canonical pathway) and the other is not dependent on b-catenin (known as the non-canonical pathway). In the canonical pathway binding of Wnt7a to its receptor (the receptor has two parts: a protein called Frizzled and a co-receptor called LRP5/6), stabilizes intracellular β-catenin by combining to a complex responsible for β-catenin degradation [79]. This complex has many components including APC (adenomatous polyposis coli) and GSK-3b (glycogen synthase kinase). The stabilized β-catenin in the cytoplasm combines with E-cadherin (cell surface protein responsible for cell-to-cell interactions) and the complex plays an important role in chondrogenesis of the developing limb. Stabilized β-catenin enters the nucleus to interact with transcription factors (especially T-cell factor or TCF) and upregulates Shh expression. Shh is initially expressed in

the absence of Wnt7a. Later, however, Shh can only be maintained in the presence of Wnt7a. The secreted Shh does not just control anteroposterior patterning (including the development of the ulnar ray). It is also the key inductive signal for Fgf4 in the AER, which acts via a biofeedback mechanism to regulate Shh expression (Fgf4 contributes to the outgrowth of the limb along the proximodistal axis), and it also induces local expression of Hox-D13, which is also required for limb patterning. Isolated suppression of Shh results in ulnar ray deficiency which is always associated with short upper limbs secondary to reduced Fgf4 activity. Animals lacking the Shh gene function have truncated hind limbs and ulnar ray deficiency in the forelimbs. The Wnt7a non-canonical pathway in the upper limb development induces the expression of Lmx-1 in the dorsal mesoderm, which is responsible for the development of dorsal hand structures (hair, nails, extensor tendons, etc.). Isolated mutation of the Lmx-1 results in nail-patella syndrome (absent nails and patella). In the Al-Qattan palmar duplication syndrome there is a mutation of Wnt7a and hence there is lack of development of dorsal hand structures (the dorsum of the hand resembles a palm with absent nails) (Lmx-1 suppression), ulnar ray deficiency (Shh suppression), short upper limbs/truncated lower limbs (severe Fgf4 and Shh suppression). There are two main down-regulating proteins of Wnt7a activity. Dickkopf-1 (Dkk1) is a Wnt antagonist (i.e., an inhibitor of the Wnt canonical pathway) and animals with null allele of Dkk1 get overexpansion of the apical ectodermal ridge (AER) with polysyndactyly. The second protein is Engrailed-1 (En-1) produced in the ventral ectoderm and acts to suppress the activity of Wnt7a. Loss of En-1 allows Wnt7a to be expressed throughout the ectoderm (i.e., along both the ventral and dorsal ectoderm). As a result, no ventral structures will develop and affected mice get duplication of dorsal feet elements with all nails exhibiting the characteristic ‘circumferential’ nail appearance of the distal ends of the digits because of the presence of a plantar nail and dorsal nail joining at tip of the digit. Furthermore, affected mice have defective cerebellums indicating that En-1 has a role in cerebellar development. In humans, bi-dorsal duplication is usually partially expressed in the little (and sometimes also the ring) fingers and more than 30 cases have been described under different terms such as ‘clam nail deformity’.

e. Wnt3/3A pathways: Wnt3 and Wnt3a are closely related. Wnt3 is expressed in the AER of the chick and is necessary for AER formation while Wnt3a is expressed in the limb ectoderm of the mouse and is required for normal limb development. In humans, homozygous Wnt3 mutation causes tetra-amelia. This is expected since the human Wnt3 is responsible for normal development of the AER which is, in turn, responsible for the outgrowth of the limb. The pathway of Wnt3/3a is similar to the canonical pathway of Wnt7a (i.e., via β-catenin). It is interesting to note that LEF-1 is the preferred factor for the Wnt3/3a pathway, while TCF is the preferred factor for the Wnt7a pathway. The normal interaction of β-catenin and LEF-1 will result in normal development of the AER, the production of Fgf8 (an important growth factor for the outgrowth of the limb) and the production of Bmp-2 from the AER. Proper chondrogenesis is known to be balanced by the antagonistic actions of Bmp2 (stimulating effect) and the Wnt7a pathway (inhibitory effect). Mis-expression of Wnt3a causes ectopic expression of AER genes for Fgf8 and Bmp2. Working in conjunction with Wnt3a in the AER is R-spondin-2. R-spondin-2 is a protein which acts synergistically with extracellular components of the Wnt pathway and also functions as a class of ligands independent of Wnt-proteins, leading to downstream activation of β-catenin-dependent genes. Finally, Fgf10 (a mesenchymal inducer for limb formation) is known to induce Wnt3a in the AER, and mice in

which both copies of the Fgf10 gene have been disrupted by gene targeting fail to develop limbs. Furthermore, Fgf10 appears to have a positive regulatory loop with Fgf8.

f. Wnt5/5a pathway: We have previously noted that Wnt7a is specifically expressed in dorsal ectoderm and Wnt3/3a in the AER. Wnt5/5a are expressed in two sites: the AER and the limb mesoderm where Wnt5a follows a proximodistal gradient in the mesoderm of the developing limb. It is important to realize that Wnt5/5a act in the early stages of regeneration of the limb bud to control the de-differentiation of stump cells giving rise to regeneration-competent cells. Therefore, it is not surprising to know that Wnt5a is essential for normal chondrogenesis. It is also important to realize that the Wnt5/5a pathway acts mostly via modulation of intracellular calcium and not via stabilization of β-catenin. In fact, the Wnt5a pathway is sometimes called 'the antagonizer' pathway of the Wnt7a canonical pathway [87]. Wnt5a pathway stimulation induces siah 2 which leads to increased b-catenin degradation in the cell. In contrast, Wnt7a canonical pathway stimulation results in decreased β -catenin degradation. As mentioned earlier, Wnt5a is strongly expressed in the distal limb bud and its expression becomes weaker as one goes proximally. Therefore, it is not surprising that experimental limbs with Wnt5a -/- have missing distal digits. In these limbs, the AER has a normal appearance but the distal limb bud has accumulation of β -catenin and lack of expression of Sox9 (an early marker for mesenchymal condensation and chondrocyte differentiation).

When considering all the patterning molecules mentioned, it can be readily noticed that only Wnt3a and Fgf10 signaling interruption could give rise to tetra-amelia, which is part of the spectrum of thalidomide embryopathy. Albeit one can speculate then that interruption of Wnt3a signaling at a certain time point during limb development could result in composite reduction deformities, which involve both the anteroposterior and the proximodistal axes (due to the role of Fgf10), similar to the deformities seen in thalidomide victims, it is improbable that the phenotypic spectrum of such insult will include phocomelia. Therefore, at this stage we have to consider an alternative developmental sequence which is corresponding to the limb deformity sequence described in thalidomide victims. The last spatiotemporal sequence left is the sequence of chondrogenesis.

Chondrocyte maturation sequence: In the limbs, where extensive growth is required for proximodistal extension of the long bones, endochondral development begins with the aggregation of undifferentiated mesenchymal cells to form condensations, which by their position, shape, and size already prefigure the future skeletal elements [88]. In the core of these condensations, cells differentiate into chondrocytes, which secrete cartilage matrix. At the periphery, cells form a perichondrial sheath that surrounds the cartilage model. Proliferation of chondrocytes and perichondrial cells, as well as deposition of new matrix, are responsible for initial growth of these skeletal elements. At a certain stage, which is specific for each element, cells in the center undergo further maturation into hypertrophic chondrocytes, a process characterized by their exit from the cell cycle, enlargement and secretion of a distinct extracellular matrix that becomes progressively calcified. As a result, immature proliferating chondrocytes become restricted to each end of the skeletal element. These changes are accompanied by direct differentiation of perichondrial cells into osteoblasts, followed in turn by the deposition of a calcified bone matrix, the "bone collar," around the cartilaginous core of the future long bone. Changes in the composition and properties of the cartilage matrix in the hypertrophic zone, allow invasion by capillaries,

accompanied by apoptosis of terminally differentiated chondrocytes, degradation of the calcified cartilage matrix, and its replacement by the trabecular bone matrix secreted by invading osteoblasts. The ordered progression of the differentiation program and the columnar arrangement of the chondrocytes result in a stratified organization where zones of proliferation, maturation, hypertrophy, calcification, and bone formation can be recognized, proceeding from the articular ends to the shaft of the bone. Continued proliferation of the less mature chondrocytes at the extremities, their differentiation into hypertrophic chondrocytes, and their replacement by trabecular bone near the center results in longitudinal growth of the bone. Continued deposition of cortical bone by the periosteum leads to radial growth. Finally, bone remodeling results from the combined activities of the osteoclasts, bone resorbing cells of hematopoietic origin, and the osteoblasts. Coordinating each of these processes is critical in determining the size, shape, and mechanical properties of the bones.

