RESEARCH ARTICLE

Modeling the proteome of a Marek's disease transformed cell line: a natural animal model for CD30 overexpressing lymphomas

Joram J. Buza[1, 2] *and Shane C. Burgess* [1, 2, 3]

[1] College of Veterinary Medicine, Mississippi State, MS, USA
[2] Institute for Digital Biology, Mississippi State University, MS, USA
[3] Mississippi Agricultural and Forestry Experiment Station, MS, USA

Marek's disease (MD) in the chicken, caused by the highly infectious MD α-herpesvirus (MDV), is both commercially important and a unique, naturally occurring model for human T-cell lymphomas overexpressing the Hodgkin's disease antigen, CD30. Here, we used proteomics as a basis for modeling the molecular functions and biological processes involved in MDV-induced lymphomagenesis. Proteins were extracted from an MDV-transformed cell line and were then identified using 2-D LC-ESI-MS/MS. From the resulting 3870 cellular and 21 MDV proteins we confirm the existence of 3150 "predicted" and 12 "hypothetical" chicken proteins. The UA-01 proteome is proliferative, differentiated, angiogenic, pro-metastatic and pro-immune-escape but anti-programmed cell death, -anergy, -quiescence and -senescence and is consistent with a cancer phenotype. In particular, the pro-metastatic integrin signaling pathway and the ERK/MAPK signaling pathways were the two predominant signaling pathways represented. The cytokines, cytokine receptors, and their related proteins suggest that UA-01 has a regulatory T-cell phenotype.

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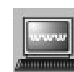

Keywords:
Insulin-like growth factor-1 / Interleukin 1 receptor accessory protein / Interleukin 10 / Lymphomatoid populosis / Regulatory T cells

1 Introduction

Marek's disease (MD) is a commercially important, rapidly progressive lymphomatous disease of chickens caused by the highly infectious MD α-herpesvirus (MDV). MD has been a valuable model for human lymphomas for almost four decades [1] and has been identified as a unique natural animal model for lymphomas that overexpress tumor necrosis factor receptor superfamily member 8 (TNFR8, also known as the Hodgkin's disease antigen or CD30) [2, 3]. CD30 overexpressing ($CD30^{hi}$) lymphomas include classical Hodgkin's, as well as non-Hodgkin's, lymphomas of viral and unknown etiology. $CD30^{hi}$ non-Hodgkin's lymphomas include anaplastic large cell lymphomas, primary cutaneous anaplastic large cell lymphoma, adult T-cell leukemia/lymphoma, peripheral T-cell lymphoma, unspecified, extranodal NK/T-cell lymphoma, nasal type, and enteropathy-type T-cell lymphoma [4]. Elevated levels of soluble CD30 in sera from patients with $CD30^{hi}$ lymphomas correlate with a poor prognosis. Similarly, MD lymphoma-bearing chickens also have soluble CD30 in their sera [3].

Correspondence: Dr. Joram J. Buza, College of Veterinary Medicine, P.O. Box 6100 Mississippi State, MS 39762, USA
E-mail: buza@cvm.msstate.edu
Fax: +1-662-325-1459

Abbreviations: DDF, differential detergent fractionation; **GO**, gene ontology; **IL**, interleukin; **IPA**, ingenuity pathways analysis; **MD**, Marek's disease; **MDCC**, Marek's disease lymphoblastoid cell line; **MDV**, Marek's disease virus; **nrpd**, non-redundant protein database; **RIPA**, radioimmunoprecipitation buffer; **SCX**, strong cation exchange; **T-reg**, regulatory T cells; **VEGF**, vascular endothelial growth factor

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The CD30^{hi} neoplastically transformed cells are minority populations in human and chicken lymphomas [2, 5, 6] and are difficult to isolate directly *ex vivo*. Because of this, work to identify the functional lineage and potential signaling pathways disregulated during neoplastic transformation routinely uses cell lines. Although some lymphomagenic herpesviruses such as human Epstein-Barr virus can transform cells *in vitro* other human herpesviruses and MDV do not. However, MDV-transformed cell cultures (MDCC) can be derived directly from MD tumors [7]. MDCC express antigens, detected by mAb-based techniques, consistent with activated T-helper-2 cells (TCR-II, CD4, CD25, CD28, CD30, CD44, and MHC-II) [8] and these antigens are also expressed by lymphomas *in vivo* [2, 9]. However, further description of the functional lineage and mechanisms of MDV transformation, either *in vitro* or *in vivo*, is severely hampered by the paucity of chicken antigen-specific mAb. Our aim here was to take advantage of the chicken genome sequence and proteomics methods to identify the functional lineage and molecular pathways disregulated during MD neoplastic transformation [10, 11].

We used differential detergent fractionation (DDF) 2-D LC MS/MS [11] to identify proteins expressed by an MDCC called UA-01. UA-01 expresses CD3, CD4, CD28, CD30^{hi}, CD44, CD45, MHC-I and MHC-II [8]. We identified 3870 cellular and 21 MDV proteins. We annotated our entire dataset using the gene ontology (GO) [7] and used hypothesis-driven modeling to define the UA-01 phenotype and to describe cellular functions including activation, proliferation, differentiation, apoptosis and other signaling pathways. We then modeled MD lymphoma physiology and host-virus interactions.

