Journal of Pharmacogenomics & Pharmacoproteomics

Yu et al., J Pharmacogenomics Pharmacoproteomics 2018, 9:2
DOI: 10.4172/2153-0645.1000181

Research Article Open Access

Gene Expression and Proteomic Profiling of Lp (a)-Induced Signalling Pathways in Human Aortic Valve Interstitial Cells

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Received date: June 2, 2018; **Accepted date:** June 22, 2018; **Published date:** 30 June, 2018

Abstract

Aortic valve stenosis is one of the most common valve diseases in the world for which there is currently no pharmacological treatment to prevent or halt disease progression. Recent genetic research has demonstrated a causal association between elevated blood levels of lipoprotein (a) (Lp (a)) and aortic valve calcification, however, the mechanisms by which Lp (a) contributes to aortic valve calcification and stenosis, is unknown. In the present study, we aimed at determining Lp (a)-induced changes in human aortic valve interstitial cells using an integrated bioinformatics approach. The Lp (a)-induced cellular pathways were analysed using microarray gene expression and proteomic data from non-stenotic human aortic valve interstitial cells. Lp (a) treatment induced osteogenic differentiation, extracellular remodeling, extracellular vesicles biogenesis, and apoptosis of human aortic valve interstitial cells. In particular, the Wnt/ β-catenin signalling pathway, a known calcification pathway contributing to aortic valve stenosis, was differentially expressed compared to non-treated cells. Lp (a) also induced the expression of 14-3-3 proteins known to regulate various signalling pathway relevant to aortic valve disease. Elucidating the mechanisms and molecular players that Lp (a) induces in the early stages of the disease to initiate aortic valve calcification could provide insight into potential pharmacological targets for the treatment of this debilitating disease.

Keywords: Aortic valve stenosis; Gene expression; Bioinformatics; Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Introduction

Aortic valve stenosis is one of the most common valve diseases in the Western world [1,2]. Narrowing of the aortic valve occurs when the valve leaflets become thickened, due to fibrosis and/or calcification, resulting in reduced leaflet mobility. As a result, blood flow is restricted which put more strain on the heart to pump adequate amounts of blood systemically. This eventually leads to heart failure. Aortic valve stenosis is a potentially fatal condition and is becoming an increasing public health burden [2]. Currently there are no medical therapies available that are able to prevent or slow disease progression. The only available treatment option is aortic valve replacement, for which not every patient is a viable candidate. Hence, there is an unmet need for pharmacological treatments that can target various aspects of disease progression [3].

Aortic valve stenosis was previously thought to be a passive degeneration due to mechanical stress from wear-and-tear. However, it has become apparent that specific active processes are also involved, and these have the potential to be targeted by medical therapeutics [4]. Aortic valve stenosis progression can be divided into two phases: (1) an initiation phase and (2) a propagation phase [5]. The initiation phase is caused by endothelial injury leading to inflammation and lipid deposition [3]. This process is similar to atherosclerosis and involves many of the same key molecular players and events [6]. Lipoprotein (a) (Lp (a)) was recently identified as an independent and causal risk factor for developing aortic valve stenosis [7]. Lp (a) is a complex human plasma lipoprotein with an undefined physiological role [8]. It consists of a low-density lipoprotein (LDL)-like particle to which plasminogen-like hepatic apolipoprotein (a) is covalently linked through a disulfide bond with apolipoprotein B-100 [7,9]. Thanassoulis et al. identified a SNP in LPA locus, the gene encoding for apolipoprotein(a), to be associated with aortic valve stenosis through elevated plasma Lp (a) levels across multiple ethnic groups. This suggested a causal relationship, and that perhaps lowering plasma Lp (a) levels may be of clinical importance. Additionally, Lp (a) is the preferential carrier of pro-inflammatory oxidized phospholipids (OxPL) [10]. Lipid deposition and oxidative stress have previously been shown to induce osteoblastic differentiation of aortic valve cells [11]. Accordingly, inhibiting this process with statins was proposed as a potential treatment option to slow or halt disease progression [12-16]. However, this treatment was unsuccessful because the patients that were targeted were already in the propagation stage of aortic valve stenosis, which is dominated by a positive feedback loop characterized by calcium deposition, further injury, and apoptosis [17,18]. This stage of aortic valve stenosis parallels processes of skeletal bone formation [19].

Despite the evidence that Lp (a) is a genetically determined causal risk for aortic valve stenosis, the exact mechanism that links Lp (a) to the progression of aortic valve stenosis is unknown. It is also uncertain whether lowering levels of plasma Lp (a) will attenuate the progression of valvular calcification, leading to aortic valve stenosis. Research has demonstrated that OxPLs carried by Lp (a) to the site of tissue injury is the cause of inflammation [20]. However, a recent study by Langsted et al. [21] showed that Lp (a) was not causally associated with low-grade inflammation, which suggests that Lp (a) likely works through a different mechanism to induce valvular calcification. Additionally, we

have recently demonstrated that Lp (a) induces osteogenic differentiation of non-stenotic human aortic valve interstitial cells (HAVICs), suggesting that Lp (a) is a potential link to trigger later stages of the disease [22].

In the present study, we investigated the gene expression and proteomic profile of HAVICs treated with Lp (a) in order to gain insight into the Lp (a)-induced mechanisms that promote progression of aortic valve stenosis, and could be potential therapeutic targets. We hypothesized that Lp (a) will be involved in inducing extracellular remodeling, calcification, and apoptotic pathways. We show that Lp (a) may be involved in initiating pathways that induce osteogenic differentiation, perhaps via the Wnt signalling cascade.