Before discussing the place of chondrogenesis in the thalidomide story, it has to be emphasized that none of the patterning molecules mentioned could explain how a single dose of thalidomide causes truncation of an entire limb or reduction of several bone elements, considering the short $t_{1/2}$ of the drug and the recovery of limb bud mesenchymal cells following considerable insults like irradiation [76]. To illustrate that, recall the two week sensitive period in humans, in which the 12-hours exposure to effective thalidomide levels represent 3.5% only of the sensitive period and therefore 3.5% of the limb development during that period. In fact, the striking effect of any dose of thalidomide on limb development, leaves only one logical explanation to that 50-year mystery, which is that thalidomide strikes only once, but this strike irreversibly changes cell fate of susceptible primordia. In other words, thalidomide is predicted to behave like a differentiating agent, much like retinoids which are also teratogenic in a dose independent manner. In considering the phenotype and the timing of the catastrophic event, speaking of already defined identities (i.e., humerus, ulna, radius etc.), which must however be prone to immature differentiation or commitment, only condensed chondrocytes could fulfill these criteria. From the chondrogenetic sequence described, it appears that premature differentiation of proliferating chondrocytes to hypertrophic chondrocytes, when already committed segments are being protected (or spared), can explain the truncation deformities induced by thalidomide. Indeed, the differentiation sequence of condensed mesenchymal elements described by Koyama et el. is largely overlapping the limb deformity sequence described in thalidomide babies. Additional support to this scenario might be gained from assessment of radiological images of thalidomide victims. Indeed, careful assessment of radiological images of thalidomide victims from the series of McCredie et al. [51], can give the impression of a “frozen” or fixed maturation state, manifested by a spectrum of maturation figures in the same image, from well-formed elements which were developed normally to formed elements with abnormal features, such as fusions or incomplete shaping as well as to completely absent segments. For example, the image in Figure 8a [51], shows truncation of the proximal part of humerus with an intact distal part and normal shapes of the ulna and fibula, which are however fused at the tips. Figure 7a shows proximal radioulnar fusion alone. Figure 10a, shows humeroulnar fusion between the proximal humerus and the distal ulna, with a relatively normal shape of the fused elements. The interpretation on the basis of premature commitment, is that the missing segments were composed initially of hypertrophic chondrocyte rudiments (which have limited elongation potential), whereas the mineralized tissue between the zeugopod bones (ulna and radius) could result from premature differentiation of intermediate zone

chondrocytes to hypertrophic chondrocytes, which were later replaced by osteoblast. The fused humeroulnar bone could result from hypertrophic differentiation of the missing segment in between the two element tips (before humeroulnar separation to two discrete elements by joint formation), leaving the growing tips fused because the prematurely committed segment in between did not elongate and was replaced by osteoblasts which were mineralized later.

Such scenario is also consistent with the pathological findings described in the postmortem (PM) examination of a thalidomide child who was born alive and survived for 13 months [89]. In that case, which was described by Odette Hagen in 1966, it was considered extremely likely that the cause of the deformities was the ingestion of thalidomide by the pregnant mother. Clinically, in addition to severe limb shortening, there was bilateral microtia (abnormally small ears), a midline facial hemangioma, and a systolic heart murmur which disappeared as the child grew older. Radiologically, the baby was considered to have one long bone and three digits in each fore limb, and one long bone with a deformed foot in each inferior limb. It was considered that her death at the age of 13 months was caused by sudden heart failure due to congenital heart disease. In the upper limbs the main defect was an absence of the upper end of the humerus on both sides. Embedded in the deltoid muscle on the right was a small piece of cartilage about 1 cm. long, which on microscopy was identified as hyaline cartilage. The only true long bone in the upper limb was the ulna, to the upper end of which was attached a mass of hyaline cartilage which seemed to represent the lower end of the humerus. The radius, thumb and index finger were absent. The glenoid fossa was absent. The muscles which usually attach to the upper end of the humerus were fused together, and this fused fibrous body was attached to the cartilaginous mass at the upper end of the ulna. The muscles which usually attach to the radius and thumb were absent. The flexor muscles associated with the ulna and medial three digits were normal, except that there was no separation of the flexor digitorum superficialis and flexor digitorum profundus. The extensor muscles were poorly developed and in the hand the tendons fused with the deep fascia. On the left the soft tissues of the two lateral digits were fused. The nerve supply of the upper limbs followed the usual basic pattern, but with certain variations. The arterial supply of the upper limbs appeared very good. The lower limbs showed basically the same defects as did the upper limbs, viz, the upper end of the proximal long bone was absent and muscles which usually attach to it were fused. The acetabulum was absent. The distal end of the femur was present, but the only muscle directly attached to it was the short head of the biceps. In the leg the fibula with its muscles and their nerves was absent on the right, but on the left was represented by a thick strip of fibrous tissue to which the peroneal muscles were attached. This strip fused with the lower end of the tibia. On both sides the soleus arose with the gastrocnemius from the rudimentary femur. On the left the extensor hallucis longus arose from the fascial strip representing the fibula and sent tendons to all five toes. The phalanges of the second and third digits were partially fused, as were the skin and subcutaneous tissue of these digits. The extensor digitorum on this side sent a tendon to the hallux which blended with the periosteum of the first phalanx. There were no other muscles or tendons associated with this digit. The internal auditory meatus was normal, but the auditory nerve ended blindly in the bone. The facial nerve continued on to emerge from the skull at the suture between the mastoid process of the temporal and the parietal bone. There was no styloid process.

In summary, both the radiological images and the PM examination revealed fused bone segments and residual rudiments, which the PM examination identified as hyaline cartilage. This finding is consistent with the suggested catastrophic differentiation insult. In order to

find which signaling pathways are controlling chondrocyte maturation and therefore can be the targets of the IMiDEs in the context of differentiation induction we have to recognize the chondrocyte maturation control.

Chondrocytes Maturation Control: At the molecular levels, it is now believed that mesenchymal condensations occur through 'identity' determination signals rather than simple 'core' formation: the humerus under the influence of retinoic acid/Meis-1 genes, and the bones of forearm/hand under the influence of Shh signaling. The zeugopod and autopod may be divided into three zones [88]. The area around the Shh contain descendants of Shh-expressing cells and is responsible for the development of the ulna, digit 5, digit 4 and part of digit 3. The next zone is under the influence of long-range Shh signaling and is responsible for the development of digit 2 and part of digit 3. The third zone is not under the influence of any Shh signaling. In this zone, the radius and digit 1 develop under the influence of other signals including Tbx5 and Sall4. Therefore, experimental mice with deletions leading to lack of Shh expression will have absence of the ulna and digits 2 to 5. The final digital patterning is a very complex process. It includes apoptotic mechanisms within the interdigital tissue and is under the influence of many factors including bone Bmps, Fgf and Hox-7. The transcription factor Msx2 is highly expressed in the interdigital zones and is thought to be a regulator in the Bmp4-mediated programmed cell death pathway. Hox gene interactions also play a role in this 'identity' process with Hox-D9, Hox-A11, and Hox-A13/D13 contributing to the development of the humerus, forearm bones, and bones of the hand respectively.

Hox genes: The 39 human HOX genes are located in four clusters (A-D) on different chromosomes at 7p15, 17q21 (corrected) 12q13, and 2q31 respectively and are assumed to have arisen by duplication and divergence from a primordial homeobox gene. Disorders of limb formation, such as hand-foot-genital syndrome, have been traced to mutations in HoxA13 and HoxD13. Hox genes are expressed in nested patterns along the proximal-distal axis of the limb bud, and mutations of these genes lead to alterations in the sizes and shapes of precartilage condensations in mice and humans, which are normally expressed in a region of the limb that will give rise to the radius and ulna. The result is severe reduction of these elements [90].