2 Materials and methods

2.1 Cell culture and protein extraction

The MDCC called UA-01 was obtained from Dr. M. Parcells (University of Delaware) and grown as described [8]. For protein isolation, 8×10^8 UA-01 cells were grown and washed twice with cold PBS. Then, two aliquots of 2×10^8 cells each were lysed using radioimmunoprecipitation analysis buffer (RIPA) [12] and two aliquots of 2×10^8 cells each were fractionated by DDF to produce four DDF fractions [11].

2.2 Proteomics

Protein lysates were precipitated using 25% TFA, resuspended in 0.1 M ammonium bicarbonate, 5% ACN and the pH adjusted to ≥7.5 using 1 M Tris pH 8.0. The protein solutions were then reduced using DTT (final concentration 5 mM; 65°C, 5 min) and alkylated with iodoacetamide (final concentration 10 mM; 30°C, 30 min). The proteins were then digested overnight using molecular biology grade porcine trypsin (Promega, Madison, WI; 50:1 final substrate:trypsin ratio; 37°C). The resulting peptides were desalted using a C18 microtrap (Microm Bioresources, Auburn, CA) and eluted using 0.1% TFA and 95% ACN solution, vacuum dried and resuspended in 0.1% formic acid. MS analysis was done by 2-D LC ESI MS/MS using a Thermo Separations P4000 quaternary gradient pump LCQ Deca XP Plus (Thermo Electron, San Jose, CA) as described previously [11]. LC was done by strong cation exchange (SCX) followed by RP-LC coupled directly in line with ESI IT mass spectrometer. Samples were loaded into an LC gradient ion exchange system (Thermo Separations P4000 quaternary gradient pump coupled with a 0.32 × 100 mm BioBasic SCX column). A flow rate of 3 μL/min was used for both SCX and RP columns. A salt gradient was applied in steps of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45,50, 57, 64, 71, 79, 90, 110, 300, and 700 mM ammonium acetate in 5% ACN, 0.1% formic acid and the resultant peptides loaded directly into the sample loop of a 0.18 × 100 mm BioBasic C18 RPLC column (Thermo Electron). The RP gradient used 0.1% formic acid in ACN and increased the ACN concentration in a linear gradient from 5 to 30% in 30 min and then 30 to 65% in 9 min followed by 95% for 5 min and 5% for 15 min. The spectrum collection time was 59 min for every SCX step. The mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by alternating between a single full MS scan followed by three MS/MS scans on the three most intense precursor masses (as determined by Xcalibur software in real time) from the full scan. The collision energy was normalized to 35%. Dynamic mass exclusion windows were set at 2 min, and all of the spectra were measured with an overall *m/z* range of 300–1700.

UA-01 cells contain cellular and MDV proteins. The nonredundant protein database (nrpd) was downloaded from the National Center for Biotechnology Institute (NCBI; 2/10/04). Using TurboSEQUEST (Bioworks Browser 3.1; Thermo Electron) we created an Avian nrpd subset exactly as described [11]. We next identified all MDV proteins in the NCBI database and manually compiled a text file of these proteins in FASTA format. Our experimental mass spectra and MS/MS spectra were then searched against both databases, including cysteine carbamidomethylation (Δmass 57.02 Da) and methionine mono- and di-oxidation (Δmass 16 and 32 Da). The peptide (MS precursor ion) mass tolerance was 1.5 Da and the fragment ion (MS/MS) tolerance was 1.0 Da. Protein identifications made with peptides that had Xcorr ≥1.5, 2.0, and 2.5 for +1, +2, and +3 charged ions, respectively, and delta Cn values of ≥0.1 [13, 14] were used for protein identification. Furthermore, the protein identifications were considered valid only when peptides were ≥ six amino acids long [11]. All protein identifications and their associated MS data have been submitted to the Proteomics Identifications Database (PRIDE; http://www.ebi.ac.uk/pride; Experiment Accession 1654 and 1655). To estimate the overall probability that peptide identifications may be incorrect we used the reverse database function in the Bioworks software to create a reverse database of the Avian and MDV protein databases. Our experimental mass spectra and MS/MS spectra were then searched against both of these reverse

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databases and filtered exactly as for the correct databases. The number of peptide identifications from the reverse databases (854) divided by the number from the correct database (13 145) estimates that the overall probability of an incorrect identification (P) is less than 0.065. This means that for any protein identified by two or more peptides, the probability of being incorrect is $<0.065^n$, where n the number of peptides used to identify the protein. In addition, P depends on the peptide length and we can use this to assign more accurate P values for proteins with single peptide identifications (Supplementary Fig. 1). We used the ΣXcorr method [15] to estimate the proportions of proteins identified from different DDF fractions.

2.3 GO annotation

All identified proteins were manually annotated based on the three organizing principles of GO, *i.e.* molecular function, biological process and cellular component [16]. We first annotated our chicken and MDV protein data using existing GO annotations and then annotated from the literature when it existed. When no annotations or literature were available and when we could identify direct orthologs by name, chicken proteins were annotated based on annotations attributed to their human orthologs. The remaining chicken proteins were then individually searched against the vertebrate entries in the nrpd. Orthologs were identified manually based on sequence identity (expected value <0.000001), conserved domain structure and conserved key residues. When these vertebrate orthologs had GO annotations, the inferred by sequence similarity (ISS) GO annotations were applied to the chicken orthologs. To obtain an overview of function in the UA-01 proteome, the Cellular Component and Biological Process annotations were classified into broad groups based on the Mouse Genome Informatics (MGI) GO-slim using the GOSlim viewer (generic GO slim set) and other tools at AgBase [17, 18].