Materials and Methods

Isolation and culture of human aortic valve interstitial cells (HAVICs)

Primary human HAVICs cell lines were generated from surgically removed fresh human aortic valve leaflets, and cultured in DMEM high glucose medium containing 10% FBS and 1x streptomycin/penicillin solution (ThermoFisher Scientific) as previously described [22,23]. Briefly, aortic valve leaflets were washed with 1x HBSS buffer (ThermoFisher Scientific), cut into small pieces and incubated in DMEM media with Collagenase Type II (Sigma, 100 U/ml) for 3 hours at 37°C in water bath with occasional vortex. The fully digested mixture was centrifuged at 500 g at 4°C for 10 minutes; the supernatant was transferred into new 50 ml tube and centrifuged at 1000 g at 4°C for 10 minutes. Cell pellets were suspended in complete DMEM, and seeded in 75 cm^2 culture flasks. HAVICs cells at passages 3 to 5 were used for all experiments. In addition, HAVICs were cultured in osteogenic medium (OSM: full DMEM medium plus 2 mM buffered phosphate, pH 7.4, final phosphate concentration ~2.9 mM). HAVICs were treated with an unoxidized, purified and unoxidized human Lp (a) containing no oxidized phospholipids at 50 ug/ml for 3 days, 10 days and 20 days with fresh medium change every 2-3 days.

Total RNA extraction and gene expression microarray analysis

Total RNAs were extracted using TRIzol (ThermoFisher Scientific) reagent combined with RNeasy kit (Qiagen), and gene expression microarray analysis were performed on HumanHT-12_V4 Expression BeadChip targeting more than 47,000 probes at the service of Genome Quebec Innovation Center (McGill University, Montreal, Canada). Gene expression data were analysed using FlexArray1.6.3 developed by McGill University and Genome Quebec Innovation Centre, and differentially expressed genes (DEGs) were generated using filter setting with Fold change (FC)>1.25 or <0.8, and P<0.05.

Proteomic profiling

Total proteins were extracted from HAVICs incubated with Lp (a) for 10 days. Total proteins were precipitated with isopropanol, washed with 0.3 M guanidine hydrochloride in 95% ethanol, and 100% ethanol. Purified proteins were kept in 100% ethanol, and used for LS-MS analysis. Briefly, protein samples were diluted in 500 µl of 50 mM Tris pH 8.0, 0.75 M urea and digested over night with 0.5 µg of Trypsin/LysC. Samples were purified by reversed phase SPE and processed by LC-MS. Acquisition was performed with a ABSciex TripleTOF 5600 (ABSciex, Foster City, CA, USA) equipped with an electrospray interface with a 25 µm iD capillary and coupled to an Eksigent µUHPLC (Eksigent, Redwood City, CA, USA). Analyst TF 1.6 software was used to control the instrument and for data processing and acquisition. The source voltage was set to 5.2 kV and maintained at 225°C, curtain gas was set at 27 psi, gas one at 12 psi and gas two at 10 psi. Acquisition was performed in IDA mode in which a 250 ms TOF MS survey scan was used to trigger 40x 35 ms MS/MS scan using dynamic exclusion of 35 sec. Separation was performed on a reversed phase HALO C18-ES column 0.3 µm i.d., 2.7 µm particles, 150 mm long (Advance Materials Technology, Wilmington, DE) which was maintained at 50°C. Samples were injected by loop overfilling into a 5 µL loop. For the 60 min LC gradient, the mobile phase consisted of the following solvent A (0.2% v/v formic acid and 3% DMOS v/v in water) and solvent B (0.2% v/v formic acid and 3% DMSO in EtOH) at a flow rate of 3 µL/min. The gradient was the following 0-36 min 2% B to 30% B, 36-46 min 30% B to 55% B, 46-50 min 55% B to 95%, hold 95% B from 50-55 min and followed by a 1.5 min post-flush at 3 uL/min at final condition. Peptide quantification was performed with Peakview 1.2 (ABSciex) using the area under the curve, protein identification was based on MASCOT search engine using UniProtKB/Swiss-Prot database.

Bioinformatics analysis

DEGs and differentially expressed proteins (DEPs) were grouped based on experiment time points, including the following samples: microarray mRNA profiling at Day 3 in DMEM and OSM (n=4), Day 10 in OSM and Day 20 in OSM (n=2); proteomic profiling at Day 10 in OSM (n=2). List of DEGs and DEPs were subjected to multiple bioinformatics analysis platforms for functional attribution and pathway network generation, the following platforms were used: g:profiler, FunRich [24]: Functional Enrichment analysis tool with Vesiclepedia incorporated.

Ethics statement

This study was carried out in accordance with the recommendations of the McGill University Health Centre Ethics Committee, and approved by Research Ethics Board Office (REB13-025-GEN). All participants were given written informed consent.

Results

Lp (a)-induced changes in expression profiles and enrichment of molecular function

In order to study the profile of DEGs, we exposed the non-stenotic HAVICs to purified human Lp (a) in either non-osteogenic or pro-osteogenic conditions, and isolated total mRNA at 3 time points: day 3, day 10, and day 20. Increased number and fold changes of DEGs were observed following prolonged Lp (a) treatment. Indeed, there were 28, 330 and 2996 DEGs at days 3, 10, and 20, respectively. Day 20 showed the largest number and fold change of DEGs, the majority of which were upregulated, and there was no common DEG found between the three time points (Figure 1A and 1B). At day 3, 216 DEGs were identified in OSM treated cells compared to DMEM medium, Lp (a) treated cells showed 28 and 91 DEGs in OSM or DMEM conditions respectively, and there are only two common DEGs (RASD1 and LOC645738) between Lp (a) induced DEGs in OSM and DMEM (Figure 1C).