Indian hedgehog: Ihh is initially expressed in chondrocytes of the early cartilaginous skeletal elements [88]. On maturation, expression becomes progressively restricted to postmitotic prehypertrophic chondrocytes adjacent
to the PTH/PTHrP-R-expressing proliferative zones. It was demonstrated that ectopic expression of Ihh in developing chick long bones induces up-regulation of PTHrP expression in the articular perichondrium, leading to delayed differentiation of the chondrocytes, and delayed and abnormal ossification, a phenotype opposite to that seen in mice homozygous for null mutations in either PTHrP or its receptor. Furthermore, addition of Hedgehog protein to limb cultures delayed chondrocyte differentiation but only if PTHrP signaling was intact. These results suggest that Ihh and PTHrP regulate chondrocyte differentiation through the establishment of a negative feedback mechanism, in which production of Ihh by prehypertrophic chondrocytes induces PTHrP expression, thereby preventing additional chondrocytes from moving down the differentiation pathway. When prehypertrophic chondrocytes fully differentiate they no longer express Ihh. Consequently, the negative

feedback mechanism is attenuated and new chondrocytes are allowed to initiate the differentiation process. Ihh signaling is also required for chondrocyte proliferation and osteoblast differentiation, independent of PTHrP signaling. Most of the $Ihh^{-/-}$ mice die at birth due to respiratory failure resulting from defects in the development of the rib cage.

Dkk1: is a member of the dickkopf family and it functions as an inhibitor of the canonical Wnt signaling. Elevated levels of Dkk1 in the BM, plasma and PB is associated with the presence of osteolytic bone lesions in patients with MM [91]. Recently the role of Dkk1 in limb development was evaluated in a transgenic mouse model overexpressing Dkk1 in the appendicular skeleton [92]. In this model, Dkk1 was expressed under the control of the mesodermal specific enhancer element of Prx1 which its expression is mainly limited to the limbs, interlimb flank, latissimus dorsi muscle, craniofacial mesenchyme, and soft-tissues of the eye during embryogenesis. Prx1-Dkk1 mice exhibit severe shortening to near absence of forelimb and/or hind limbs when compared to their wild type counterparts. Importantly, all of the skeletal deficiencies observed were of the longitudinal type as in all cases absence of distal elements was always associated with reduction of proximal elements. I addition, two-thirds of mice showed some degree of forelimb abnormality, while one-third exhibited hind limb abnormalities and all mice with hind limb abnormalities had some degree of forelimb anomaly. No abnormalities of the cranial skeleton or viscera were noted. The wide spectrum of phenotypes observed from almost normal appearing mice to mice with severely affected and nearly absent limbs was correlated with the degree of Wnt/β-catenin inhibition. Interestingly, Dkk1 expression was readily observed in the osteoblasts lining the bones of Prx1-Dkk1 mice but not in wild-type controls. In BM-derived mesenchymal stem cells from 8-week old Prx1-Dkk1 mice (severe phenotype) there was an almost 80% decrease in total β-catenin in comparison to wild type. Using a β-catenin/TCF-responsive reporter (pGL3-OT/OF), in BM-mesenchymal cells from wild-type cells, β-catenin nuclear activity increases proportionally to the amount of exogenous β-catenin. In contrast the ability of BM-mesenchymal cells from Prx1-Dkk1 to similarly upregulate β-catenin nuclear activity is significantly reduced in direct correlation to the phenotype (i.e., the more severe phenotype the less β-catenin nuclear activity). In addition mesenchymal stem cells from Prx1-Dkk1 mice had limited proliferative ability, but normal differentiation potential.

A Unifying Explanation for all the Thalidomide Effects

Of the various signaling pathways discussed, only Ihh interruption could fully explain the thalidomide embryopathy, whereas mis-expression of Dkk1 resulted in truncation deformity sequence along the proximodistal axis [92]. Ihh keeps condensed chondrocytes undifferentiated and capable of proliferating and premature interruption of Ihh signaling will induce terminal differentiation of the affected segments to hypertrophic chondrocytes, leaving ungrowing chondrocyte rudiments instead of growing bone segments. Ihh mutations are associated with brachydactyly [93] and brachydactyly was also observed in thalidomide treated monkey [94]. However, differentiation of mesenchymal cells to osteoblasts depends on canonical Wnt signaling so a feedback loop must exist between Wnt and hedgehog pathways to ensure one way commitment. Therefore, primary or secondary modification of Wnt signaling by thalidomide might be also the common thread, which connects between the embryopathy, REM-sleep, and the antimyeloma activity of the IMiDEs. For example, in myeloma the overexpression of Dkk1 is associated with myeloma bone disease [91]. In

considering the fact that thalidomide is not directly cytotoxic to primary MM cells, together with the findings that endothelial and stromal cells and especially osteoclasts are critical supporters of primary MM cells and can rescue MM cells from apoptosis in vitro [95], my speculation is that thalidomide induces (terminal) differentiation or commitment of endothelial and mesenchymal cells as well as of monocytes, which reduce their ability to support MM cells. Endothelial cell differentiation was already noticed after thalidomide treatment of congenital and acquired vascular malformations. Finally, differentiation of the MM cells themselves is also an option, considering the observation that co-culture of BM-derived mesenchymal stromal cells revert MM cells to less differentiated phenotype by the combined activities of adhesive interactions and interleukin-6 [96]. Thus, it is possible that the antimyeloma activity of the IMiDEs is partly mediated via maturation of BM mesenchymal cells, leading in turn to maturation of the MM cell phenotype from immature to more mature and so less proliferative. The connection between REM-sleep and Wnt signaling, was demonstrated by Millstein et al. using a computationally efficient statistical approach that was applied to relate sleep patterns to gene expression patterns in mouse hypothalamus. The results yielded a set of 11 transcripts associated with 24 h REM sleep. Two of the corresponding genes, Sfrp1 and Sfrp4, are involved in Wnt signaling and several others, Irf7, Ifit1, Iigp2, and Ifih1, have links to interferon signaling. Interestingly, in our GEP analysis of MM cells, Ifit1 and related genes were induced considerably after exposure of the cells to lenalidomide in vitro (unpublished data).

The Thalidomide Binding Site

In 2010, Takumi Ito et al. reported that CRBN, a protein encoded by a candidate gene for mild mental retardation, is a primary target of thalidomide teratogenicity [97]. To purify the bound protein they used ferriteglycidyl beads and after extensive washing their eluate contained two specifically bound polypeptides. After proteolytic digestion and tandem mass spectrometry CRBN and damaged DNA binding protein 1 (DDB1) were identified, respectively and confirmed by immunoblotting. Moreover, in 293T cells CRBN was found to function as a subunit of E3 ubiquitin ligase complex and undergoes autoubiquitination in a Cul4A- and DDB1-dependent manner.

These results suggested that thalidomide inhibits E3 function of the CRBN-containing complex by directly binding to CRBN. To examine the effects of thalidomide on zebrafish development, they transferred dechorionated embryos to media containing different concentrations of thalidomide at 2 hours post fertilization (hpf) and allowed them to develop for 3 days. It was found that in thalidomide-treated embryos, formation of the proximal endoskeletal disc of the pectoral fin was severely inhibited at 75 hpf, and otic vesicle size was significantly reduced at 30 hpf. In addition, the zebrafish CRBN orthologous gene, zCRBN, whose product has ~70% identity to human CRBN was found to interact with DDB1 and was affinity-purified from zebrafish embryos as a major interactor with thalidomide. Embryos injected with an antisense morpholino oligonucleotide for zCRBN exhibited specific defects in fin and otic vesicle development, similar to those of thalidomide-treated embryos. For example, the size of otic vesicles was reduced by half in the knockdown embryos. At the molecular level, Fgf8a expression in the AER was severely reduced or absent in thalidomide-treated 48-hpf embryos, whereas shh expression in the ZPA was affected negligibly. They speculated the existence of an inhibitor to Fgf8 production which is a possible downstream target of thalidomide and the CRBN-containing E3 complex.

