International Journal of Hematological Disorders, 2014, Vol. 1, No. 1A, 1-6
Available online at http://pubs.sciepub.com/ijhd/1/1A/1

DOI:10.12691/ijhd-1-1A-1

Maturation of the Thalidomystery

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Received September 25, 2014; Revised November 11, 2014; Accepted December 09, 2014

Abstract Despite years of investigations, the mechanism of action of thalidomide and its derivatives is still unsolved. Recently we elaborated on the basis of the current literature data, including x-ray images of thalidomide victims, a hypothesis that premature differentiation of developing limb elements underlies the teratogenic effects of thalidomide and may also account for the antitumoral activity of the IMiDs. To examine the feasibility of this theory, we searched within gene expression profile datasets, which analyzed the changes induced by the IMiDs in myeloma cells, for supportive evidence. As expected, our analysis revealed a reproducible upregulation of a cluster of differentiating genes induced after either in-vivo or in-vitro treatment of the tumor-cells with thalidomide/ lenalidomide. This finding debates the long standing dogma that ascribed the teratogenic effect of thalidomide to apoptosis or toxic injury to limb bud mesenchyme/blood-vessels and places the IMiDs in line with differentiating teratogens like retinoids and related compounds.

Keywords: *Thalidomide, lenalidomide, dexamethasone, gene expression profile, PLSCR1, differentiation*

Cite This Article: Yossi Cohen, Odit Gutwein, Osnat Garach-Jehoshua, Adina Bar-Chaim, and Abraham Kornberg, "Maturation of the Thalidomystery." *International Journal of Hematological Disorders*, vol. 1, no. 1A (2014): 1-6. doi: 10.12691/ijhd-1-1A-1.

1. Introduction

Thalidomide was developed in the late 1950s for the treatment of morning sickness during pregnancy. However, several years after its introduction, more than 10,000 babies were born with severe birth defects and the drug was withdrawal from the markets. Nevertheless, after years of interrogation, in 2006, thalidomide was approved by the FDA for the treatment of multiple myeloma (MM) and in the year 2010 a thalidomide binding protein, called cereblon was discovered. Despite this, the molecular pathways modulated by the IMiDs and the mechanism of the embryopathy are still unresolved. Recently, we elaborated a new theory to explain the teratogenic effects of thalidomide, based on: 1. The stereotypical sequence of limb defect accumulation along the severity scale, which partially overlaps the spatiotemporal differentiation sequence of the developing bone elements. 2. The coexistence of well-formed bone segments side by side to absent, distorted or incompletely shaped nearby segments, that many times remained unseparated from adjacent elements (e.g., radius from ulna; humerus from ulna) and the whole structure kept growing in size while keeping the shape and proportions fixed (e.g., with normal elongation of fused humero-ulnar elements or continues growth of residual radio-ulnar segment with age). 3. The dose independence but timing dependence of the malformation extent and distribution [1]. All these characters are consistent with a premature differentiation casualty of prospective bone elements with fixation of their temporary shape and proportions, while sparing the already patterned segments. Moreover, induction of differentiation or exit from a proliferating state can be also the explanation for the antimyeloma activities of the IMiDs, either via direct activity of the drug on the tumor cells proper or alternatively by changing the fate or function of their supportive stromal cells, endothelial cells or monocytes/ osteoclasts. Maturation can be also the explanation for the activity of lenalidomide in the 5q- dysplasia, which is associated with maturation arrest. To examine this theory further and search for differentiation markers induced in response to IMiD treatment, we began analyzing gene expression profile (GEP) changes in bone marrow samples from untreated patients with multiple myeloma (MM) before and after thalidomide/lenalidomide treatment in vitro and also followed the clinical response of these patients to lenalidomide. However, because the teratogenic and the antimyeloma activities of thalidomide have been attributed to drug metabolites which are generated in vivo after microsomal processing of the parent drug in a species specific manner [2], we finally decided to rely principally on the published dataset from the Seattle/Little Rock's study, which analyzed the GEP in paired bone marrow samples taken before and 48-hours after administration of thalidomide, lenalidomide or dexamethasone to MM patients [3]. In that study, newly diagnosed patients received dexamethasone (n = 45) or thalidomide (n = 42); in the case of relapsed MM, microarray data were obtained prior to (n = 36) and after (n = 19) lenalidomide administration. The analysis by the