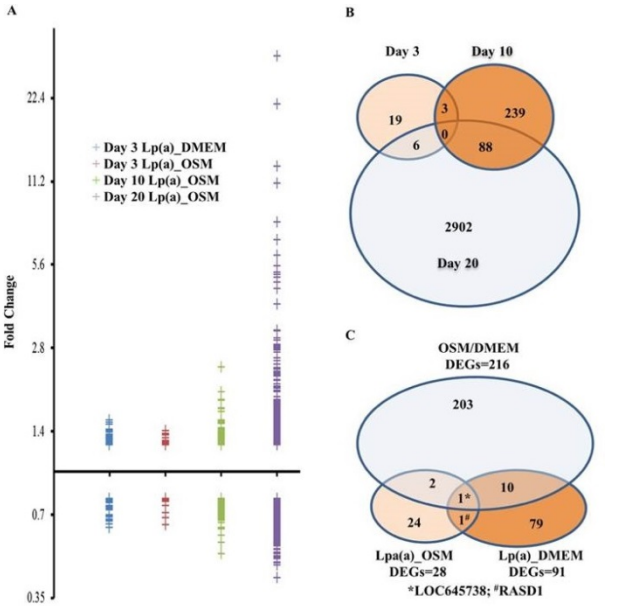


Figure 1: Prolonged Lp (a) treatment showed increasing effects on HAVICs gene expression.

To elucidate the differences in protein expression induced by Lp (a), we analyzed total proteins from day 10 Lp (a)-treated non-stenotic HAVICs in pro-osteogenic conditions, compared to non-treated HAVICs. We identified 508 proteins in total from Lp (a) treated HAVICs and control HAVICs. A total of 147 proteins were exclusively identified in Lp (a)-treated HAVICs; with only 23 proteins were exclusively identified in untreated HAVICs. Among the 338 commonly identified proteins both in the Lp (a)-treated and control HAVICs, we identified 54 upregulated (fold change>1.5) and 43 downregulated (fold change>0.5) proteins in the Lp (a)-treated HAVICs. A total of 311 mapped proteins identified were found in the Vesiclepedia database (Figure 2A).

We hypothesized that genes enriched in the presence of Lp (a) treatment would be involved in structural remodeling, calcification and apoptotic pathways. We therefore profiled the of top enriched genes, based on gene ontology (GO) terms to uncover the molecular functions that the highest percentage of genes were involved in at day 10 and 20 of Lp (a) treatment. We observed high similarities of molecular function in the gene expression data at day 10 and day 20 (Table 1). In addition, several microRNAs like miR-130a, miR-154, miR-216A and miR-302B were enriched in D20 (Table 2).

Days	Biological process	p-value	No. of gene enriched	GO:term ID
Day10	Adherens junction	0.0318	30	GO: 0005912
	Apoptotic process	0.000706	87	GO: 0006915
	Extracellular exosome	6.15E-11	141	GO: 0070062
	Extracellular matrix organization	0.0104	28	GO: 0030198
	Extracellular vesicle	8.95E-11	141	GO: 1903561
	Membrane-bounded organelle	6.41E-06	385	GO: 0043227
	Response to cytokine	0.00724	46	GO: 0034097
	Response to endoplasmic reticulum stress	0.0157	21	GO: 0034976
	RNA binding	0.0166	73	GO: 0003723
	Cellular response to interferon-gamma	0.0183	40	GO: 0071346
Day20	RNA binding	0.00258	291	GO: 0032559
	Cytokine-mediated signalling pathway	6.24E-06	139	GO: 0019221
	Apoptotic process	0.00123	346	GO: 0006915
	Cell surface receptor signalling pathway	7.46E-07	515	GO: 0007166
	Extracellular matrix organization	1.80E-06	1884	GO: 0043227
	Cytokine-mediated signalling pathway	0.0397	40	GO: 0001959
	Response to stress	0.00201	692	GO: 0006950

Table 1: Top enriched molecules functions based on GO terms at day 10 gene expression data.

Target ID	Definition	Fold change
miRLET7F1	microRNA let-7f-1 (miRLET7F1), microRNA.	0.73
miR759	microRNA 759 (miR759), microRNA.	0.77
miR648	microRNA 648 (miR648), microRNA.	0.79
miR642	microRNA 642 (miR642), microRNA.	1.25
miR638	microRNA 638 (miR638), microRNA.	0.8
miR586	microRNA 586 (miR586), microRNA.	1.28
miR582	microRNA 582 (miR582), microRNA.	0.71
miR581	microRNA 581 (miR581), microRNA.	0.8
miR548M	microRNA 548m (miR548M), microRNA.	1.35
miR548A2	microRNA 548a-2 (miR548A2), microRNA.	0.73
miR504	microRNA 504 (miR504), microRNA.	0.78
miR421	microRNA 421 (miR421), microRNA.	0.8
miR363	microRNA 363 (miR363), microRNA.	0.8
miR339	microRNA 339 (miR339), microRNA.	1.4

miR302F	microRNA 302f (miR302F), microRNA.	0.75
miR302B	microRNA 302b (miR302B), microRNA.	0.68
miR216A	microRNA 216a (miR216A), microRNA.	0.8
miR1978	microRNA 1978 (miR1978), microRNA.	1.27
miR1976	microRNA 1976 (miR1976), microRNA.	0.7
miR194-2	microRNA 194-2 (miR194-2), microRNA.	1.29
miR192	microRNA 192 (miR192), microRNA.	1.3
miR1826	microRNA 1826 (miR1826), microRNA.	0.78
miR15B	microRNA 15b (miR15B), microRNA.	1.28
miR154	microRNA 154 (miR154), microRNA.	1.28
miR1321	microRNA 1321 (miR1321), microRNA.	1.29
miR130A	microRNA 130a (miR130A), microRNA.	1.27
miR1290	microRNA 1290 (miR1290), microRNA.	1.34
miR1288	microRNA 1288 (miR1288), microRNA.	0.75
miR1276	microRNA 1276 (miR1276), microRNA.	0.79
miR1253	microRNA 1253 (miR1253), microRNA.	0.79
miR1252	microRNA 1252 (miR1252), microRNA.	0.8
miR1245	microRNA 1245 (miR1245), microRNA.	1.34
miR1228	microRNA 1228 (miR1228), microRNA.	1.25

Table 2: List of differentially expressed microRNAs after 20 days of Lp (a) treatment.