In myeloma cells, acquired deletion of CRBN was found to be the primary genetic event determining isogenic MM1.S cell lines to be sensitive or resistant to lenalidomide and pomalidomide. Gene expression changes induced by lenalidomide were dramatically suppressed in the presence of CRBN depletion, further demonstrating that CRBN is required for lenalidomide activity. Downstream targets of CRBN include interferon regulatory factor 4 (IRF4) previously reported to also be a target of lenalidomide. Patients exposed to, and putatively resistant to, lenalidomide had lower CRBN levels in paired samples before and after therapy. In summary, CRBN was found to be an essential requirement for IMiD activity and a possible biomarker for the clinical assessment of antimyeloma efficacy [98]. In addition, in GEP data from the HOVON-65/GMMG-HD4 trial high cereblon expression was associated with better survival in patients with newly diagnosed MM treated with thalidomide maintenance. In this patient set, increase of CRBN gene expression was significantly associated with longer progression-free survival. In contrast, no association between CRBN expression and survival was observed in the arm with bortezomib maintenance [99]. Likewise, in MM patients with which were treated with lenalidomide and dexamethasone, there was significant association between baseline CRBN expression and the response to therapy [100]. More recently, two articles reported independently that lenalidomide promotes the CRBN-dependent destruction of Ikaros proteins [101]. Therefore, it will be interesting to check in the future possible regulation of Ihh by the CRBN complex. However, as argued by Ito et al., cereblon may not be the exclusive thalidomide binding protein.

Two alternative binding proteins for the IMiDEs, if indeed Ihh serves as a primary target of these agents, may include Cyp26a1 and Cytochrome P450 oxidoreductase (POR). Both of these proteins were implicated in teratogenesis and are also known to bind small chemical compounds. In mice, Cyp26a1 knockout exhibits spina bifida and tail truncation as a consequence of patterning defects, and also homeotic transformation of anterior vertebrae has been described. Cyp26b1 knockout mouse embryos display craniofacial defects and reduced limbs. Homozygous mutants die immediately after birth owing to respiratory distress before vertebral defects become obvious, and hence no other bone defects have been reported. In zebrafish, the effects of Cyp26 enzymes have been studied in the context of hindbrain and neural crest patterning. The zebrafish *stocksteif* mutant, exhibits severe over-ossification of the entire vertebral column. *stocksteif* encodes cyp26b1, a cytochrome P450 member that metabolizes retinoic acid. The mutant is completely phenocopied by treating 4 dpf wild-type embryos with either retinoic acid or the pharmacological Cyp26 blocker R115866, thus identifying a role for retinoic acid and *cyp26b1* in osteogenesis of the vertebral column. In conclusion, Cyp26 might be another possible binding site for thalidomide in analogy to the binding of the differentiating factor retinoic acid to the same cytochrome P450 members [102].

In considering the dependence of Ihh in cholesterol biosynthesis, another possibility is that thalidomide perturbs the interaction between Ihh and associated sterol. Cholesterol has been shown to be necessary for the activity of members of the hedgehog family. After being synthesized intracellularly, the N-terminal half of hedgehog proteins becomes conjugated with cholesterol by an autocatalytic reaction that simultaneously cleaves off the inactive C-terminal polypeptide. The mature active hedgehog protein, which is about half the size of full-length hedgehog, can then be anchored to the cell membrane and eventually released from the secreting cell. The inhibition of cholesterol biosynthesis is associated with congenital malformations. Triparanol, an inhibitor of desmosterol Delta24 reductase, produces a high

rate of limb malformations in rat fetuses exposed at gestational day 10 to a single oral dose (150-200 mg/kg) given to the pregnant dam. The inhibitor induces the accumulation of desmosterol and zymosterol in embryo tissues. Comparison of the animal model with human syndromes, including limb osseous and skeleton perturbations, suggests a combination of desmosterol and Delta8 unsaturated sterols as being involved in the deleterious influence on limb bone formation [103]. The relations between both the cytochrome system and cholesterol biosynthesis with teratogenicity is exemplified by POR. POR is the electron donor for microsomal cytochrome P450 enzymes and other non-P450 enzymes. Targeted deletion of POR expression in mice leads to a variety of embryonic defects, including bone abnormalities. In addition, POR mutations in humans are associated with impaired steroidogenesis and skeletal malformations. Rat chondrocytes transfected with POR-specific short interfering RNAs exhibited decreased cell proliferation and differentiation and induced apoptosis. In addition, the reduced expression of POR in chondrocytes caused decreased intracellular cholesterol content. The addition of cholesterol in the culture medium prevented the POR small interfering RNA (siRNA)-mediated effects on chondrocyte proliferation, differentiation, and apoptosis. Interestingly, POR siRNA-transfected chondrocytes exhibited reduced Ihh expression, with such effect being neutralized by cholesterol. Lastly, recombinant human/mouse Ihh prevented the POR siRNA-mediated effects on chondrocyte proliferation, differentiation, and apoptosis. These findings suggested that the bone malformations associated with defective POR activity are due to reduced cholesterol synthesis and, in turn, reduced Ihh expression in chondrocytes [104].

Finally, a variety of compounds regulate downstream hedgehog signaling and by this way may also the IMiDEs act. Normally, hedgehog signaling is initiated by the binding of hedgehog ligands (Shh, Ihh, and Dhh) to the 12TM Patched1 (Ptch1) receptor, resulting in accumulation and activation of the G protein-coupled receptor-like protein Smoothened (Smo) within the primary cilium. How Smo regulates Gli transcription factor function remains unclear, but this process involves the scaffolding protein Suppressor of Fused (Sufu), which can directly inhibit the Gli proteins and facilitate their proteolytic processing into N-terminal repressors. Smo is perhaps the most “druggable” target within the hedgehog pathway, and Smo inhibitors have demonstrated efficacy in murine tumor models and human clinical trials [105].

Conclusion Remarks

- Thalidomide is not acting via inhibition of angiogenesis, at least in myeloma. This was clearly shown using BM microvascular density (MVD) measurements in thalidomide treated patients.
- Thalidomide is a known differentiating agent for blood vessel endothelium. This property has been utilized clinically to treat congenital and acquired vascular malformations.
- The teratogenicity of thalidomide can be explained by premature differentiation of limb bud chondrocytes during the sensitive period.
- Interruption of Indian hedgehog/Dkk1 signaling pathways can be the direct cause of thalidomide teratogenicity, which may be mediated by thalidomide binding to cytochrome members or disruption of sterol-Ihh relationships.
- Terminal differentiation of BM monocytes and/or mesenchymal cells, which support MM cells can be responsible for the antimyeloma activities of the IMiDEs

Proteasome Inhibitors

The proteasome is the main nonlysosomal endoprotease enzyme complex present in the cytoplasm and nucleus of all eukaryotic cells. It plays a critical role in the degradation of most short-lived intracellular proteins that control cellular events such as cell cycle, transcription, DNA repair, cell death, signal transduction, metabolism, morphogenesis, differentiation, antigen presentation and neuronal function. The proteasome is also responsible for protein quality control by eliminating damaged and abnormal proteins. Its structure is hollow and cylindrical and this 26S enzymatic complex is assembled from at least 66 proteins with the help of a number of chaperone proteins. It is composed of the catalytic 20S core and two 19S or 11S regulatory units at either ends. The catalytic 20S core is organized into a stack of four seven-subunit rings, with the top and bottom rings formed by seven polypeptides, termed the α-subunits, and the two inner rings of seven β-subunits. Poly-ubiquitination drives the interaction between the 19S (11S) and 20S particles. It requires the activity of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). The mechanisms behind polyubiquitination are still being hotly debated. According to one mechanism, each ubiquitin is added sequentially to the growing chain (termed ‘‘elongation‘‘). Other authors support the idea of a poly-ubiquitin chain that is preformed and then added to the target protein. Ubiquitin receptor proteins have an N-terminal ubiquitin-like (UBL) domain, and the 19S regulatory particle has an affinity for and recognizes these UBL domains. After the poly-ubiquitinated protein is recognized by 19S, the substrate protein is unfolded by hydrolases so that it may enter the narrow gate of the 20S particle and then be degraded. The 19S regulatory particle is divided into two subcomplexes called the base and the lid. The base consists of six AAA+ ATPases and three non-ATPase polypeptide chains. The lid includes at least nine non-ATPase polypeptide chains that help remove ubiquitin from the substrates. The lid and base connection is stabilized by the Rpn10 subunit. The base of hexameric ring of 6 ATPases in the 19S regulatory particle facilitates the opening of the 20S gate and is responsible for substrate recognition, deubiquitination, unfolding and translocation into the core particle. However, lack of structural data makes it difficult to understand how the 19S subunits are arranged and how the 19S helps with the opening of the 20S gate. The 20S catalytic core has a narrow gate at the center of the α-subunit ring where substrate proteins enter into this proteolytic degradation chamber. The gate is closed when the N-termini of the α-subunits interact, blocking substrate entry into the chamber. The 19S regulatory particle functions to help open the gate at the center of the α-subunit by interacting with this α ring and facilitate substrate access to the proteolytic chamber. The proteolytic chamber of the 20S core has three types of catalytic activities: chymotrypsin-like, trypsin-like, and caspase-like. These activities are presented by β5, β2, and β1 subunits, respectively. In immune cells the β1, β2, and β5 subunits, which are constitutively expressed, are replaced by β1i, β2i, β5i induced subunits to compose the immunoproteasome. Compared to the normal eukaryotic proteasome, the immunoproteasome has increased chymotrypsin-like and trypsinlike activities which assists in antigen processing. Instead of the 19S regulatory particle, the immunoproteasome utilizes the 11S regulatory particle, in place of 19S, to facilitate the opening of the 20S gate and stimulates substrate unfolding and translocation. Similar to 19S, the 11S binds to the 20S core particle through the