authors, which was based on false discovery rate (FDR) indicated that dexamethasone and thalidomide induce both common and unique GEP changes in tumor cells, with many of them being related to oxidative stress and cytoskeletal dynamics. Some of the changes were also predictive for the outcome of newly diagnosed MM patients receiving tandem transplants. Thalidomide-altered genes also changed following lenalidomide exposure and predicted event-free and overall survival in relapsed patients receiving lenalidomide as a single agent. To get further insight into the GEP changes of interest to our hypothesis, we extracted the entire raw data (which was available for 42 patients who were treated with dexamethasone, 40 patients in the thalidomide arm and 15 patients in the lenalidomide arm) and examined the average changes in GEP on each treatment arm. This analytical strategy was associated however with two major pitfalls. First, certain genes like *DDIT4*, *IRF4*, *TNFSF10*, *DKK1* and *FOS* changed extremely in both directions, being among the top upregulated genes is some of the cases and among the top downregulated genes in others so the average changes missed the individual changes, possibly resulting from spontaneous fluctuations unrelated to the treatment or alternatively from a rebound phenomenon between drug doses (Supplementary Table 1). Second, an unacceptable bias was noticed in the case of disproportional change in the expression of any gene relative to the average change. For example *SPP1* and *CCND2* were induced >135 folds and >117 folds, respectively following thalidomide treatment of case #237, resulting in average fold changes (FC) of 27.2 and 18.8, respectively as compared to 2.66 and 2.51, respectively after exclusion of this case. To overcome this bias, we sorted the fold changes for each case separately and ranked them from 1 (most upregulated gene) to 12,625 (most downregulated gene). On this scale, the average position of *SPP1* and *CCND2* relative to all other genes were 4075 and 410, respectively whereas the most upregulated gene after either thalidomide or lenalidomide treatment was *PLSCR1*, which encodes for phospholipid scramblase (Figure 1 and Supplementary Table 2). *PLSCR1* was also among the top 100 upregulated genes in 13 of the 40 (32.5%) thalidomide treated cases and in 11 of the 15 (73.3%) lenalidomide treated cases (Supplementary Table 1). Likewise, *PLSCR1* was induced x2.55 folds in the bone marrow sample from our lenalidomide responsive patient, after treatment in vitro with lenalidomide (0.5 µg/ml) but not in the sample which was treated with thalidomide (0.8 µg/ml) neither in the samples from our two lenalidomide refractory cases which were treated in vitro with lenalidomide (Supplementary Table 3). As already mentioned, some of the activities of thalidomide depend on in vivo modification of the parent drug [2] and thus the significance of the findings when using this compound in vitro is unclear. Phospholipid scramblase 1 was originally identified as a type II transmembrane protein that mediates the calcium-dependent bidirectional movement of membrane phospholipids. It was also identified as a substrate for several kinases, including c-Abl, c-Src, and protein kinase Cδ (PKCδ). In addition, *PLSCR1* plays potential roles in hematopoiesis and leukemogenesis. *PLSCR1*$^{-/-}$ bone marrow cells exhibit defective myeloid proliferation and differentiation in response to stimulation by selected growth factors. Moreover, *PLSCR1* RNA levels correlate with significantly longer overall survival in acute myeloid leukemia (AML), and *PLSCR1* gene expression has been identified as a new prognostic factor in AML. All-trans retinoic acid (ATRA), an effective differentiation-inducing agent of acute promyelocytic leukemia (APL) cells, elevates *PLSCR1* expression in ATRA- sensitive APL cells NB4 and HL60, but not in maturation-resistant NB4-LR1 cells. ATRA- and phorbol 12-myristate 13-acetate (PMA)-induced monocytic differentiation is accompanied by increased *PLSCR1* expression, whereas only a slight or no elevation of *PLSCR1* expression was observed in U937 cells differentiated with dimethyl sulfoxide (DMSO), sodium butyrate, or vitamin D3. Cell differentiation with ATRA and PMA, but not with vitamin D3 or DMSO, results in phosphorylation of PKCδ, and the PKCδ -specific inhibitor rottlerin nearly eliminates the ATRA- and PMA-induced expression of *PLSCR1*, while ectopic expression of a constitutively active form of PKCδ directly increases *PLSCR1* expression. Finally, decreasing *PLSCR1* expression with small interfering RNA inhibits ATRA/PMA-induced differentiation [4]. More recently it was shown that Wogonoside, the main flavonoid component derived from the root of the Chinese herb medicine Scutellaria baicalensis, can effectively inhibit the proliferation of several cancer cell lines and induce granulocytic differentiation and upregulation of *PLSCR1* expression in AML cells. Wogonoside also promoted *PLSCR1* trafficking into the nucleus and facilitated its binding to the inositol 1,4,5-trisphosphate receptor 1 (*IP3R1*) promoter, thus increasing the expression of *IP3R1*. Finally, inhibition of *PLSCR1* expression with small interfering RNA partially blocked Wogonoside-induced cell cycle arrest and differentiation and disturbed the Wogonoside-associated molecular events [5,6]. Similarly, after treatment of U937 cells with the flavonoid III-10 the cells differentiated into monocyte-like cells in association with upregulation of the differentiation-related proteins *PLSCR1* and promyelocytic leukemia protein (PML) [7]. Moreover, III-10 stimulated *PLSCR1* and PML probably through activation of PKCδ. In ovarian carcinoma cells, both IFN-2α and Aresnic trioxide modulate *PLSCR1* mRNA levels. In turn, *PLSCR1* modulates aspects of the Aresnic trioxide cellular response. Finally, *PLSCR1* is also expressed in maturing chondrocytes and the latter cells show scramblase activity [8].