There were only 27 common genes/proteins observed between day10's DEGs and DEPs (Figure 2B), which showed the discordance between RNA *vs* protein in this homologous sample. Since proteomic data more accurately represent the changes upon Lp (a) treatment, we focused our bioinformatics analysis more on DEPs. Our proteomic analysis revealed high enrichment of proteins involved in calcium binding, phosphate metabolism, RNA-binding (Tables 3 and 4) and vesicle biogenesis (Yu et al., In press). With regards to RNA-binding proteins, which are also known to be implicated in calcification pathways, 38 were found to be upregulated and 31 were downregulated. Most of the identified DEPs were also mapped in the Vesiclepedia database (Figure 2A).

Gene ID	Description	Fold change
AARS	Alanyl-tRNA synthetase	UP
ATIC	5-aminoimidazole-4-carboxamide-ribonucleotide formyltransferase	UP
CLIC1	Chloride intracellular channel 1	UP
DDX17	DEAD (Asp-Glu-Ala-Asp) box helicase 17	UP
DDX39B	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B	UP
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked	UP
EEF1B2	Eukaryotic translation elongation factor 1 beta 2	UP
EIF2S3L	Eukaryotic translation initiation factor 2 subunit 3-like protein	UP
EIF5A	Eukaryotic translation initiation factor 5A	1.94
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	1.84
HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	UP
HNRNPH2	Heterogeneous nuclear ribonucleoprotein H2 (H')	2.23
HNRNPM	heterogeneous nuclear ribonucleoprotein M	UP
NARS	Asparaginyl-tRNA synthetase	UP
NONO	Non-POU domain containing, octamer-binding	2.66
RNH1	Ribonuclease/angiogenin inhibitor 1	1.71
RPL10	Ribosomal protein L10	1.42
RPL11	Ribosomal protein L11	2.29
RPL15	Ribosomal protein L15	3.05
RPL19	Ribosomal protein L19	1.44
RPL23	Ribosomal protein L23	UP
RPL24	Ribosomal protein L24	1.38
RPL5	Ribosomal protein L5	2.24
RPL7	Ribosomal protein L7	1.39
RPL9	Ribosomal protein L9	UP
RPS12	Ribosomal protein S12	1.98
RPS13	Ribosomal protein S13	UP
RPS14	Ribosomal protein S14	1.4
RPS19	Ribosomal protein S19	3.38
RPS25	Ribosomal protein S25	1.33
RPS27A	Ribosomal protein S27a	1.77
RPS3A	Ribosomal protein S3A	2.55
RPS5	Ribosomal protein S5	UP
RPS6	Ribosomal protein S6	UP
RPS8	Ribosomal protein S8	UP
SERBP1	SERPINE1 mRNA binding protein 1	UP
SFPQ	splicing factor proline/glutamine-rich	UP
TAF15	TAF15 RNA polymerase II	UP

Table 3: Enrichment of up regulated RNA-binding proteins identified at Day 10 proteomic data.

Gene ID	Description	Fold change
CRIP2	Cysteine-rich protein 2	0.3
EEF1D	Eukaryotic translation elongation factor 1 delta	0.62

EIF4A2	Eukaryotic translation initiation factor 4A2	DOWN
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	0.62
HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	0.23
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	0.57
RPL10A	Ribosomal protein L10a	DOWN
RPL12	Ribosomal protein L12	DOWN
RPL17	Ribosomal protein L17	0.27
RPL18	Ribosomal protein L18	0.65
RPL21	Ribosomal protein L21	0.5
RPL23A	Ribosomal protein L23a	0.29
RPL29	Ribosomal protein L29	0.4
RPL3	Ribosomal protein L3	0.69
RPL34	Ribosomal protein L34	0.27
RPL35	Ribosomal protein L35	0.3
RPL35A	Ribosomal protein L35a	0.24
RPL36AL	Ribosomal protein L36a-like	0.38
RPL4	Ribosomal protein L4	0.62
RPL8	Ribosomal protein L8	0.61
RPLP0	Ribosomal protein, large, P0	0.39
RPS10P5	Ribosomal protein S10 pseudogene 5	0.52
RPS15	Ribosomal protein S15	DOWN
RPS18	Ribosomal protein S18	0.61
RPS20	Ribosomal protein S20	0.25
RPS21	Ribosomal protein S21	0.28
RPS26P11	Ribosomal protein S26 pseudogene 11	0.45
RPS29	Ribosomal protein S29	DOWN
RPS7	Ribosomal protein S7	DOWN
RRBP1	Ribosome binding protein 1	0.51
TUFM	Tu translation elongation factor, mitochondrial	0.37

Table 4: Enrichment of down regulated RNA-binding proteins identified at Day 10 proteomic data.