C-terminal tails of its subunits and causes α-ring conformational changes to open the proteolytic gate.

Bortezomib (velcade), is a dipeptide that contains a boronic acid instead of a carboxylic acid at the C-terminus. Bortezomib's boronic acid reversibly binds to the chymotrypsin-like β5 subunit of the catalytic chamber of the 20S particle and inhibits proteasome function [106]. Carfilzomib contains an α,β-epoxyketone moiety that assists in the formation of a morpholino adduct with the N-terminal threonine residue in the proteasome, which inactivates proteasome function irreversibly [107].

One of the earliest explanations for the antimyeloma activity of proteasome inhibition ascribed the cytotoxicity to the accumulation of IkB, the inhibitor of the antiapoptotic factor NFkB, which is implicated in B-cell malignancies. [108-110]. However, more recent data debated this thought because direct inhibitors of NFκB reduce the proliferation of MM cells markedly less effectively than bortezomib [111-112]. Alternative proapoptotic proteins, which are proteasome degraded, were also considered as possible candidates. For instance, the proapoptotic molecule NOXA is induced 20-to-60-folds in certain cancer cells after exposure to proteasome inhibitor [113]. Other groups attributed the antitumoral effects to the inhibition of HIF-1α and repression of HIF-1 transcriptional activity with attenuation of the release of VEGF [114]. However, in the recent years, much of the attention has been directed towards the endoplasmic reticulum (ER) stress response and the accumulation of misfolded proteins destined to be degraded by the proteasome [115-116]. In general, increased misfolded protein levels initiate the unfolded protein response (UPR) signaling, which in turn stimulates splicing of inactive XBP1 [117] whereas spliced XBP1 (XBP1s) modulates genes which are responsible for the ER-associated degradation (ERAD) (e.g., EDEM), ending-up within the proteasome. XBP1s also induces genes which enhance protein folding such as p58IPK and a variety of ER chaperones [118]. Although UPR activation is intended to return protein homeostasis and is normally functions in plasma cell differentiation and survival by induction of various ER chaperones and folding enzymes [119-121], under prolonged and uncompensated ER stress the UPR ends with cellular apoptosis, known as terminal UPR [122-124]. The latter insult is mediated by the proapoptotic transcription factor CHOP (also known as GADD153 and DDIT3), which is induced via PERK and ATF6 pathways. CHOP causes downregulation of BCL2, and thereby leading to caspase-dependent apoptosis [125-126]. In head and neck squamous cell carcinoma cells (HNSCC), bortezomib induces apoptosis through induction of ER stress along with the generation of ROS, which lead to caspase activation whereas inhibition of NFkB alone was not sufficient to induce apoptosis [127]. Additional support to the proapoptotic potential of exaggerated ER stress came from the association between the levels of immunoglobulin chain production and the sensitivity to proteasome inhibition in subclones of the human IgG-secreting myeloma cell line JK-6L and in subclones of the murine myeloma cell line Ag8 transfected with an expression plasmid encoding the μ heavy (H)–chain [128]. Likewise, proapoptotic factors of the ER stress response were induced to a greater extent in subclones producing high levels of μH-chains as compared to subclones producing no μH-chains. Conversely, MM cells became bortezomib resistant through inhibition of unfolded protein accumulation by acquired mutations of the PSMB5 gene which prevented the catastrophic ER stress [129].

In order to find which of the cytotoxic mechanisms mentioned are consistent with the molecular changes appearing in primary tumor cells after treatment with proteasome inhibitors, we compared recently the gene expression profile (GEP) changes, evolving in BM

samples from MM patients with bortezomib responsive versus bortezomib resistant myeloma in response to bortezomib treatment in vitro (unpublished data). The MM cases selected had extreme BM infiltration with tumor cells (> 90%), which omitted the need for cell separation procedures, known to modify the expression of numerous genes due to spontaneous switch in their expression in vitro [29]. The microarray data is available in the public Gene Expression Omnibus (GEO), accession number GSE51940. So far, we examined BM samples from an overall group of five MM cases, three of which responded to velcade/dexamethasone (Vel/Dex) combination whereas two failed various velcade regimens; one with newly diagnosed myeloma showing primary resistance to Vel/Dex as reflected by increase in his paraprotein levels and persistence of > 95% plasma cells in repeated BM examination after 6 injections of velcade (days 1, 4, 8, 11, 29, 32, 36, 39); the second refractory case was heavily pre-treated and initially responded to Vel/Dex with VGPR (very good partial response) followed by long term remission after consolidation stem cell transplantation (SCT) with melphalan (200 mg/m2). However, after almost 3-years the patient progressed and received second line treatment with lenalidomide/dexamethasone (Len/Dex) but was refractory to this regimen, then he partially responded to retreatment with Vel/Dex and thereafter received salvage therapy with two cycles of VD-PACE (velcade combined with dexamethasone, platinol, adriamicin and etoposide) followed by second SCT while in VGPR. Once again, after 6 months the patient progressed but now also showing absolute resistance to bortezomib regimens, without any response to Vel/Dex and to VD-PACE treatments, and soon he developed new plasmacytomas, pancytopenia, hypercalcemia, renal failure and rapid increase in his paraprotein levels in the presence of > 95% plasma cells in the marrow. At this stage he entered the study and his BM sample was obtained.

The BM samples from our five MM cases (three bortezomib responsive and two resistant) were cultured and treated in vitro with bortezomib at a concentration of 2 μg/ml, and after 6-8 hours incubation the treated and control cells were released from the bottom wells and fixed with liquid nitrogen. As compared with the control samples, the GEPs analysed from the bortezomib treated samples showed dramatic upregulation of a large subset of genes, mostly encoding for heat shock proteins (HSPs) or related ER stress chaperones, and this observation was common to virtually all five samples examined, irrespective of the clinical response of the patient to bortezomib regimens. A partial list of the upregulated genes includes: HSPA6, HSPA1A, HSPA1B, CHRNA5, HSPB1, HSPH1, DNAJB1, SERPINH1, HSPA7, BAG3, HSP90AA4P, DNAJA4, HSP90AA6P, HSPA4L and HSP90AA2. In contrast, neither NOXA nor HIF-1α, GADD45A, GADD45B, GADD45G, TNFRSF10B, FAS, FASLG, DAP3, CASP8, CASP7 or CASP1, which were all implicated in the apoptosis induced by bortezomib [130-131], were induced markedly in any of the BM samples examined in our series, and no BCL2 repression could be recognized. Furthermore, although the proapoptotic signaling molecule CHOP (DDIT3), which is considered to be induced and activated by ER stress [126-127], was induced to somewhat greater extent in our bortezomib sensitive versus resistant cases, the differences in expression were too small to explain the clinical differences.