2.4 Functional modeling

To model our datasets, we compared the number of proteins that were either agonistic or antagonistic for biological processes that included differentiation, activation, proliferation, cell cycle, quiescence, apoptosis, anergy, senescence, angiogenesis and cell migration. The GO identification numbers included are shown in Supplementary Table 1. Transcription factors from UA-01 cells that have not been previously reported from T-cells were identified by manual curation of literature cited in PubMed. Finally we modeled the biological role of the UA-01 soluble factors and receptors based on their function including mechanisms for tumor escape from host immunity. We used Ingenuity Pathways Analysis (IPA) (Ingenuity Systems) to identify biological functions/diseases, and associated signaling pathways, significantly associated with the dataset. Currently, IPA accepts gene/protein ID from human, mouse or rat only. Thus, we developed an in-house program (ProteinMapper) to convert all except five chicken protein GI numbers into those of corresponding human orthologs (using blastp at NCBI; E-value <0.000001). These GI numbers were then uploaded into IPA. Fischer's exact test was used to calculate the probability of each biological function/disease or pathway being assigned by chance and accepted only those with >99% confidence [19].

3 Results

3.1 Proteomes

We identified 3870 cellular (Supplementary Table 2A) and 21 MDV proteins (Supplementary Table 2B). Only 37.5% of proteins were identified based on a single-peptide and this compares favorably to data reported by others [10, 20]. Overall, 44.37% of cellular proteins were found only in DDF fractions, 29.40% were only in the RIPA buffer extract and 26.23% were found in both. Out of the 1717 proteins identified exclusively from DDF fractions, 35.99% were contributed exclusively by DDF-1; 34.71% by DDF-2; 13.51% by DDF-3; 2.15% by DDF-4; and 13.64% were identified in more than one fraction (Supplementary Fig. 2A). The MDV proteins had a similar partitioning between DDF and RIPA buffers (Supplementary Fig. 2B). Approximately half (14 396) of the 29 498 proteins in the chicken NCBI nrpd (02/04/2006, Genome Build 1.1) are anticipated either by *de novo* gene prediction algorithms or based on sequence homology but their expression at protein level has never been confirmed. At the time of writing, there were 29 655 chicken protein entries in the NCBI (04/04/2006, Genome Build 1.1), 48.55% of which were annotated as "predicted" based on sequence homology [21] and 8.45% annotated as "hypothetical" because they could not be related to other proteins of known structure and function [22]. Our results provide the first experimental confirmation of 3150 predicted and 12 hypothetical chicken proteins (Supplementary Table 2A) and, in addition to allowing us to model MD lymphomagenesis, our data are fundamentally useful for chicken genome annotation.

Tumorigenesis may result from events that follow genetic damage to two types of genes; proto-oncogenes and tumor suppressor genes [23]. Although so far only one protein, the MDV putative "oncoprotein" Meq has been established as contributing to MD lymphoma transformation [24–26], we also identified nine proto-oncogenes and two tumor suppressors expressed by UA-01 (Table 1). We identified 84 transcription factors (Supplementary Table 3); 67 were "PREDICTED" and we have experimentally confirmed the expression of these proteins for the first time. Furthermore, 8 out of 17 known transcription factors have never before been reported in T-cells.

3.2 Comparison with previously reported proteins

Previously, only nine proteins were known to be expressed by MDCC [8] and we have extended this number by over 400-fold. We identified seven out of these nine proteins: CD3,

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Table 1. Proto-oncogenes and tumor suppressors

GI no.	Protein name	Type	Action
15080692	Platelet-derived growth factor (sis)	Oncogene	Growth factor
439763	Epidermal growth factor receptor	Oncogene	Growth factor receptor
5075112	Ras	Oncogene	Signal transducer
212355	C-myc	Oncogene	Transcription factor
280734	Bcl-2	Oncogene	Programmed cell death regulator
50758553	RB1 (retinoblastoma protein)	Tumor suppressor	Control cell division
50740200	P53	Tumor suppressor	Cell suicide
50794283	MAS pro-oncogene	Oncogene	Membrane receptor?
50761210	Mel transforming oncogene	Oncogene	Member of Ras superfamily
50806658	EMSI (cortactin)	Oncogene	Signal transducer
50733592	Dek oncogene (Insulin-like growth factor 2, Somatomedin A)	Oncogene	Growth factor