The second most upregulated gene at 48-hours after thalidomide treatment in the Seattle/Little Rock dataset, based on the average fold change position described, was the glutamate transporter *ARL6IP5*. This gene was also among the top 100 upregulated genes in 10 of the 40 thalidomide treated cases and in 6 of the 15 lenalidomide treated cases. *ARL6IP5* (JWA) has been recently shown to be important for differentiation induced by some chemicals, including ATRA. In HeLa cells, the inhibition of proliferation and the induction of apoptosis by ATRA are dependent on *ARL6IP5* expression [9]. *ARL6IP5* is also induced by the synthetic retinoid 12-O-tetradecanoylphorbol 13-acetate, N-4-hydroxy-phenyl-retinamide and by arsenic trioxide. In HeLa and MCF-7 cells, treatment with arsenic trioxide produced apoptosis in a dose-dependent manner in parallel to increase in *ARL6IP5* expression [10]. In the human myeloid leukemia HL-60 cells, concomitant with the progressive cell

differentiation, *ARL6IP5* expression was up-regulated by ATRA in a dose- and time-dependent manner while inhibition of *ARL6IP5* expression by RNA interference partially blocked ATRA-induced differentiation and growth inhibition of HL-60 cells. Pre-treatment with PMA, a PKC activator, decreased ATRA-mediated differentiation, together with downregulation of *ARL6IP5* expression. In addition, Arsenic trioxide enhanced the cellular differentiation induced by low dose ATRA concurrent with the enhancement of *ARL6IP5* expression, suggesting that up-regulation of *ARL6IP5* expression is essential for ATRA-induced differentiation of HL-60 cells [11].