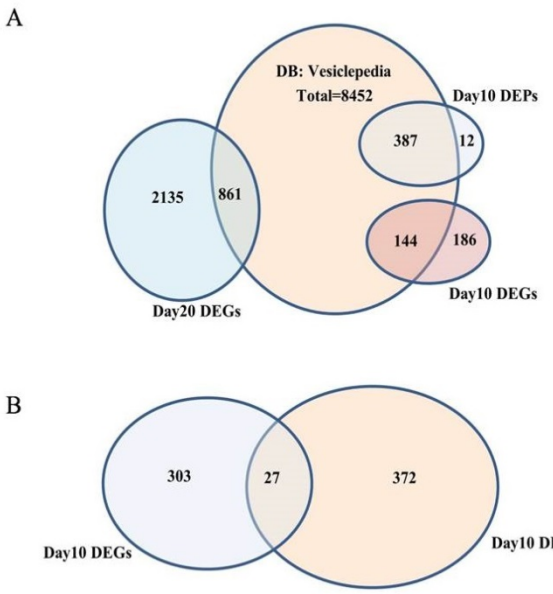


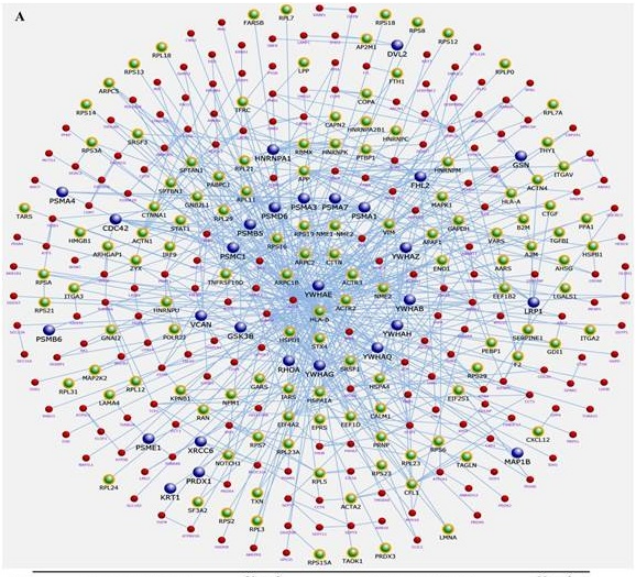
Figure 2: Number of DEGs and DEPs in HAVICs treated with Lp (a).

Biological pathways induced in response to Lp (a) treatment

Our gene expression and proteomic profiles provide global views of major functional changes in Lp (a)-induced HAVICs. However, we wanted to offer more biological insight by also profiling biological pathways in response to Lp (a). We analyzed the gene/proteomic expression profiles at days 10 and 20 of Lp (a) treatment to elucidate the top enriched biological pathways. Although there is no dominant pathway identified, we found similar biological pathway enrichment among gene/proteomic data sets, including integrin family, glypican, TRAIL, VEGF signalling, EGF receptor, mTOR, E-cadherin, TGF-beta, and WNT signalling (Figures 3 and 4). Many of the top similar pathways were mapped, including extracellular remodeling, calcification, and apoptotic pathways.

WNT signalling pathways induced by Lp (a) treatment

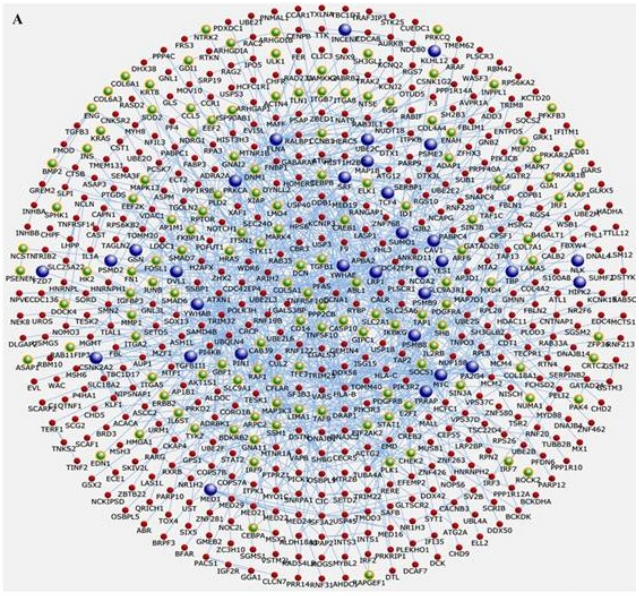
Our finding that members of the Wnt signalling pathway were differentially expressed is of particular interest, as the Wnt pathway is known to be implicated in aortic valve calcification [3,23]. Many DEGs were found to be involved in the Wnt signalling network at day 10 (Figure 3) and day 20 (Figure 4) following Lp (a)-treated HAVICs. At day 10, Wnt signalling genes, like 14-3-3 proteins (YWHAQ, YWHAE, YWHAB, YWHAG, YWHAZ, and YWHAH), GSK3B, DVL2, VCAN and RHOA were identified and many more were found at day 20, like BMP2, DVL1, FZD7, ITGA2, LRP1, MARK1, MSX2, MYC, NOTCH1, SUMO1, TGFB1, TGFB1I1, TAGLN2, YWHAB and YWHAE, etc.



B

Biological pathway	No. of genes	Biological pathway	No. of genes
AP-1 transcription factor network	45	IFN-gamma pathway	97
Arf6 signaling events	96	Integrin family cell surface interactions	103
BMP receptor signaling	18	mTOR signaling pathway	96
CDC42 signaling events	54	p38 MAPK signaling pathway	16
Class I PI3K signaling events	96	PDGF receptor signaling network	96
E-cadherin signaling events	29	Proteoglycan syndecan-mediated signaling	99
EGFR-dependent Endothelin signaling	96	TGF-beta receptor signaling	21
Endothelins	96	TRAIL signaling pathway	101
ErbB receptor signaling network	97	VEGF and VEGFR signaling network	97
Glypican pathway	98	Wnt signaling network	19

Figure 3: Interaction network and top biological pathways mapping of Day 10 DEGs and DEPs.