Because induction of HSPs is one of the most typical changes induced by proteasome inhibition [132-134], the remarkable in vitro induction of HSPs in our bortezomib resistant cases, and so the lack of in vitro resistance to proteasome inhibition, implies that unlike the resistance acquired in mutant subclones, which is secondary to impaired drug binding [135-140], in the MM cases presented the clinical resistance resulted probably from other

mechanisms. One possibility is reduced downstream activation of proapoptotic pathways like p38 and ATF6 [141], Bax and Bak [142], generation of ROS [143-144] or dysregulation of the intracellular calcium [145] following proteasome blockade. Another possibility is that the antitumoral activity is dissociated from the UPR activation, and this option can be tested directly by the response to direct ERAD inhibitors such as Eeyarestatin I [146]. The third possibility is that the clinical resistance results from reduced BM levels of the drug. The last possibility represents also the major limitation of our findings, because the bortezomib concentration used in our cultures was much higher (~20 folds) than the usual peak plasma levels in humans (89-120 ng/ml). However, bortezomib is rapidly and widely distributed in tissues and the mean 24-hour total radioactivity levels (TR) of ^{14}C-bortezomib was found to be 43.5, 30.5 and 27.8 folds higher in the BM versus plasma of Sprague-Dawley rats after the first, third and fourth dose of the drug in a biweekly schedule, respectively [147]. Likewise, the area under the concentration-time curve (AUC; μg-eq. h/g) of TR between 0 and 72 hours post ^{14}C-bortezomib injection ($AUC_{0\text{-}72\ h}$) was 31.7 and 24.1 folds higher in the BM versus plasma after the first and the fourth dose, respectively. Thus, it is still reasonable that the usual bortezomib levels existing in the marrow of treated patients are similar to the concentration used in our cultures, although this issue requires confirmation.

In summary, the mechanism of the antimyeloma activity of proteasome inhibitors is still undefined. The ER stress response seems to be critical, though preserved proteasome inhibition capacity, which elicited potent in vitro induction of HSPs was not predictive for clinical activity of bortezomib in MM patients. On the other hand, the excellent clinical response to bortezomib regimens, despite the remarkable in vitro induction of HSP transcripts in our bortezomib responsive cases, doubts the role attributed to HSP inhibitors in overcoming bortezomib resistance [148-150], though the in vitro observations might be misleading. Another option to overcome resistance to proteasome inhibitors is to combine them with direct ERAD inhibitors [151]. Finally, among all the GEP changes observed after in vitro treatment of primary MM cells with bortezomib, only matrix metalloproteinase-12 (MMP12) was expressed differently in bortezomib responsive versus bortezomib resistant cases. Specifically, MMP12 was induced 6.55, 18.62 and 7.0 folds higher in the BM samples from our bortezomib sensitive cases versus 0.48 and 1.08 folds in the samples from the two bortezomib resistant cases, as compared to the corresponding controls. So far, MMP12 has not been implicated in MM, but is known to participate in tissue remodelling, and is also strongly associated with tumor invasion and pathologies like emphysema and aortic dissection.

Targeted Therapy Using Monoclonal Antibodies

Plasma cells are characterized by the co-expression of CD138 and CD38, which allows their identification in flow cytometry. These terminally differentiated B-cells may lose the expression of surface CD19 and that of CD20 while retaining CD27. When malignant, they can gain a number of other markers such as CD28, CD33, CD56, or CD117 and lose CD27 [152].

The successful assimilation of monoclonal antibodies into the arsenal of treatments for a variety of malignancies, including B cell neoplasms, has encouraged developing similar targeted therapy against myeloma. This effort however has been encountered with difficulties, mainly because of the heterogeneity of the tumor clone, and the obscure phenotype of the cell subset which attains self-renewal capacity. Two terms have been adopted to discriminate specific subset of cells within the malignant plasma cell clone; the first was clonotypic cells, identified by the sharing of the same immunoglobulin heavy chain (IgH) rearrangement as the monoclonal plasma cell population residing in the BM. More recently clonogenic and myelomagenic cell counterparts have been also recognized, likely by an inspiration from the leukemia stem cell research. The existence of clonotypic, non-plasma cells in the blood of MM patients, was firstly described by Leif Bergsagel and Linda Pilarski et al. who showed that in myeloma, certain blood B cells have identical IgH rearrangements as expressed by the BM plasma cells. Specifically, DNA from purified blood B cells and BM plasma cells, taken at the same time, was amplified using Allele-Specific Oligonucleotid (ASO) polymerase chain reaction (PCR) method. In 10/16 patients, a single IgH rearrangement was amplified from the BM plasma cells, and in all the 10 cases the same IgH rearrangement was also amplified from the purified blood B cells [153]. In addition, a significant proportion of the clonotypic CD19+ B cells, aberrantly expressed both CD38 and CD56 [154]. The discovery of a distinct blood B cell population, which is expressing the same IgH rearrangement as the malignant plasma cell clone, readily fueled speculations as to the existence of a malignant B cell compartment with stem-cell like properties, feeding the malignant plasma cell compartment. To quantify the clonotypic B cell subset residing in the circulation, Szczepek and Pilarski et al. analyzed sorted B cells derived from blood of MM patients and healthy donors, using in situ RT-PCR with patient-specific primers. The average proportion of clonotypic B cells in the blood from the patients examined was 66% +/- 4% (range, 9% to 95%), while not detectable among normal blood samples [155]. Whereas the existence of clonotypic B cells was also confirmed by other groups, the proportion of these cells in the blood of MM patients became a subject of debate. Thus, Chen and Epstein from the Arkansas group, estimated the average proportion of CD19+ B lymphocytes in the blood mononuclear cells of nine MM patients as 6% (range, 0.2% to 12.1%), and only a minority of which (range, 0.04% to 0.8%) were clonally related to the MM cell clone [156]. This controversy was later addressed in a phase III clinical trial, conducted by the Eastern Cooperative Oncology Group (ECOG). The study group included 13 newly diagnosed patients with myeloma, and the results excluded any correlation between the number of CD19+ B cells (range, 5% to 51%), and the number of clonal cells in the PB (range, .009% to 3.6%), as estimated by quantitative ASO-PCR. In fact, low CD19+ B cell was associated with clinical stage III disease, while higher levels of CD19+ cells were associated with a longer overall survival. In addition, high CD19+ levels also predicted a better response to treatment and longer event-free survival. There was also a strong and inverse correlation between the level of CD19+ cells at the diagnosis and the incidence of infections within the first 2 months. The number of deaths from infections was also significantly higher in the low versus high CD19+ group. Finally, CD19+ cell quantity was an independent prognostic factor, in addition to plasma cell labeling index, beta2-microglobulin, hemoglobin, and plasmablastic morphology. Taken together, higher CD19+ quantities were associated with a favorable outcome, with no apparent relationship to circulating tumor cells [157]. In line with the ECOG findings, Luque et al. also found that most B lineage cells in the blood of MM patients are polyclonal, and only a minor

proportion is clonotypic [158]. However, in contrast to the phenotype described by Pilarski et al., Luque et al. characterized the blood clonotypic cells as expressing CD19- /CD38++/ CD45-/dim CD138++ and monoclonal cytoplasmic immunoglobulin, while being negative for pan-B antigens (CD19, CD20, CD24, DR), surface immunoglobulin and certain adhesion molecules like CD62L, CD18 and CD11a. This phenotype is closely related to the phenotype of malignant plasma cells in the marrow, with the exception of the CD56 antigen. Clinically, clonotypic cells showed a remarkable drug resistance, and could be detected also after treatment albeit in very low proportions (median, 0.004%), and most of which were $CD19^{-}$ [159]. Clonotypic cells were also detected after high dose therapy and autologous SCT, and the proportion of clonal $CD19^{+}$ cells ranged between 0.1% and 6.1% (mean, 1.0%) in the cases examined [160].

