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Significant pathways response to osmotic stimuli in the Intervertebral disc

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Abstract. – BACKGROUND: The intervertebral disc (IVD) is a special tissue localized in the middle of vertebrae and it often experiences osmotic environment to accommodate applied mechanical forces.

AIM: Here we aim to confirm significant pathways associated with IVD osmotic regulation.

MATERIALS AND METHODS: Two different pathway analyses which are named component-based approach and protein-protein interaction (PPI)-based approach are used to identify the significant pathways from the related databases.

RESULTS: The results showed that neuroactive ligand-receptor interaction pathway was specifically identified based on traditional significant pathway analysis. New significant pathway analysis indicated that ErbB signaling pathway, nucleotide excision repair, progesterone-mediated oocyte maturation pathway, and alpha-Linolenic acid metabolism pathway, etc. were all associated with osmotic regulation. Importantly, progesterone-mediated oocyte maturation pathway was only significant in the hyper-osmotic dataset, however, alpha-Linolenic acid metabolism pathway in the hypo-osmotic dataset.

CONCLUSIONS: Our results suggest that IVD accommodation mechanism is important in response to osmotic stimuli.

Key Words:
Intervertebral disc, Significant pathway enrichment, Neuroactive ligand-receptor interaction.

Introduction

The intervertebral disc (IVD) is an avascular and alymphatic tissue and comprises three morphologically distinct zones, which are nucleus pulposus (NP), annulus fibrosus (AF), and transition zone (TZ)[1]. IVD cell can secrete a complex extracellular matrix that contains the highly sulfated proteoglycan, through its interaction with cations, providing the tissue with its unique osmotic properties[2].

One of the primary responses of disc cells to variations in local osmolarity is a change in regulatory cell volume[3]. Disc cells adapt to these osmotic shifts by remodeling their cytoskeletons and catalyzing the transport of osmotically active molecules and water through the cell membrane[4]. For example, the expressions of aggrecan and type II collagen were demonstrated up-regulated in TZ under hypo-osmotic conditions. Gene expressions of small proteoglycans, biglycan, and decorin were up-regulated in TZ cells following incubation in either hypo- or hyper-osmotic media. But the same genes were down-regulated in NP cells under either hypo- or hyper-osmotic conditions[5]. Identically, human cells which were cultured over 5 days would have an increased expression of aggrecan and collagen II in both NP and AF cells under increasing osmolarity condition. In contrast, collagen I expression was decreased at high osmolarity in both cell types[6].

Further analysis indicates that cells respond to osmotic stress with altered biosynthesis through a pathway that may involve calcium (Ca^{2+}) as a second messenger. IVD cells respond to hyper-osmotic and hypo-osmotic stress by increasing the concentration of intracellular calcium (Ca^{2+}) through a mechanism involving F-actin[7,8]. In addition, there were also some reports indicating hypertonic saline injection[9] or iontophoresis combined with Long's bone-setting manipulation[10] could effectively treat IVD protrusion.

Boyd et al[11] also identified many different expressed genes in response to increased osmolarity by microarray analysis. Our results suggest that we combined the traditional methods and the PPI-based approach[12] to further explore the more comprehensive pathways for the IVD in the hyper and hypo-osmotic stimuli.

Materials and Methods

Data Sources

We downloaded all the pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG)[13]

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and protein-protein interaction (PPI) datasets from human protein reference database (HPRD)[14] and biological general repository for interaction datasets (BIOGRID)[15] database.

Then an ensemble protein-protein interaction network was constructed by integrating two above existing PPI databases in human. A total of 326,119 unique PPI pairs were collected in which 39,240 pairs are from HPRD and 379,426 pairs are from BIOGRID.

We generated the IVD gene expression profile on hyper-osmotic, hypo-osmotic and iso-osmotic stimuli of GSE1648.

The limma method[16] was used to identify differentially expressed genes (DEGs). The original expression datasets from all conditions were processed into expression estimates using the robust multiarray averaging (RMA) method with the default settings implemented in Bioconductor, and then the linear model was constructed. The DEGs were chosen if the value has more than 2 folds change and *p*-value is less than 0.05.

Traditional Significant Pathway Analysis

The pathway annotations were based on KEGG annotations. The *p*-value for the pathway enrichment was calculated based on a hypergeometric distribution.

We used the DAVID[12] (the database for annotation, visualization and integrated discovery) bioinformatics resources for the pathway enrichment analysis. Here we reported the significant pathway enrichments for groups which contained at least two members and *p*-value was less than 0.1.

New Significant Pathways Analysis Based on PPI Datasets

Firstly, to determine the co-expressed significance of a gene pair in disease cases, we used the Pearson correlated coefficient (PCC) test to calculate the *p*-value.