Gene Symbol	Relative FC Thal PLSCR1>5000	Relative FC Dex	Relative FC Len	Relative FC Thal	Relative FC Thal PLSCR1<100
PLSCR1	10615	12110	1	1	1
ARL6IP5	1325	2054	21	2	3
FGL2	161	3724	57	3	44
ANXA6	2144	355	119	4	27
DKK1	1897	942	61	5	30
CLN3	3706	475	1108	6	29
ITGB7	1463	12551	145	7	4
DYNLT1	7002	11484	4569	8	65
GAS6	1487	11734	75	9	52
RAB13	290	2212	81	10	48
PON2	1904	171	87	11	32
GRN	6670	8226	431	12	69
DNAJA1	9987	12049	229	13	8
FCGR2B	1301	263	3	14	18
CASP1	1201	5929	15	15	16
TUBA4A	4498	1832	99	16	17
E2F3	6785	3695	88	17	7
CASP4	3020	544	27	18	12
CAPN2	4800	8	17	19	9
DENND3	97	11179	256	20	81
CASP1	181	3869	19	21	38
IFI30 /// PIK3R:	4115	12327	400	22	31
NR3C1	261	11833	595	23	124
IFI30 /// PIK3R:	1132	12328	380	24	15
LPCAT4	2039	1438	25	25	53
HLA-F	6273	12480	64	26	46
GSTO1	4752	3668	78	27	39
GAS6	822	11766	76	28	95
RNPEP	9411	5692	142	29	50
ID2 /// ID2B	8763	12063	200	30	2
FCGR2A /// FC(	2464	95	11	31	22
PBXIP1	8499	5417	68	32	171
DNAJA1	11684	12363	248	33	14
ARF3	9862	111	2	34	20
MEF2C	1033	11238	12309	35	154
TOP1	6993	12233	9671	36	254
ARHGEF2	3710	3331	235	37	222
LSP1	19	103	1006	38	136
PHF15	908	108	20	39	35
CTSZ	21	8894	322	40	386
GPX1	7	6217	158	41	409
SMTN	1	60	325	42	1001
CLINT1	10374	135	33	43	49
LOC10099649(	5635	9646	6226	44	389
CIB1	7568	2571	510	45	74
ANXA4	8282	1535	45	46	10
IKZF1	753	7443	176	47	155
DUSP3	7339	807	227	48	40
RABGAP1L	9086	8516	30	49	26
CD180	7278	2440	175	50	90
STK10	3588	1181	53	51	11
PRKCI	7637	5206	3898	52	169
MDK	2388	12587	311	53	121
GAS6	851	11750	110	54	71
UNC13B	7636	12420	132	55	110
BST2	5240	9698	721	56	102
GADD45B	10785	11677	16	57	6
DUSP4	42	7114	234	58	184
ST3GAL5	2019	1634	65	59	63
ATP10D	6852	8956	1665	60	158
HARS2	6710	657	1504	61	375
CAMSAP2	5120	10678	44	62	47
MORF4L2	11608	11391	8643	63	101
TRIM38	9	538	1949	64	602
PNP	9168	12590	363	65	362
LY6E	377	1710	41	66	128
CCNC	12105	12566	66	67	84
IKZF1	874	10647	348	68	137
DPYD	10173	849	625	69	37

Figure 1. The most upregulated genes at 48-h after treatment

Fold changes (FC) of each case from the Seattle/Little Rock dataset were sorted and ranked in a decreasing order from 1 (most upregulated gene) to 12,625 (most downregulated gene). The position of any gene in this scale was used to calculate the average FC position in each treatment arm and the most upregulated genes in the thalidomide arm are presented (blue column) and compared to the average position of the same genes in the other treatment arms. *PLSCR1* was the most upregulated gene after thalidomide (Thal) and lenalidomide (Len) but not dexamethasone (Dex) administration. For additional information see the text.

Other reproducibly induced genes included *RAB13*, which is highly upregulated during differentiation of human peripheral blood monocytic cells into osteoclasts [12] and *MEF2C*, which is essential for endochondral bone development. *MEF2C*, controls bone development by activating the gene program for chondrocyte hypertrophy [13]. Genetic deletion of *MEF2C* or expression of a dominant-negative *MEF2C* mutant in endochondral cartilage impairs hypertrophy, cartilage angiogenesis, ossification, and longitudinal bone growth in mice. Conversely, a super activating form of *MEF2C* causes precocious chondrocyte hypertrophy, ossification of growth plates, and dwarfism. Endochondral bone formation is exquisitely sensitive to the balance between *MEF2C* and the corepressor histone deacetylase 4 (*HDAC4*), such that bone deficiency of *MEF2C* mutant mice can be rescued by an *HDAC4* mutation, and ectopic ossification in *HDAC4* null mice can be diminished by a heterozygous *MEF2C* mutation. Interestingly, *PLSCR1* and *ARL6IP5* were upregulated in the Seattle/Little Rock series by either thalidomide or lenalidomide but not dexamethasone, whereas *MEF2C* was induced by thalidomide only.