The idea that the clonotypic cell subset constitutes the main proliferative population in myeloma, was rebutted by their minor proportions as estimated by most groups, as well as by the positive correlation shown by the ECOG between the quantity of blood CD19+ cells and the overall survival, event free survival and lower infection rate. Further skepticism regarding the ability of clonotypic B cells to replenish the BM following treatment, arose from attempts to eradicate this cell reservoir. The first documentation of targeted therapy, directed against residual CD19+/CD20+ clonotypic B cells, which survived HDT was reported by Gemmel et al., who gave B-cell directed consolidation therapy to a patient with plasma cell leukemia in remission after HDT [161]. Prior to antibody therapy, the proportion of clonotypic cells in the PB mononuclear cell fraction, enriched for CD20+ cells, was estimated as 0.093% by PCR assay, based on limiting dilutions. However, despite effective depletion of CD20+ cells from the PB and from the BM following rituximab consolidation (375 mg/m2 on days 1, 8, 15 and 22), the tumor load in boat compartments increased progressively, and by day 120 a relapse was diagnosed clinically and the patient had in the PB 0.65% CD38++/CD138+/CD20- plasma cells, with no detectable CD20+ B cells. This observation excluded, at least for that patient, the possibility that the clonotypic B cell subset represents a major proliferative compartment. More recently, Zojer et al. tested the efficacy of a single course of rituximab monotherapy (375 mg/m2 on days 1, 8, 15 and 22) in patients with relapsed myeloma [162]. The rational for that study was the identification of a population of clonotypic CD20+ B-cells that are believed to be precursors of malignant plasma cells. Before treatment, CD20 was expressed on the cell surface of 10% and 50% of the BM cells from two of the ten patients enrolled. Following rituximab treatment, none of the patients achieved an objective response and no clinical benefit could be recognized, despite the significant reduction in the numbers of circulating B lymphocytes. In another small trial, rituximab given as maintenance therapy to MM patients after autologous SCT, resulted in a shortened time to relapse thereby leading to early termination of the trial [163].

In conclusion, the majority of published studies showed that the clonotypic blood population, detected either before or after treatment, remains consistently minor and relatively static. The clonal diversity seen can be settled with the fact that translocations involving the immunoglobulin locus (14q32), are an early non-transforming event common to MM and MGUS, introduced at the memory B-cell level [164]. Thus, it is not surprising that clonotypic B cells are also detectable in MGUS [165] and therefor might simply represent an earlier stage population, along the ontogenetic course of the tumor clone. On the other hand, in symptomatic MM patients, the persistence of clonotypic cells after treatment or increase in their levels after transplant did correlate with reduced overall survival [166-168]. A possible

explanation for the discrepancy mentioned between the "static" behavior of the clonotypic cell population, which shows no expansion even during full blown disease and the unfavorable outcome associated with its persistence, can be that this cell subset is indeed 'innocent' by itself, but its persistence after therapy reflects resistance of the entire clone. An alternative explanation is that the clonotypic blood population does contribute to the tumorigenic actively, but rather than doing so by expansion of the non-plasma cell clonotypic subset, as originally suspected, the expansion is concealed and manifested by gradual replacement of lost plasma cells in the marrow by the non-plasma cell counterparts of the clone, followed by switch in their phenotype after BM engagement, towards the typical mature phenotype. Such scenario may explain the difficulties mentioned to determine the phenotype of the clonotypic cells, being local environment dependent. It may also explain the resistance of the disease to rituximab when given as consolidation or maintenance therapy after HDT, because rituximab can only eliminate the CD20 positive subset of the clone, and not BM engaged or the circulating CD20 negative counterparts identified by Luque et al. [158]. I personally prefer the second explanation, no matter what is the exact phenotype of the clonotypic or clonogenic cells. This is because it is very likely that the relapse initiates from a 'dormant' or hibernated reservoir of clonotypic cells, which are protected from most cytotoxic regimens including HDT, due to their rare biological activities (e.g., replication, metabolism, turnover, recycling, macromolecule repair), and thus reduced dependence on cell resources like ribosomes and proteasome, leading by that to reduced susceptibility to corresponding inhibitors. Indeed, in our experience we were able to maintain primary MM cells for months ex-vivo with minimal supplements to cultures and interestingly, these static cells acquired somewhat different morphology, with less basophilic cytoplasm and less eccentric nuclei (29; and unpublished data).

The phenotype of the clonogenic (or myelomagenic) cells, was firstly reported by Pilarski et al., who identified myeloma initiating cells in granulocyte-colony-stimulating factor (G-CSF) mobilized grafts from symptomatic MM patients. Specifically, after intracardiac injection of the mobilized cells from 3 of 7 patients to mice, the mice developed disease symptoms with a latency of 103 to 157 days, characterized by overall morbidity, hind leg paralysis, and/or boney changes, including easily fractured long bones and macroscopic loss of red marrow. In addition, human B and plasma cells were detected in the BM, spleen, and blood of the mice. Likewise, injection of MM cells directly into the murine sternal BM, led to lytic bone lesions, BM plasma cells, and a high frequency of clonotypic cells in the femoral BM. Finally, the myeloma progenitors underwent self-renewal in murine BM, as demonstrated by the transfer of human myeloma to a secondary recipient mouse [169]. Later, this group showed that injection of CD34-enriched autografts from either fresh or cryopreserved mobilized blood also generates myeloma in NOD/SCID mice. Specifically, the enriched grafts from six of six patients were myelomagenic, as evident by engraftment of clonotypic cells and/or the development of lytic bone lesions. Also the intrasternal route of injection of enriched progenitor fractions led to clonotypic cells in the femoral BM and bone lesions at distant skeletal locations, confirming dissemination of myelomagenic cells. The highly purified CD34+ fractions (separated by immunomagnetic method), contained on the average, 31% clonotypic MM cells and 31% DNA aneuploid cells. Given the lack of morphologically identified plasma cells among enriched progenitors, it was concluded that the xenografting myelomagenic component is unlikely to be plasma cells and appears to be CD34+ CD45 low, similar to normal myeloid progenitors [170]. In line with that conclusion,

Matsui et al. found that CD138- cells from clinical MM samples, were clonogenic both in vitro and in NOD/SCID mice, whereas CD138+ cells were not [171]. Specifically, purified CD138+/CD34− and CD138−/CD34− cells from BM mononuclear cells of 4 MM patients were injected intravenously into NOD/SCID mice. In contrast to CD138+ cells which did not engraft any of the mice, the CD138− cells from one of the patients engrafted 3 of 3 animals, as evident by the presence of human CD138+ cells with light chain restriction, matching the original patient sample within the murine BM and PB. Further analysis of clonotypic CD138- cells from both cell lines and clinical samples, revealed their phenotypic resemblance to post germinal center B cells, suggesting that MM "stem cells" are CD138- B cells, with the ability to replicate and subsequently differentiate into malignant CD138+ plasma cells. In contrast, Hosen et al. showed recently that CD138- cells and CD138+ cells can be both clonogenic [172]. Specifically, CD138- /CD19- /CD38++ plasma cells from 3 out of 9 MM cases propagated in SCID-rab mice model, while CD138+ plasma cells from 4 out of 9 cases propagated too, although more slowly than CD138- cells. In contrast, no engraftment of CD19+ B cells was detected in either SCID-rab mice or in NOD/SCID IL2Rγc(-/-) mice.

As noticed, the controversy encountered in relation to the clonotypic cell phenotype continued with the phenotype of the clonogenic cells. According to Pilarski et al. CD34- enriched grafts are myelomageous while Matsui et al. demonstrated the clonogenic potential of CD138−/CD34− cells and Hosen et al. showed this potential in both CD138- /CD19- /CD38++ and CD138+ cells. These differences could result from the different model used and technical variations. However, another possibility, which was already mentioned, is that the phenotypic variation observed is not necessarily methodological, but represents true clonal diversity, or more intriguingly is the consequence of transition (reversible or not) between two or more 'maturation' states, from the so called 'stem-state' to more proliferative states, possibly depending on timing and local microenvironment. The clonal diversity option is consistent with the cancer stem cell concept and is considered attractive by the potential to eradicate the tumor clone completely by only targeting the minor stem cell counterpart. However, such assumption looks somewhat naïve and elimination of cancer stem cells was never achieved even in core binding AML, where the transforming event can be solitary, the genetic diversity very rare, and the tumor cells "synchronized" in the same maturation arrest stage (due to specific translocation). Also in chronic myeloid leukemia (CML), which is a bona fide single "hit" tumor (t 9;22), there are no evidence for the existence of a minor subset of clonogenic cells, which replenish the tumor clone. Thus, instead of speaking of a distinct subset of cancer stem cells, it is possible that the tumor cells have plasticity up to a certain ontogenetic stage, and can de-differentiate or transit between 'dormant' and more proliferative states (with corresponding phenotypes), leading by that to erroneous conclusions. The existence of a transient 'dormant' state is also predicted from the response to long term maintenance therapy, for example in acute promyelocytic leukemia patients (receiving ATRA + 6-Meraptopurine) or in MM patients (receiving IMiD or proteasome inhibitor maintenance). If this is really the case, then targeted therapy using monoclonal antibodies can be the best option to eliminate the 'dormant' subsets of cells within the malignant clone, provided that their phenotype will be clarified. However, until the exact phenotype of myeloma initiating cells will be determined, the available antibodies will continue to be used empirically.