Then we mapped those *p*-values to the nodes and edges in the PPI network collected from the HPRD[14] and BIOGRID[15] database. The following formula is used to define the function combined with statistical significance by a scoring scheme. The detail description could be found in Liu et al[17].

$$S(e) = f\,[\mathrm{diff}\,(x), \mathrm{corr}\,(x, y), \mathrm{diff}\,(y)]$$

$$= -2 \sum_{i=1}^{k} \log_e (p_i)$$

The diff(x) and diff(y) are differential expression assessments of gene x and gene y, respectively. Corr (x, y) represents their correlation between gene x and gene y. f is a general data integration method that can handle multiple data sources differing in statistical power. Where k = 3, p1 and p2 are the *p*-values of differential expression of two nodes, p3 is the *p*-value of their co-expression.

$$Sp = \sum_{e \in P} s(e)$$

Then, to estimate the significance of the pathways, we randomly sampled 10^6 times of the same size pathways in the edges of pathway network and calculated their overlapping scores. The frequency of scores that are larger than Sp was used as the significant *p*-value of pathway P to describe its importance.

Results

For the GSE1648 dataset of IVD cells treated with 293 mOsm/kg H_2O, a total of 723 genes were detected as DEGs, in which 484 were in hypo and 304 were in hyper, using the limma method.

Traditional Significant Pathway Analysis

The DAVID with the DEGs was used to find the significant pathways. At last, two datasets of hypo and hyper datasets' significant pathways were detected. Only one pathway neuroactive ligand-receptor interaction (hsa04080) was enriched in the two sets with the $p = 0.06$ in hyper and $p = 0.0009$ in hypo. The total result of $p < 0.1$ list is in the Table I.

New Significant Pathway Analysis Based on PPI Datasets

The pathway neuroactive ligand-receptor interaction (hsa04080) was not significant in our new significant analysis because the *p*-value is more than 0.1. We also used the standard deviation of the population (Sp) to evaluate the importance of pathways. Total 16 pathways were detected with the $p < 0.05$ and node comprising of at least 2 members.

We found total 18 significant pathways in hyper-osmotic stimuli or hypo-osmotic stimuli. Progesterone-mediated oocyte maturation (hsa04914) was significant in the hyper-osmotic

Table I. Significant pathway analysis using the DAVID.

Data	Term	*p*-value	FDR
hyper	hsa00982: Drug metabolism	0.006803	7.71972
hyper	hsa04730: Long-term depression	0.010621	11.80897
hyper	hsa00980: Metabolism of xenobiotics by cytochrome P450	0.028712	29.02668
hyper	hsa05414: Dilated cardiomyopathy	0.032816	32.47639
hyper	hsa00040: Pentose and glucuronate interconversions	0.046891	43.1767
hyper	hsa04080: Neuroactive ligand-receptor interaction	0.060174	51.82856
hyper	hsa00910: Nitrogen metabolism	0.072857	58.94721
hyper	hsa05410: Hypertrophic cardiomyopathy (HCM)	0.082985	63.92561
hyper	hsa00830: Retinol metabolism	0.087169	65.81583
hyper	hsa04350: TGF-beta signaling pathway	0.088646	66.46095
hypo	hsa04080: Neuroactive ligand-receptor interaction	9.45E-04	1.087845
hypo	hsa04640: Hematopoietic cell lineage	0.005131	5.775059
hypo	hsa05332: Graft-versus-host disease	0.006842	7.631889
hypo	hsa05320: Autoimmune thyroid disease	0.020702	21.48712
hypo	hsa04940: Type 1 diabetes mellitus	0.041015	38.38715
hypo	hsa05222: Small cell lung cancer	0.046725	42.49898
hypo	hsa05200: Pathways in cancer	0.049288	44.26165
hypo	hsa04650: Natural killer cell mediated cytotoxicity	0.055683	48.4466
hypo	hsa04060: Cytokine-cytokine receptor interaction	0.0651	54.08822

Data mean the datasets is from hyper-osmotic stimuli or hypo-osmotic stimuli. FDR: Flight Data Recorder Analysis. DAVID: the database for annotation, visualization and integrated discovery.

dataset but not in the hypo-osmotic. The pathway alpha-linolenic acid metabolism (hsa00592) was significant in the hypo-osmotic dataset but not in the hyper-osmotic dataset (Table II).