Importantly, when the analysis of the thalidomide arm was restricted to the subset of 13 cases whose *PLSCR1* position was < 100 (the average *PLSCR1* induction of this group was 3.81 folds as compared to 2.15 folds in the thalidomide arm as a whole), the majority of the top upregulated genes from the thalidomide arm remained so and continued to overlap the top upregulated genes from the lenalidomide arm (in which arm *PLSCR1* was among the top 100 upregulated genes in 73.3% of cases). In contrast, when the analysis of the thalidomide arm was restricted to the 7 cases whose *PLSCR1* position was >5000 (in the scale from 1 to 12,625) (the average fold change for *PLSCR1* in this subset of patients was 0.86), the highly modulated gene list changed completely and now it had almost nothing in common with the induction signature characterized the lenalidomide and thalidomide arms including the subset of thalidomide treated patients whose *PLSCR1* position was < 100. Therefore, it is obvious that *PLSCR1* is part of a cluster of genes whose expression is strongly associated and context specific. The specificity of the *PLSCR1* associated signature is also reflected by the absence of this signature in the dexamethasone arm (Figure 2). However, even when *PLSCR1* induction was maximal, individual *PLSCR1* associated genes were not always concordant and many times some of them did not change at all or even changed in the opposite direction. The explanation for this observation can be that many of the changes induced by the IMiDs are sequential and/or transient and therefore not necessarily exist simultaneously. The analysis of the 13 samples with maximal *PLSCR1* induction separately exhibited also the association of *PLSCR1* with additional relevant genes. For example, *ID2/ID2B* progressed from average position 30 in the thalidomide arm (and average fold change induction of 2.09) to position 2 (and average fold change induction of 3.58) in the subset of thalidomide treated patients whose *PLSCR1* position was < 100. *ID1* and *ID2* are retinoic acid responsive genes [14] and *ID2* controls chondrogenesis during maxillary morphogenesis [15]. Likewise, the position of *IGF1*, which is another retinoic acid responsive gene [16], progressed from 808 to

21, and that of *SP100*, which is part of the PML nuclear body [17] from position 91 to 25. Moreover, both *SP100* and *SP110* (another PML nuclear body transcript) were induced after lenalidomide treatment in vitro in our lenalidomide responsive case (x2.21 folds and 3.74 folds, respectively). The highly induced gene list also included *FGL2*, which encodes for prothrombinase [18]. The contribution of *FGL2* to the hypercoagulable state associated with IMiD administration seeks further investigation.

Dex/Len	Dex/Thal	Dex/Thal PLSCR1<100	Dex/Thal PLSCR1>5000	
12110.0	12110.0	12110.0	1.1	PLSCR1
86.6	1793.0	3137.8	8.6	ITGB7
2.5	1435.5	176.7	1.6	DYNLT1
156.5	1303.8	225.7	7.9	GAS6
65.3	1241.3	84.6	23.1	FGL2
97.8	1027.0	684.7	1.6	ARL6IP5
52.6	926.8	1506.1	1.2	DNAJA1
19.1	685.5	119.2	1.2	GRN
30.8	560.3	397.6	3.0	IFI30 /// PIK3R2
43.7	559.0	138.0	115.2	DENND3
19.9	514.5	95.4	45.3	NR3C1
32.4	513.7	821.9	10.9	IFI30 /// PIK3R2
195.0	480.0	271.3	2.0	HLA-F
154.8	420.2	123.9	14.3	GAS6
60.3	402.1	6031.5	1.4	ID2 /// ID2B
395.3	395.3	370.6	4.9	CASP1
49.9	374.6	883.1	1.1	DNAJA1
1.3	339.8	48.2	1.7	TOP1
0.9	321.1	73.0	10.9	MEF2C
40.5	237.5	104.0	5.3	MDK
94.1	225.8	112.9	1.6	UNC13B
27.6	222.4	23.0	423.5	CTSZ
27.3	221.2	46.1	7.6	RAB13
1.5	219.2	24.8	1.7	LOC100996496 /// SFPQ
106.8	217.6	165.5	13.8	GAS6
42.0	217.4	527.9	0.5	E2F3
729.8	204.9	1946.2	1.1	GADD45B
40.1	196.3	113.8	0.6	RNPEP
34.7	193.7	34.8	1.4	PNP
15.4	188.4	31.4	0.5	DKK1
190.4	187.6	149.6	1.0	CCNC
203.6	184.2	101.8	21.4	CASP1
1.3	180.8	112.8	1.0	MORF4L2
20.1	175.0	18.8	1.6	CCNC
283.9	173.8	327.5	0.9	RABGAP1L
13.5	173.2	95.1	1.9	BST2
242.7	172.2	227.2	2.1	CAMSAP2
79.7	169.3	31.7	0.6	PBXIP1
42.3	158.4	48.0	9.9	IKZF1
30.6	156.6	77.7	12.2	IKZF1
39.3	151.6	15.2	888.1	GPX1
188.0	149.4	116.6	7.5	TSPAN7
5.4	149.3	56.7	1.3	ATP10D
39.0	147.4	436.8	1.4	SAMHD1
1.7	144.8	16.8	5.3	HMGN4
46.5	140.6	60.4	2.9	STK3
47.0	135.9	94.1	0.8	GSTO1
147.7	133.6	179.4	1.1	MOXD1
103.9	132.3	45.5	10.9	MDK
98.2	123.4	63.6	1.6	IFIT1
64.9	122.8	368.3	4.3	TNFSF10
30.4	122.7	38.7	169.4	DUSP4
20.4	122.4	213.6	3.4	NR3C1
45.4	120.6	58.5	1.4	CAV2
2.1	119.8	43.7	1.4	PTGES3
33.4	118.1	76.9	3.1	RAB8A
9.2	117.7	75.3	1.0	C5orf15
69.7	114.7	31.0	49.5	TNFRSF13B
7.3	114.6	92.2	1.4	RAB8A
18.5	114.5	107.8	0.4	TUBA4A
1.1	100.7	19.4	2.0	MEF2C
1.3	100.1	30.8	0.7	PRKCI