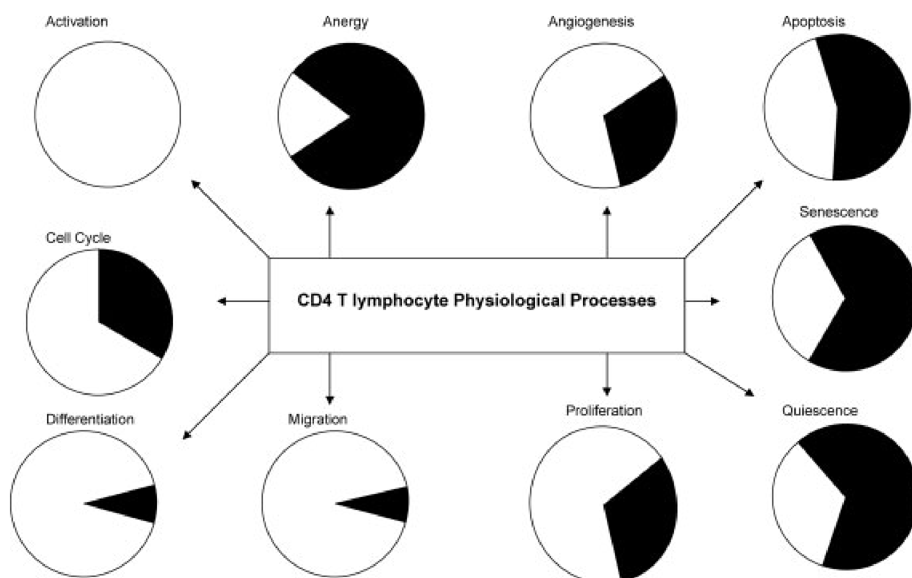

Figure 1. Potential $CD4^+$ T lymphocyte biological processes.

CD44, CD45, TCR-II, MHC-I, MHC-II and CD30. All were present in DDF fractions with the exception of TCR-II, which was only found in the RIPA buffer sample. Since DDF-2 primarily extracts membrane proteins [11, 27], the six membrane proteins identified would be expected to be most abundant in DDF-2. All six proteins were present in DDF-2 and four proteins were exclusive to DDF-2. Although MHC-I was present in three fractions (DDF-1, DDF-2 and DDF-3), most (54%) was present in DDF-2. Similarly, MHC-II was present in DDF-1, -2, and -3, but was equally distributed between DDF-2 and DDF-3. These distributions are consistent with the biology of MHC-I and MHC-II [28]. The UA-01 cell line is known to express CD4, CD28 [8] and the MDV oncogene Meq [29], however, we did not identify these proteins. This may be because only three CD4 peptides, one CD28 peptide (a signal peptide not present on mature protein) and one Meq peptide could theoretically be detected by 2-D LC ESI MS/MS (Supplementary Table 4).

3.3 GO annotation

Out of the 3870 cellular proteins identified, we were able to annotate 3188 with GO terms for molecular function, biological process or cellular component. For MDV proteins, 2 out of 21 proteins had existing GO annotations for molecular function, biological process and cellular component. Almost one-third (30%) of the cellular proteins were membrane proteins, consistent with the estimated proportion of membrane proteins encoded by the genome [30, 31].

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3.4 Functional modeling

Our GO-based modeling shows that UA-01 cells are activated, differentiated, proliferative with progressive cell cycle, angiogenetic and metastatic but oriented away from apoptosis, anergy, quiescence and senescence (Fig. 1). Transcription factors location in the cell is one determinant of their activity: 8 were found exclusively in the nuclear fraction, suggesting active involvement in transcription at the time of sampling. These 8 transcription factors are important in development, regulation of cell growth, mitosis, cell proliferation, signal transduction, protein folding and DNA damage repair. Cell signaling occurs through soluble factors, as well as transmembrane proteins and their receptors. We identified 29 soluble factors: 3 with chemokine activity, 4 with cytokine activity, 9 with growth factor activity, 12 with hormonal/neuropeptide activity and 1 uncharacterized (Table 2A). Slightly more than half (15) of the soluble factors are predicted. Furthermore, 12 of these soluble factors have never been reported in T cells in any species. We identified 26 receptors: 9 cytokine receptors, 9 hormone receptors, 6 growth factor receptors and 2 chemokine receptors (Table 2B).

All 21 MDV proteins were from the MDV capsid, tegument, and envelope and the major function was viral genome replication, assembly, egress, and release during active MDV replication. This is not surprising given that 5–10% of cells in any MDCC [8, 32] like MD lymphomas *in vivo* [2], are productively infected. We also identified UL49, which is important for MDV growth in cell cultures [33] and may be present because we used a cultured cell line.

Cell signaling is critical for cells to communicate with, and effect, their environment. Out of the 3870 proteins uploaded for analysis, the IPA software identified 1916 “focus genes” that were eligible for generating networks and 1910 focus genes that were eligible for generating biological functions/diseases and associated pathways (signaling/biochemical). A total of 49 functions/diseases were significantly represented in the UA-01 proteome (Fig. 2). The top 10 functions/diseases (ranked based on number of focus genes per function/disease), the associated top 10 signaling pathways and the number of respective focus genes are shown in Table 3. The integrin signaling pathway was the top pathway represented in the UA-01 proteome (Fig. 3). Proteins from our dataset that mapped to the integrin signaling pathway are described in Supplementary Table 5.

4 Discussion

MD is a unique naturally occurring animal model for CD30^{hi} human lymphomas [3], however, very little is known about MD lymphomagenesis, especially the host genes involved in maintenance of the neoplastically transformed state and lymphoma growth [34]. Furthermore, although MDV genes have been implicated in MD lymphomagenesis [24–26] these genes alone are insufficient for lymphoma growth *in vivo*