B

Biological pathway	No. of genes	Biological pathway	No. of genes
AP-1 transcription factor network	75	Integrin family cell surface interactions	182
Arf6 signaling events	165	mTOR signaling pathway	165
BMP receptor signaling	33	p38 MAPK signaling pathway	26
Class I PI3K signaling events	165	PDGF receptor signaling network	166
EGFR-dependent Endothelin signaling	165	Proteoglycan syndecan-mediated signaling	177
Endothelins	167	TGF-beta receptor signaling	38
ErbB receptor signaling network	166	TRAIL signaling pathway	171
Glypican pathway	173	VEGF and VEGFR signaling network	171
IFN-gamma pathway	166	Wnt signaling network	32

Figure 4: Interaction network and top biological pathways mapping of Day 20 DEGs.

The 14-3-3 protein family

The 14-3-3 protein family has seven members (β, γ, ε, σ, ζ, τ, η), 14-3-3 proteins are a family of conserved regulatory molecules that are expressed in all eukaryotic cells. 14-3-3 proteins have the ability to bind many functionally diverse signalling proteins, such as kinases, phosphatases, and trans membrane receptors (Mackintosh 2004). A total of 6 out of 7 14-3-3 family proteins were found differentially expressed in day 10 proteomic data, and these were YWHAZ, YWHAQ, YQHAH, YWHAG, YWHAE, and YWHAB (Table 5). These 14-3-3 proteins were found at the center of mapped biological pathways including WNTs signalling pathway identified (Figures 3 and 4), and further analysis revealed that the same 14-3-3 proteins were also involved in most of the top signalling pathways identified among DEGs (Figure 5 and Table 6).

Name	Description	Fold Change
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5 monooxygenase activation protein, zeta	1.68
YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta	0.65
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta	0.63
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma	0.62
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon	0.76
YWHAB	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta	0.54

Table 5: Protein expression fold changes of 14-3-3 proteins at day 10 Lp (a)-treated proteomic data.

Biological pathway	No. of genes
Beta1 integrin cell surface interactions	77
Integrin family cell surface interactions	77
TRAIL signalling pathway	76

Proteoglycan syndecan-mediated signalling events	75
ErbB receptor signalling network	74
IFN-gamma pathway	74
IGF1 pathway	74
VEGF and VEGFR signalling network	74
Alpha9 beta1 integrin signalling events	73
Arf6 signalling events	73
Class I PI3K signalling events	73
EGFR-dependent Endothelin signalling events	73
Endothelins	73
Glypican pathway	73
GMCSF-mediated signalling events	73
IL3-mediated signalling events	73
IL5-mediated signalling events	73
LKB1 signalling events	73
mTOR signalling pathway	73
Nectin adhesion pathway	73
PAR1-mediated thrombin signalling events	73
PDGF receptor signalling network	73
PDGFR-beta signalling pathway	73
Signalling events mediated by focal adhesion kinase	73
Thrombin/protease-activated receptor (PAR) pathway	73
Metabolism of RNA	48
CDC42 signalling events	42
Integrin-linked kinase signalling	39
AP-1 transcription factor network	36
Wnt signalling network	32
E-cadherin signalling in the nascent adherens junction	22
N-cadherin signalling events	19
ALK1 pathway	17
Regulation of cytoplasmic and nuclear SMAD2/3 signalling	17
TGF-beta receptor signalling	17
TNF alpha/NF-kB	15
BMP receptor signalling	14

Table 6: List of enriched biological pathways sharing 14-3-3 proteins.

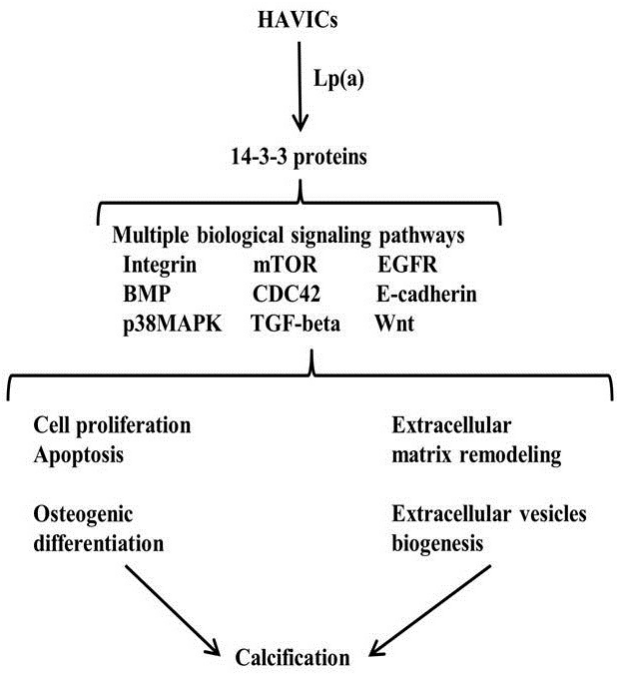


Figure 5: Graphic illustration of biological mechanism of Lp (a) induced calcification on HAVICs.

Discussion

By using an integrated bioinformatics approach to analyse and interpret proteomic and gene expression data, we demonstrate that non-stenotic HAVICs treated with purified Lp (a) show differences in gene and protein expression when compared to untreated non-stenotic HAVICs. These results further confirm previous studies done in our lab which demonstrated for the first time that Lp (a), caused remodeling and calcification of normal HAVICs, in a manner resembling the phenotype seen in calcified human aortic valves [22]. Together, our data provides further insight into the currently unknown mechanisms by which Lp (a) promotes progression of aortic valve stenosis.