The available monoclonal antibodies which are currently under clinical assessment, will be now briefly mentioned, starting with the anti CD138 (Syndecan-1) antibody. CD138 is a cell surface receptor for epidermal growth factor ligands, hepatocyte growth factor, Fgf and extracellular molecules such as collagen and fibronectin. It also mediates cell-to-cell adhesion and it was showed to be a differentiation antigen and a co-receptor for MM growth factors. CD138 is expressed on the majority of malignant plasma cells, and at lower level also on normal plasma cells. It is continues to be expressed on MM cells after repeated treatments, thus it can be targeted also in advanced stage disease. In a Phase I/II study, 52% of the patients responded to BT062, an anti CD138-drug conjugated antibody with at least a stable disease, and 4% achieved a PR, when the drug was given weekly for 3 weeks in each 4 week cycle [173]. Two additional surface antigen targets are CS1 (SLAMF7) and CD38. CS1 is a glycoprotein expressed strongly on the surface of normal and malignant plasma cells and elotuzumab is a fully humanized IgG1 anti CS1 monoclonal antibody. As a single agent elotuzumab showed no objective response, although 26% of 34 evaluable patients stayed with a stable disease. Nevertheless, elotuzumab is currently examined in a phase III trial in combination with lenalidomide owing to preclinical evidence for synergism between these agents [174]. CD38 is a type II transmembrane glycoprotein, involved in receptor-mediated adhesion, signaling events and intracellular calcium mobilization. It is relatively highly and uniformly expressed in MM cells, while under normal conditions it is expressed at low levels on lymphoid and myeloid cells. Daratumumab, an anti CD38 antibody showed in dose-escalation study, a marked reduction in paraprotein and BM plasma cells [175]. Such activity was not previously demonstrated with other single-agent monoclonal antibody. Another surface antigen supposedly expressed ubiquitously and preferably on the surface of MM cells was identified recently using comparative GEP [176]. In the above mentioned and in other series GPRC5D expression was extremely higher in primary MM cells than in normal BM cells or non-myeloma tumor cells (e.g., AML, B cell lymphoma cells). This finding justifies further investigation.

Targeting Extracellular Factors

MM cells are strictly dependent on the BM niche for growth and survival. Apart from supplying essential growth factors and nutrient support the BM offers the proper physical conditions required for vital cell-cell interactions among its various inhabitants. Accordingly, targeted therapy which is directed against critical extracellular factors or surface adhesion molecules seems to be an extremely promising approach to threat myeloma. Moreover, unlike the behavior of malignant cells which “escape” therapy due to clonal evolution, soluble and adhesion molecule targets are less likely to “escape” from an antibody. In myeloma, one of the most reasonable targets for such therapy, is soluble factors which drive myeloma bone disease, especially Dkk1 and RANKL. Dkk1 functions as a soluble inhibitor of Wnt/β catenin pathway, and is overexpressed in a subset of MM patients with osteolytic bone lesions. The overexpression of Dkk1 blocks the differentiation of osteoblast precursors and inhibits anabolic bone formation, thus promoting bone resorption. Preclinical studies showed that neutralizing Dkk1 and/or enhancing Wnt/β catenin signaling may be effective in treating bone pathologies [177-178]. BHQ880 is a fully human anti-Dkk1 monoclonal antibody; it increases osteoblast differentiation and bone formation, neutralizes inhibitory effects of MM

cells on osteoblastogenesis, reduces IL-6 secretion and inhibits cell adhesion and tumor growth both *in vitro* and in mouse models. These preclinical results provided the rationale for a phase I/II trial evaluating BHQ880 in combination with zolendroic acid and anti-myeloma therapy in patients with relapsed/refractory MM. Preliminary data of phase I trial showed increases in bone mineral density in some patients with a good safety profile [179]. Another ongoing phase II study in patients with previously untreated smoldering MM at risk for progression showed no significant direct anti-myeloma effects but evidence for anabolic bone activity was seen in some patients using novel imaging [180]. RANKL is a type II membrane protein and a member of the tumor necrosis factor (TNF) superfamily. The binding of RANKL to its receptor, RANK increases osteoclastogenesis and activation of osteoclasts. Osteoprotegerin is a natural soluble decoy receptor of RANKL, and it is able to prevent excessive bone resorption by modulating RANKL effects. Denosumab is a fully human monoclonal antibody with high affinity and specificity for RANKL. It was developed to treat patients with skeletal disease mediated by osteoclasts, and it was evaluated in different trials in patients with MM or metastatic bone cancer. None of the documented studies reported any objective response in terms of reduction in serum M protein level. However, the stabilization of disease in some subjects suggested a possible cytostatic effect through alteration of the microenvironment. The available results showed that denosumab was effective in decreasing rapidly bone resorption, measured by changes in serum and urine N-telopeptide X levels. In a recent evaluation of three phase III studies, although similar overall survival and progressive disease were reported, denosumab was significantly more effective than zoledronic acid in preventing or delaying first skeletal-related event (including pathological fracture, radiation to bone, surgery to bone, spinal cord compression), as well as in preventing skeletal complications and hypercalcemia associated with this malignancy [181]. Another MM specific target is the cytokine IL6. IL6 is produced predominantly by the BM stromal cells, and is upregulated by multiple cytokines; it plays a critical role in the proliferation and survival of MM cells. It stimulates at least three different signaling pathways (Ras/MEK/ERK, JAK2/STAT3 and PI3K/Akt cascades). Moreover, IL6 protects against apoptotic cell death induced by several agents, thus conferring resistance to chemotherapeutic agents, including bortezomib and melphalan, and to corticosteroid-induced apoptosis. The combination of the anti IL6 chimeric monoclonal antibody siltuximab (S) directed against IL6 and dexamethasone showed clinical activity, including responses in patients refractory to dexamethasone [182]. Ongoing clinical trials evaluate the addition of S to other agents. The addition of S to the VMP (bortezomib-melphalan-prednisone) regimen was studied in 106 patients who were randomized to receive 9 cycles of VMP or VMP+S (11 mg/kg every 3 weeks) followed by S maintenance. Baseline characteristics were well balanced except for IgA subtype and 17p deletions. With a CR rate of 27% on S+VMP and 22% on VMP, the study did not confirm its hypothesis that the addition of S would increase CR rate by at least 10%. Overall response rate was 88% on S+VMP and 80% on VMP and at least VGPR rates were 71% and 51% (P=0.0382), respectively. Median progression-free survival (17 months) and 1-year overall survival (88%) were identical in the two arms, so it was concluded that the addition of S to VMP did not improve CR rate nor long-term outcomes [183]. Finally, like in any tumor, angiogenesis is also necessary for MM cells. VEGF-A is a pro-angiogenetic factor, overexpressed in MM that also works as growth factor for MM cells, triggers MM cells migration, increases IL6 production and inhibits maturation of dendritic cells. Bevacizumab is a humanized monoclonal antibody directed against VEGF-A. In preclinical

studies, bevacizumab inhibited activation of signaling cascades in MM cells, reduced MM and endothelial cells proliferation and inhibited angiogenesis. In a phase II trial on relapsed/refractory patients, only one of 6 patients receiving bevacizumab as a monotherapy responded to treatment while bevacizumab in drug combination led to results similar to single-agent thalidomide, with a substantial lack of efficacy [184].

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