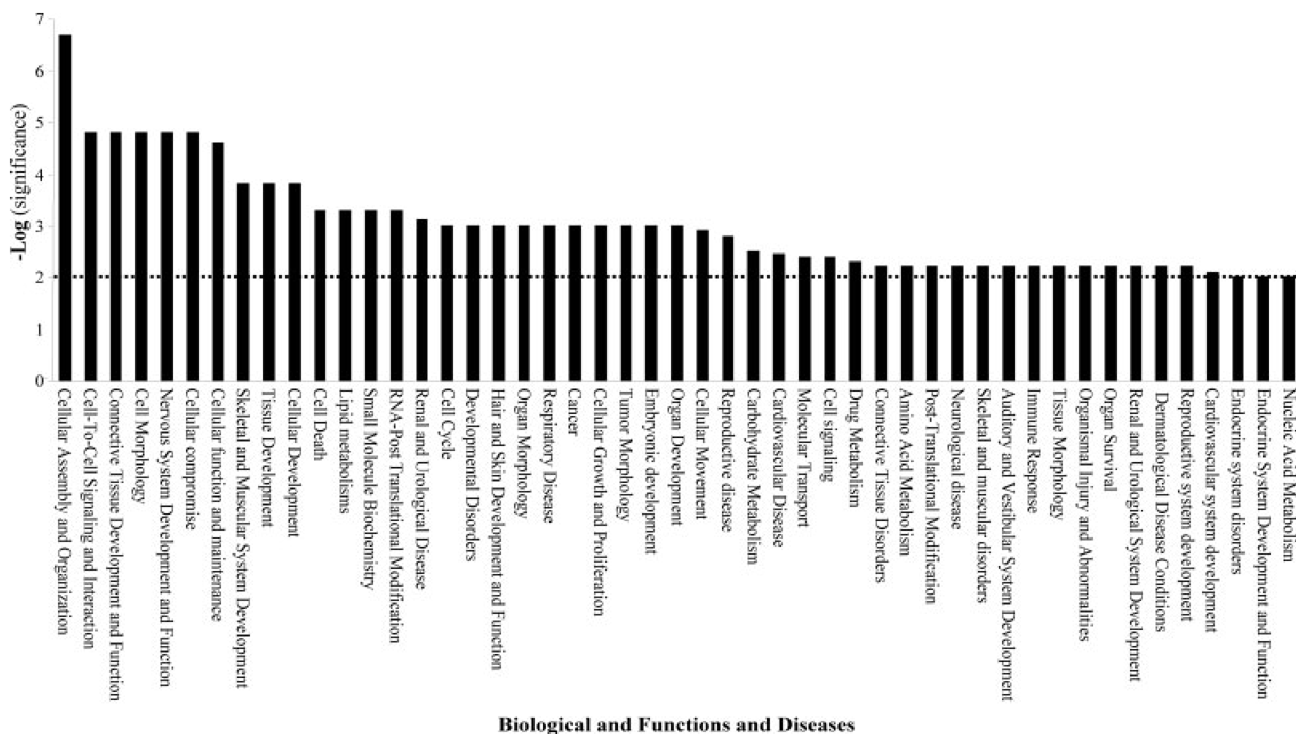

Figure 2. Functions and/or diseases represented by the UA-01 proteome.

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Table 2. Soluble factors and receptors for soluble factors

	GI no.	Name	Prior detection in T-cells	Molecular function
A.	45382647	Jun-suppressed chemokine	No	Chemokine activity
	50757159	PREDICTED: similar to complement component C5	No	Chemokine activity
	50758004	PREDICTED: similar to chemokine ah294	Yes	Chemokine activity
	8919963	Interleukin 18	Yes	Cytokine activity
	33414148	Macrophage migration inhibitory factor	Yes	Cytokine activity
	47087161	IL-12p35 subunit	Yes	Cytokine activity
	50746435	PREDICTED: similar to Multisynthetase complex auxiliary component p43	Yes	Cytokine activity
	51173886	Interleukin-10	Yes	Growth factor activity, cytokine activity
	2494455	Fibroblast growth factor-8 precursor (FGF-8) (HBGF-8)	No	Growth factor activity,
	2696219	Neurocrescin	No	Growth factor activity
	15080692	Platelet-derived growth factor A chain short form type 1 precursor	Yes	Growth factor activity
	30038758	Fibroblast growth factor 9	Yes	Growth factor activity, Heparin binding
	50728065	PREDICTED: similar to hepatocyte growth factor /scatter factor	Yes	Growth factor activity
	50746517	PREDICTED: similar to vascular endothelial growth factor C	Yes	Growth factor activity
	50748904	PREDICTED: similar to Glia maturation factor beta (GMF-beta)	Yes	Growth factor activity
	462404	Insulin-like growth factor II (IGF-II)	Yes	Growth factor activity, hormone activity
	50761761	PREDICTED: similar to lens epithelium-derived growth factor	Yes	Transcriptional coactivator activity
	1836006	Prepro-insulin-like growth factor-II; prepro-IGF-II	No	Hormone activity
	17061833	Prepro-chicken-II-type gonadotropin-releasing hormone	Yes	Hormone activity
	26453322	Prepro-orexin	No	Neuropeptide hormone activity
	50728878	PREDICTED: similar to PMCH protein	No	Melanin-concentrating hormone activity
	50730436	PREDICTED: similar to FLJ45273 protein	No	Neurohypophyseal hormone activity
	50737758	PREDICTED: similar to Proenkephalin A precursor	Yes	Neuropeptide hormone activity
	50740149	PREDICTED: similar to Secretogranin I precursor (SgI) (Chromogranin B)	No	Hormone activity
	50747750	PREDICTED: similar to adrenomedullin precursor	Yes	Neuropeptide hormone activity
	50805286	PREDICTED: similar to KIAA0556 protein, partial	No	Hormone activity
	50806810	PREDICTED: similar to Coatomer protein complex subunit alpha	No	Hormone activity
	50761710	PREDICTED: similar to relaxin 3 preproprotein; insulin-like 7	Yes	Hormone activity
	62849	Prepropeptide for arginine vasotocin and copeptin	No	Neurohypophyseal hormone activity, hormone activity
B.	439763	Epidermal Growth Factor Receptor, EGF-R	Yes	Epidermal growth factor receptor activity
	4884676	Receptor tyrosine kinase precursor	Yes	Epidermal growth factor receptor activity
	10720134	Neogenin	No	Vascular endothelial growth factor receptor activity
	50744690	PREDICTED: similar to TAK1	Yes	Transforming growth factor beta receptor
	50750085	PREDICTED: similar to receptor tyrosine kinase precursor	Yes	Epidermal growth factor receptor activity
	50785358	PREDICTED: similar to hypothetical protein DKFZp686O1689	No	Transforming growth factor beta receptor activity
	6094488	Orphan nuclear receptor NR2E1 (Nuclear receptor TLX)	No	Steroid hormone receptor activity
	15777197	Photoreceptor-specific nuclear receptor	No	Steroid hormone receptor activity
	50745617	PREDICTED: similar to leucine-rich repeat-containing G	Yes	Protein-hormone receptor activity
	50746311	PREDICTED: similar to mineralocorticoid receptor delta	No	Steroid hormone receptor activity