Gene expression profiling revealed DEGs over the course of 20 days of Lp (a) treatment, with the greatest differences in gene expression occurring at day 20. This suggests that Lp (a) contributes to an alteration in gene expression in HAVICs, alluding to the potentially deleterious effects caused by elevated circulating plasma Lp (a) levels. Furthermore, there were no genes that were shared at the three time points used in this study, demonstrating the presence of a mechanistic progression induced by Lp (a), potentially towards development of an osteoblastic phenotype. In particular, at day 3, RASD1 was commonly differentially expressed between Lp (a)-treated and control non-stenotic HAVICs in both pro-osteogenic and non-osteogenic conditions, suggesting that Lp (a) was responsible for the difference in expression of this gene. RASD1 is a member of the Ras superfamily of GTPases, which can be induced by dexamethasone [25]. It acts as an activator of G-protein signalling and participates directly as a nucleotide exchange factor for Gi to Go. Previous research has identified a variant in RASD1 loci as one of the shared genetic

susceptibilities for ischemic stroke and coronary artery disease (CAD). It was also shared between large artery disease (atherosclerosis) and CAD, making it a useful clinical marker [25]. RASD1 exerts its action by modulating multiple signalling cascades, and its upregulation in breast [26] and prostate [27] cancer led to increased apoptosis. Moreover, RASD1 expression negatively correlated with cardiac hormone atrial natriuretic factor (ANF) secretion in volume overloaded atria, an effect of stress-induced hypertrophy in CAD, thus contributing to disruption of cardiac hormone homeostasis [28]. Lp (a) is also elevated in CAD, and aortic valve stenosis is known to have many mechanistic similarities with CAD and atherosclerosis. Thus, Lp (a) may play a role in abnormal RASD1 expression in these disease states.

The most enriched genes at day 10 and 20, specifically extracellular matrix genes, vesicles, and microRNAs, were involved in structural remodeling. Analysis of the top enriched biological pathways at these time points further confirm that extracellular remodelling and calcification pathways were upregulated, in addition to apoptotic pathways at later time points. Extracellular remodelling is a major downstream effect of cells undergoing osteogenic differentiation that induce valve interstitial cells, myofibroblasts, to develop an osteoblast-like phenotype [29]. This process is mediated particularly by TGF-β receptor signalling, in addition to inflammatory cytokines, TNF receptor signalling, and insulin-like growth factor, all of which were observed to be enriched at both of these time points [30-32]. Matrix vesicles contribute to calcification by carrying and depositing calcium crystals [33]. MicroRNAs are roughly 22 nucleotides long, single-stranded molecules that function to regulate gene expression by binding to mRNA, usually in the 3'UTR, to decrease mRNA translation and stability [34], microRNAs have also been found to play an important role in cardiovascular diseases [35-39]. We have found high enrichment of microRNAs after 20 days of Lp (a) treatment in HAVICs. Among those identified were miR-130a and miR-15B, which are known to regulate Wnt signalling [40,41] and are identified as biomarker of atherosclerosis obliterans [42]. miR-154 was found to be a biomarker for CAD [43]; and miR216A has roles in osteogenesis and cholesterol efflux via PI3K/AKT pathway [44,45]. Some of the microRNAs identified are also implicated in the pro-fibrotic TGF-β signalling pathway and found to be involved in fibrosis by targeting extracellular matrix structural proteins and enzymes involved in cellular remodeling. In aortic valves, miRNAs were implicated in the osteoblastic transition to induce myofibroblast proliferation and resistance to apoptosis [46,47].

Extracellular vesicles calcification has been emerging as the major cause of isotopic calcification including aortic valve calcification. Extracellular vesicles contain high levels of calcium and calcium binding proteins. In our present study, we found enrichment of genes/proteins involved in calcium binding, phosphate metabolism, vesicle biogenesis upon Lp (a) treatment, including Fetuin-A/ASHG), Annexin (A1/2/4/5/6/11), Integrin (α2/3/v and β), Calpain1/2/S1, Calponin2/3, and S100 calcium binding proteins (A10/11/16/4/6). Extracellular vesicles may also mediate calcification in diseased heart valves as they are generally loaded with microRNAs that target osteogenic differentiation. Our results suggest that Lp (a) may be involved in induction of early signalling events that leads to extracellular structural remodeling and calcium deposition via extracellular vesicle formation as shown in our recently published study [22].

Analysis of proteomic data at day 10 for enriched molecular function and top biological pathways was consistent with the profile of DEGs, as many of the pathways were also involved in the structural remodeling and calcification pathways mentioned above. Furthermore, calcium-binding related proteins were one of the groups of abundantly identified proteins. Specifically, RCN1, RCN3, CALU, CALR, and CALM1 are members of the EF-hand calcium binding protein family, and they are involved in calcium binding and storage in the endoplasmic reticulum. Proteins in this family have been implicated in several processes, some of which include bone mineralization and cell signalling. Identification of calcium-binding proteins in Lp (a) treated HAVICs further supports the involvement of Lp (a) in inducing calcification processes, as valve calcification mimics skeletal bone formation and shares many of the same key molecular players [48]. At the same token, phosphate-related proteins are important in many signalling cascades, like Wnt signalling, further confirming the prevalence of Lp (a)-induced signalling events. RNA-binding proteins, among the most abundant proteins identified, are involved in the induction of collagen synthesis and development of cardiac fibrosis, through the TGF-β pathway whose involvement in aortic valve calcification is well established [49]. Other features of aortic valve calcification are angiogenesis and apoptosis. We observed enrichment of VEGF and VEGF receptor signalling pathways at the gene expression and protein level in day 10 Lp (a) treated HAVICs [19]. Moreover, gene expression and proteomics data showed prevalence of apoptotic pathways, which was expected based on the known progression of aortic valve stenosis to allow for more calcium deposition.