Figure 2. *PLSCR1* associated signature

Listed are the genes which showed the highest Dex/Thal ratios, that is the ratio between the average position of the assigned gene in the Dex arm versus its position in the Thal arm; the higher the ratio the greater the difference in gene modulation relative to the Dex arm (used as control). Also shown are additional Dex ratios for comparison. As illustrated, there was a considerable overlap in gene modulation among the Len arm, Thal arm and the Thal subset of patients whose *PLSCR1* was < 100. Dex, dexamethasone; Thal, thalidomide; Len, lenalidomide.

CD27 was the most downregulated gene in the thalidomide arm (position 12,625, fold change 0.84) and nearly so in the lenalidomide arm (position 12,622, fold change 0.60). In contrast, in the dexamethasone arm *CD27* was upregulated (position 82, average fold change induction = 1.50). Expression of *CD27* was associated with increased clonogenic capacity and engraftment of human myeloma cells in immunodeficient nonobese diabetes/severe combined immunodeficient (NOD/SCID) mice during both primary and secondary transplantations [19]. However, the most downregulated gene in both the lenalidomide arm and the thalidomide subset of patients whose *PLSCR1* position was < 100 was *AMPD1*. This gene encodes for adenylate deaminase, which is mutated in various muscular abnormalities. *AMPD1* was also down regulated after treatment of OCI/AML2 cells with ATRA, with decrease in the signal intensity from 69.7 in the control cells to 44.6 after ATRA and to 31.9 after ATRA + Valproic acid treatment [20]. In the same dataset (GDS1215), ATRA induced the expression of *PLSCR1*, *ARL6IP5* and *ITGB7*, similar to their induction in the lenalidomide arm and in the thalidomide subset of patients whose *PLSCR1* position was < 100 in the Seattle/little Rock dataset. Finally, the most specific dexamethasone modulated gene in the Seattle/Little Rock series was the Prolyl hydroxylase *P4HA*, which was among the top 100 induced genes in 23 of 42 dexamethasone treated cases and its induction was limited to dexamethasone.