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Table 2. Continued

GI no.	Name	Prior detection in T-cells	Molecular function
50747146	PREDICTED: similar to peroxisome proliferative activated receptor gamma	Yes	Steroid hormone receptor activity
50750834	PREDICTED: similar to nuclear receptor subfamily 4, group A, member 2	Yes	Steroid hormone receptor activity, DNA binding
50755222	PREDICTED: similar to hypothetical protein	Yes	Steroid hormone receptor activity, Glucocorticoid receptor activity
50760419	PREDICTED: similar to orphan nuclear receptor FOR1	No	Steroid hormone receptor activity
50805535	PREDICTED: hypothetical protein XP_430438	No	Lutropin-choriogonadotropic hormone receptor activity
24940152	Putative common cytokine receptor gamma chain b	Yes	Interleukin-2 receptor activity, interleukin-7 receptor activity, interleukin-4 receptor activity
28950399	CD30 protein	Yes	Cytokine receptor activity
50752281	PREDICTED: similar to Interleukin-1 receptor accessory protein precursor	Yes	Interleukin-1 receptor activity
50755753	PREDICTED: similar to interleukin-4 receptor alpha-chain	Yes	Hematopoietin/interferon-class (D200-domain) cytokine receptor activity, interleukin-4 receptor activity
50755755	PREDICTED: similar to novel interleukin receptor	Yes	Interleukin-21 receptor activity
50755757	PREDICTED: similar to Interleukin-9 receptor precursor (IL-9R)	Yes	Interleukin-9 receptor activity
50760152	PREDICTED: similar to Interleukin-10 receptor alpha chain precursor	Yes	Interleukin-10 receptor activity, hematopoietin/interferon-class (D200-domain) cytokine receptor activity
50761482	PREDICTED: similar to gp130-like monocyte receptor	No	Hematopoietin/interferon-class (D200-domain) cytokine receptor activity cytokine receptor activity
50794919	PREDICTED: similar to Tumor necrosis factor receptor superfamily member 11B precursor	Yes	Cytokine receptor activity
50732854	PREDICTED: similar to C-C chemokine receptor 8 like	Yes	C-C chemokine receptor activity
50741692	PREDICTED: similar to C-C chemokine receptor type 6	Yes	C-C chemokine receptor activity

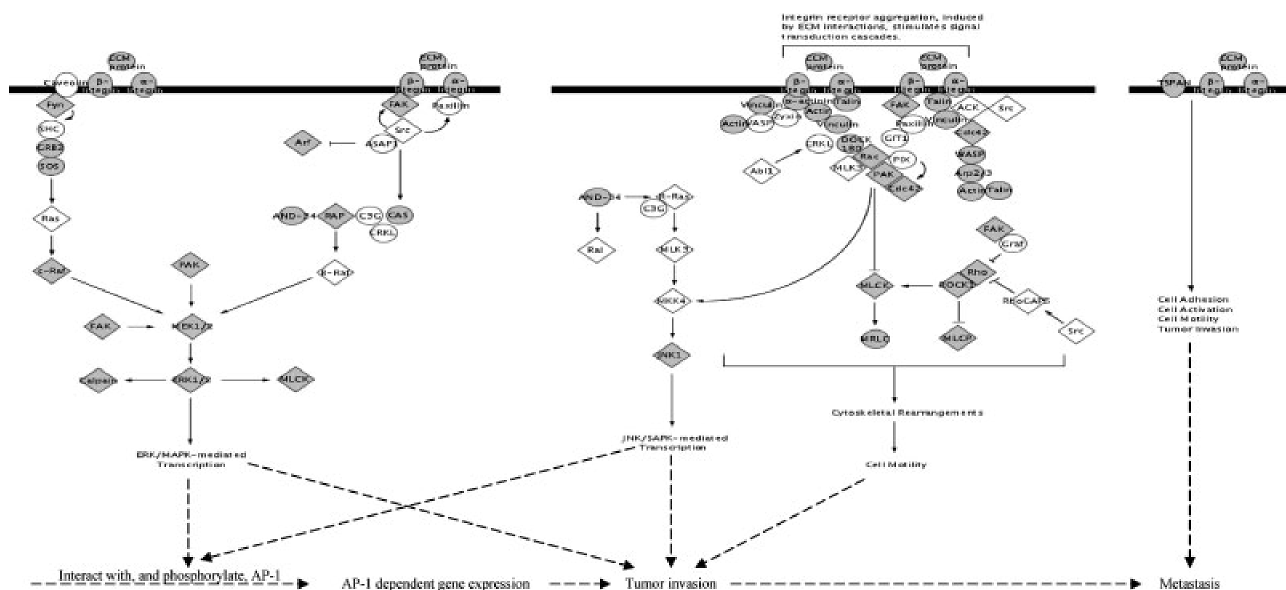

Figure 3. Integrin signaling pathway.