Interestingly, day 10 and 20 Lp (a) treated HAVICs in pro-osteogenic conditions showed differential expression of genes involved in the Wnt signalling network. In accordance, proteomic analysis at day 10 also identified many proteins involved in Wnt signalling. It has been previously described that Wnt/ β-catenin signalling is one of the calcific pathways involved in the later propagation stages of aortic valve stenosis, which is characterized by a positive feedback loop of calcium depositions and tissue injury. Wnt ligand binding to frizzled and LDL receptor-related protein 5 receptors activate Wnt/ β-catenin and calcium pathways which are implicated in osteogenic differentiation. TGF- β 1 can also stimulate osteogenic differentiation of mesenchymal progenitor cells by inducing nuclear translocation of β-catenin, and this process is upregulated in response to mechanical stress [50,51]. In support of the above findings, we have recently shown that Wnt ligands are upregulated in the later stages of aortic valve stenosis [23]. Moreover, HAVICs treated with non-canonical Wnt5a, Wnt5b, and Wnt11 resulted in significant calcification that was similar to the crystallinity seen in calcified human aortic valves [23,52]. The results of our present study could give insight into the potential link between the presence of Lp (a) throughout life and during the initiation phase to trigger progression into the symptomatic later phase of the aortic valve stenosis.

Human 14-3-3 proteins are a family of conserved regulatory molecules that are expressed in all eukaryotic cells. 14-3-3 proteins have the ability to bind to a multitude of functionally diverse signalling proteins, including kinases, phosphatases, and transmembrane receptors. 14-3-3 proteins interact with a wide spectrum of proteins including kinases, phosphatases, transmembrane receptors, transcription factors, biosynthetic enzymes, cytoskeletal proteins, signalling molecules, apoptosis factors, and tumor suppressors. Its regulatory role has been implicated in various signalling pathways including Wnt signalling, RTK/Ras signalling, canonical Hippo

signalling, TGF/SMAD signalling, PI3K/PDK/AKT, integrin and Ca2+/calmodulin-dependent protein kinase (CaMK) pathway, cell cycle regulation and actin signalling [53-61]. 14-3-3 proteins have emerged as new drug discovery targets [62-64]. Inorganic phosphate and other phosphate-containing molecules can serve as regulators of 14-3-3/phosphate interactions. Inorganic phosphates induce dissociation of complexes formed by phosphorylated HspB6 and 14-3-3γ or 14-3-3ζ, [65]. 14-3-3 expression was observed in the valvular spongiosa in degenerated aortic disease and aorta specimens from patients with large vessel vasculitides [59,66]. Of interest, 14-3-3 proteins have been shown to bind directly to β-catenin [67,68], and both molecules were found to be co-expressed in extracellular vesicles and were thought to activate Wnt signalling [54]. Indeed, 14-3-3 proteins were shown to interact with canonical Wnt signalling by binding to disheveled-2 (dsh-2) and GSK-3β [54], and disrupts β-catenin binding to the β-catenin degradation complex, resulting in increased level of Wnt signalling [69]. Our present study demonstrated enrichment of molecules involved in extracellular matrix and vesicle biogenesis, phosphate/calcium binding, and our previous data showed that HAVICs produce endogenous Apo (a) and Lp (a) treated HAVICs undergo apoptosis and extracellular vesicle biogenesis [22]. These findings allude to a potential mechanism by which Lp (a) exert its' multi-signalling pathways effects through 14-3-3 proteins in valvular cells.

Although the present study provides evidence for Lp (a)-induced aortic valve stenosis, it does not take into account aortic valve stenosis onset in other clinical contexts. As we have shown, Lp (a) produces a cascade of events involving multiple biological pathways, leading to a calcified phenotype in HAVICs. However, it is likely that aortic valve stenosis in other clinical contexts are caused by multiple risk factors and mechanisms, some of which may be congenital while others may be more clinical such as male sex, smoking, high LDL and metabolic syndrome [70].

Overall, although many of the mechanistic processes that occur during osteogenic differentiation and calcification are known, including involvement of BMP-2, Wnt signalling, calcium phosphate deposition, and subsequent apoptosis, it is unclear what induces their activation. The differential expression and presence of many of these known molecules and mechanisms after Lp (a) treatment provides evidence that the mechanism of Lp (a) action may be the link to induce aortic valve calcification.

Conclusions

In this study, we demonstrate the effect of Lp (a) on normal aortic valve interstitial cells using gene expression microarray profiling and proteomic analysis. Lp (a) has been causally associated with aortic valve stenosis, however, the mechanism by which Lp (a) exerts its effect to promote disease progression is unknown. Our study suggests that Lp (a) may exert its effects on cell proliferation/apoptosis, extracellular matrix remodeling, extracellular vesicles biogenesis and osteogenic differentiation by regulating multiple 14-3-3 proteins regulated signalling pathways. Our results provide further insight into the aortic valve stenosis disease progression, and new potential targets for drug therapy.

Supplementary materials

Gene expression data: GEO accession number GSE101155. The following are available online at www.mdpi.com/link, Supplemental Table 1: List of DEGs (Day 3, 10 and 20) used for bioinformatics analysis. Supplemental Table 2: List of DEPs (Day 10) used for bioinformatics analysis. Supplemental Table 3: Original normalized proteomic data (Day 10) used for bioinformatics analysis.

Acknowledgments

This work was supported by the Canadian Institutes of Health Research, and Natural Sciences and Engineering Research Council of Canada.

Author Contributions

BY performed experiments, data analysis and contributed to manuscript writing. HK analysed the data and drafted the manuscript; KK, GT, RC, BV, JG contributed to tissue collection and revised the manuscript. AS designed the experiments, reviewed, analysed and interpreted the data, and finalized the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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