Although our original data could add only little to the authentic ("in vivo" quality) information existing in the large dataset from Seattle/Little Rock, the whole genome platform we used can still add to the whole picture. In principle, the in vitro GEP changes evolved in the bone marrow sample from our lenalidomide responsive patient at 24-hours after incubation of the cells with either thalidomide or lenalidomide could be divided into three main groups: 1. Non protein coding genes. 2. Interferon inducible genes. 3. Other genes. In contrast, most of the above changes were absent in the samples from the two lenalidomide resistant cases. The upregulated non protein coding genes included small nucleolar RNA (*SNORD*), small Cajal body-specific RNA (*SCARNA*), micro RNAs and histone modifiers (supplementary Table 3). These genes regulate the expression at the transcriptional, translational and posttranslational levels epigenetically. *SNORD61* was the mostly induced gene after thalidomide treatment in vitro (x27.3 folds) and it was also induced (x2.97 folds) after lenalidomide treatment. Other upregulated genes included *SNORD115-42* (induced 7.2 folds after thalidomide and 6.2 folds after lenalidomide treatment) and the histone modifier *HIST2H2AC* (3.78 folds induction following thalidomide treatment). *SNORD115* and SNORD116 gene clusters believed to play key roles in the fine-tuning of serotonin receptor (5-HT2C) pre-mRNA processing and in the etiology of the Prader-Willi Syndrome, respectively [21]. Among the interferon inducible genes were *IFIT1*, *CXCL10*, *IFI44L*, *XAF1*, *FCGR2B*, *LCE1B*, *S100A10*, *STAT1* and *TNFSF10*.

In summary, our analysis gives support to the hypothesis that both the teratogenic and the antitumoral effects of the IMiDs are mediated via differentiation induction, which involves molecular pathways regulated by ATRA, Arsenic trioxide and other differentiating agents, possibly with involvement of epigenetic changes. Maturation induction also plays role in the activity thalidomide in hereditary vascular malformations [22]. The identified *PLSCR1* associated signature may help to predict the clinical response to the IMiDs and to discriminate between IMiD responsive versus IMiD refractory tumor cells. In this regard our prediction is that the 13 Seattle/Little Rock patients whose *PLSCR1* position was >5000 were less responsive to thalidomide and if so it will be possible to personalize the treatment.

Our analysis also gives the rational for combining the IMiDs with classical differentiating agents in both IMiD responsive tumors (e.g., 5q- dysplasia, MM) and ATRA/ Arsenic trioxide responsive tumors (e.g., APL). Finally, premature differentiation (and the *PLSCR1* associated signature) may prove to be a common pathway for the teratogenic effects of thalidomide, retinoids, valproic acid and related agents.

2. Methods

The bone marrow samples we used were cultured and analyzed by microarray as described elsewhere [23,24]. The study was approved by Asaf Harofhe Institute Review Board, Zerifin, Israel and patients provided written informed consent.

Acknowledgments

The authors thank Professor Dov Zipory for the training of the first author in the Weitzmann Institute of Science, Dr Izhar Hardan from Meir Medical Center for his kind technical support, Dr Relly Forer, Dr Inna Vulin and Dina Volodarsky from Dyn diagnostics Ltd for professional microarray and Bioinformatic service.

Author Contribution

YC designed the experiments, processed the samples, analyzed the data and wrote the article. All other authors assisted in patient recruitment.

Conflict of Interest Disclosure

All authors declare no conflict of interest

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Supplementary Information

Supplementary Table 1: The top modulated genes

The top 100 upregulated (red) and 100 downregulated (green) genes (of overall 12,625 analyzed genes) in the Seattle/Little Rock dataset at 48-h following treatment of MM patients with dexamethasone (Dex), lenalidomide (Len) or thalidomide (Dex). In the red and green columns

appearing the number of cases who exhibited the radical gene modulations (of overall 42 cases who received Dex, 15 who received Len and 40 who received Thal). Note that many of these genes, like *STAT1* were both upregulated and downregulated extremely in different cases, possibly due to a rebound phenomenon between doses.

Supplementary Table 2: The most modulated genes at 48-h after treatment

Fold changes (FC) of each case from the Seattle/Little Rock dataset were sorted and ranked in a decreasing order from 1 (most upregulated gene) to 12,625 (most downregulated gene). The position of any gene in this scale was used to calculate the average FC position in each treatment arm and the 500 most upregulated and 500 most downregulated genes in the thalidomide arm (column oo) are presented and compared to the average position of the same genes in the other treatment arms. Thal, thalidomide; Len, lenalidomide; Dex, dexamethasone.

Supplementary Table 3: The most modulated genes after in vitro treatment

Bone marrow samples from a patient who responded to lenalidomide clinically and two patients who did not respond were cultured for 24-h with or without thalidomide or lenalidomide. The list includes the upregulated genes for which the fold changes (FC) were > 2 and the downregulated genes for which the FC were < 0.5. Note the marked induction of non-protein coding genes using thalidomide and induction of interferon inducible genes using either compound.