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Table 3. The number of cellular proteins present in the top ten functions/diseases and their respective top ten signaling pathways

Functions/ Disease	Signaling pathways	Integrin signaling pathway	ERK/MAPK signaling	IGF-1 signaling	Chemokine signaling	FGF signaling	B-Cell receptor signaling	Apoptosis signaling	P13K/AKT signaling	VEGF signaling	T-Cell receptor signaling
Cellular assembly and organization		30	10	5	6	8	8	6	6	5	9
Cell-cell signaling and interaction		26	11	10	6	7	6	9	7	9	10
Connective tissue development and function		19	11	10	6	10	10	6	7	8	
Cell morphology		36	20	13	11	16	14	10	11	11	12
Nervous system development and function		22	9	7	6	7	6	4			5
Cellular compromise		10	4	3	4			5	2		
Cellular function and maintenance		16	10	7	5	6	8				5
Skeletal and muscular system development and function		17	5	7	6	8	8	5	6	7	
Tissue development		20	8	8	5	7	5	8	7	9	8
Cellular development		31	16	10	10	10	8	9	9	12	9

[35]; host genes must be important [2]. Accumulating evidence suggests that, although not acutely transforming *per se*, the MDV Meq protein is oncogenic [24–26]. Because Meq induction is associated with transcriptional up-regulation of genes involved in growth and anti-apoptosis as well as down-regulation of pro-apoptosis genes [24, 26], we interrogated the UA-01 proteome for proteins that promote and inhibit apoptosis, proliferation, cell cycle, activation, quiescence, senescence, anergy, differentiation, angiogenesis and cell migration. Our data indicate that UA-01 cells are activated and proliferative with a progressive cell cycle, angiogenetic, as well as metastatic. However, the UA-01 proteome is anti-apoptotic, -anergic, -quiescence and -senescence. Together, this is consistent with neoplastic-transformation. Furthermore, although de-differentiation is classically considered to be an indicator of malignancy, UA-01 cell line is considered well-differentiated [8] and our data agree overall. However, and similar to T-cells during their ontogeny, we verify that UA-01 cells co-express CD4 and CD8 [8] and show that the CD8 antigen is retained predominantly in the cytosol.

Survival of the neoplastically transformed cells in MD depends on the local lymphoma environment [2]. One major mechanism by which tumor cells interact with stroma and non-transformed infiltrating cells is through soluble factors [36]. We identified chemokines, cytokines, growth factors, neuropeptides, and hormones, which may influence the immune response and pathology, associated with MD. Chemokines direct the migration of leucocytes to sites of inflammation [5, 37]. We identified three chemotactic chemokines. (i) Jun-suppressed chemokine (JSC, also known as CXCL14) is 60% amino acid identical to the human and mouse chemokine BRAK, which is selectively chemotactic for monocytes [5]. Notably monocyte/macrophage infection is part of early lytic MD pathogenesis [38] and monocytes have a role in MD tissue lesions [38] as well as definitive MD lymphomas [9]. JSC was identified from jun-transformed avian fibroblasts and we speculate that, because of Meq's known heterodimerization with c-jun, jun-suppressed chemokine may be regulated directly by Meq. (ii) Chemokine ah294, which is 57% amino acid identical to human chemokine RANTES, which is chemotactic not only for monocytes but also for memory T-helper cells (*i.e.* the phenotype of the transformed cells in MD lymphomas) [2, 3] and eosinophils. (iii) Complement component C5, which is chemotactic to polymorphonuclear leucocytes (PMN) [5, 37]. Notably, the avian equivalent of PMN is the heterophil and again, heterophils are part of the heterogeneous lymphoma environment [39–41]. However, while attraction of these cells to the tumor site could help in the inflammation process and killing of tumor cells it may also expose immune cells to lymphoma-derived immunosuppressive factors and allow lymphoma growth.

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We identified the cytokines: IL-10, 12, and 18 and cytokine receptors: IL-1 receptor accessory protein (IL-1RAcP), IL-4R, IL-9R and IL-10R α-chain. GO-based modeling suggests a T helper-2 bias and increased cell proliferation. From a classical reductionist perspective, this seems counterintuitive because IL-12 and IL-18 are potent inducers of interferon (IFN)-γ, a potent pro-T helper-1 cytokine. We identified IFN γ mRNA from UA-01 by RT-PCR (data not shown) and, although we did not identify IFN-γ protein in this proteomics dataset, we did identify interferon-induced 35-kDa protein (IFP35), interferon gamma receptor 2, interferon regulatory factor (IRF)-3 and -4. However, the pattern of T helper-1 and T helper-2 cytokine co-synthesis in our dataset is characteristic of antigen-specific CD4$^+$ CD25 (IL-2Rα)$^+$ regulatory T-cells (T-reg) [42] and we suggest that UA-01 cells are more similar to regulatory, rather than T helper-2 T-cells.

Tumors must evolve immune escape mechanisms and some specific immune evasion mechanisms are postulated for MD lymphomas [2, 43–45]. The transformed cells in MD lymphomas having a T-reg phenotype is a potent immune escape mechanism. In addition, UA-01 cells express IL-10 and vascular endothelial growth factor (VEGF). IL-10 specifically suppresses anti-tumor immunity by augmenting T helper 2 responses, inhibiting cytotoxic T lymphocyte differentiation and cytokine production, down-regulating tumor cell cytotoxic potential, inhibiting antigen presentation and down-regulating costimulatory molecules [46, 47]. VEGF mediates tumor immune escape by interfering with functional maturation of professional antigen presenting dendritic cells from their hematopoietic progenitors [46, 48]. In addition, VEGF is a cell growth factor and promotes angiogenesis and metastasis [49]. We identified eight more proteins from UA-01 that promote cell growth and proliferation, and one specifically, fibroblast growth factor-8, directly promotes tumor invasion. Furthermore, the neuropeptides and hormones in our UA-01 proteome may also be involved in tumor growth and pathology [50, 51].

Our UA-01 proteome had many other pathways important for MD pathophysiology such as T-cell signaling, tumor cell invasion, metastasis, cell growth, proliferation and survival. However, the integrin and ERK/MAPK pathways were the top two that we identified. During cancer, altered integrin expression and function contributes extensively to invasion and migration (metastasis) of cancer cells [52]. We suggest that MDV-lymphoma transformation induce changes in integrins that facilitate lymphoma metastasis. This hypothesis is consistent with observation made by others; it was demonstrated that Marek's disease-transformed lymphoma cell lines that are highly metastatic *in vivo* show increased expression of integrins [53]. Recently, it has been shown that constitutive expression of MDV telomerase in the chicken fibroblast cell line DF-1 resulted in enhanced anchorage-independent cell growth and increased expression levels of integrin alpha5 [54]. Tumor invasion and metastasis is relevant in MD because lymphomas are found in multiple loca-

Table 4. Comparison of the UA-01 cell line and human CD30$^+$ T-cell lymphomas for expression of different immunophenotyping and other features (markers) used for lymphoma classification

Marker	UA-01	ALCL[a)]	C-ALCL[b)]	ATLL[c)]	PTL, unspec[d)]	LyP[e)]	NK/T, nasal[f)]	TCL, enteropathy[g)]
CD30	+	+	+	-/+	-/+	+	-/+	+/-
CD4	+	+/-[h)]	+	+/-	+/-	+	-	-
CD45	+	+/-						
CD3	+, cytoplasmic	-/+[i)]	+			+	+, cytoplasmic	+
CD2	NF[j)]	+/-					+	
CD43	NF	+/-						
CD25	+	+	+	+/-		+		
MHC-II	+		+			+		
Perforin/GranzymeB	+	+	+	-			+/-	+
ALK	NF	+/-		-				
EMA	NF	+/-						
Viral etiology	+, MDV	-	-	+, HTLV-1	-	-	+, EBV	-

a) Anaplasmic large cell lymphoma.
b) Primary cutaneous anaplasmic large cell lymphoma.
c) Adult T-cell leukemia/lymphoma.
d) Peripheral T cell lymphoma, unspecified.
e) Lymphomatoid papulosis.
f) Extranodal NK/T cell lymphoma, nasal type.
g) Enteropathy-type T cell lymphoma.
h) +/- = Majority positive.
i) -/+ = Minority positive.
j) NF = Not found.

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tions [9]. More interestingly, integrin signaling alone or integrin signaling through the ERK/MAPK or JNK/SAPK interaction with AP-1 [55-63] contributes to tumor metastasis [52, 64]. Meq heterodimerizes with c-jun to form an AP-1 transcription factor [65] and thus Meq may contribute to metastasis in addition to transformation. Other signaling pathways in our model include: insulin-like growth factor-1 signaling, essential in metastasis [66, 67], chemokine signaling implicated in angiogenesis and tumor-cell invasion [68], FGF signaling promoting angiogenesis and metastasis [69], P13K/AKT signaling important for cancer cell survival, invasion, and angiogenesis [70], and VEGF signaling implicated in the endothelial cell-specific factor signaling pathway required for pathological angiogenesis, including tumor neovascularization [71].

We have proposed that MD is as a unique natural animal model for $CD30^{hi}$ lymphomas [3] and our large proteomics dataset allows to compare an MDCC with human $CD30^{hi}$ T cell lymphomas in more detail. The REAL/WHO system of lymphoma classification is limited to a set of phenotypes defined only by CD45, CD4, CD2, CD3, CD25, CD43, MHC-II, perforin/granzyme B, EMA, ALK and viral etiology [72] (Table 4). UA-01 shares phenotypic features with anaplastic large cell lymphoma, primary cutaneous anaplastic large cell lymphoma, adult T-cell leukemia/lymphoma, peripheral T-cell lymphoma, unspecified, lymphomatoid populosis, extranodal NK/T-cell lymphoma, nasal type and enteropathy-type T-cell lymphoma. We suggest that MD is not a model for a single human lymphoma but rather it is a general model for $CD30^{hi}$ T-cell lymphomas.

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