Discussion

Using these two analysis methods, we identified some significant pathways associated with osmotic regulation. Of them, neuroactive ligand-receptor interaction pathway was specifically identified based on component-based approaches, which only consider differential genes. However, PPI-based approach analysis indicated that ErbB signaling pathway, nucleotide excision repair, progesterone-mediated oocyte maturation pathway, and alpha-linolenic acid metabolism pathway, etc were all associated with osmotic regulation. In addition, progesterone-mediated oocyte maturation pathway was only significant in the hyper-osmotic dataset, but alpha-linolenic

Table II. Significant pathway of hyper and hypo.

Path	Description	Size	Node	Edge	Hyper-score
hsa04012	ErbB signaling pathway	87	9	23	2183357
hsa04060	Cytokine-cytokine receptor interaction	265	103	430	5670118
hsa04062	Chemokine signaling pathway	189	118	800	8056954
hsa04140	Regulation of autophagy	35	14	88	6549952
hsa04330	Notch signaling pathway	47	32	715	10027686
hsa05322	Systemic lupus erythematosus	142	19	147	4477446
hsa03420	Nucleotide excision repair	44	31	227	3590759
hsa00534	Glycosaminoglycan biosynthesis – heparan sulfate	26	4	12	773239.4
hsa04310	Wnt signaling pathway	151	46	121	1936303
hsa04920	Adipocytokine signaling pathway	67	8	26	728663.4
hsa04623	Cytosolic DNA-sensing pathway	56	3	6	252394.5
hsa04621	NOD-like receptor signaling pathway	62	38	291	2188181
hsa00460	Cyanoamino acid metabolism	7	2	6	116999.3
hsa03030	DNA replication	36	2	7	4416.183
hsa04914	Progesterone-mediated oocyte maturation	87	3	3	23.87489
hsa00592	alpha-linolenic acid metabolism	19	2	3	8.762666

Table III. The last 2 rows red marked indicate the different pathways in two datasets.

Path	Description	Hyper-*p* value	Hypo score	Hypo-*p* value
hsa04012	ErbB signaling pathway	0	2183321	0
hsa04060	Cytokine-cytokine receptor interaction	0	5670002	0
hsa04062	Chemokine signaling pathway	0	8056997	0
hsa04140	Regulation of autophagy	0	6549977	0
hsa04330	Notch signaling pathway	0	10027605	0
hsa05322	Systemic lupus erythematosus	0	4477340	0
hsa03420	Nucleotide excision repair	0.0001	3590658	2.00E-05
hsa00534	Glycosaminoglycan biosynthesis - heparan sulfate	0.00071	773242.3	0.00075
hsa04310	Wnt signaling pathway	0.0028	1936261	0.00294
hsa04920	Adipocytokine signaling pathway	0.01153	728683.5	0.01086
hsa04623	Cytosolic DNA-sensing pathway	0.01482	252387.4	0.0145
hsa04621	NOD-like receptor signaling pathway	0.01554	2187850	0.01543
hsa00460	Cyanoamino acid metabolism	0.01478	117022.9	0.01547
hsa03030	DNA replication	0.04287	4391.62	0.04292
hsa04914	Progesterone-mediated oocyte maturation	0.02973	15.19603	0.10441
hsa00592	alpha-linolenic acid metabolism	0.49682	19.19977	0.04823

acid metabolism pathway in the hypo-osmotic dataset. There were few interactions between two pathway methods and, therefore, comprehensive analysis may be effective for signaling pathway identification.

There is evident that epidermal growth factor receptor (EGFR:ErbB) signaling pathway has a crucial function to contribute to the regulation of osmo-adaptive responses. Cell swelling (hyposmolarity, isosmotic urea, hyperosmotic sorbitol) or shrinkage (hyperosmotic NaCl or raffinose) in NP cell induces matrix metallo protease (MMP)-dependent activation of EGFR, likely via shedding of transformin growth factor-α (TGF-α), and downstream activation of Ras and the mitogen-activated protein (MAP) kinases p38 and extracellular-signal-regulated kinases (ERK)1/2, which further stimulate TonEBP transactivation activity[18,19]. Activated TonEBP binds to the tonicity responsive enhancer element (TonE) of various osmoprotective genes, such as aldose reductase, taurine transporter (TauT) and betaine/γ-aminobutyric acid(GABA) transporter[20,21], and then these genes expression may result in taurine-glycine receptor and GABA-GABAR interaction, namely, neuroactive ligand-receptor interaction.

IVD is a special tissue that permits motion between vertebrae. Therefore, NP cells secrete matrix macromolecules, which serve to accommodate applied mechanical forces by elevating the osmotic pressure. However, the hyperosmotic status and avascular supply lead to anaerobic metabolism in NP cells and then decreased pH[22]. In order to adapt to acidotic and hyperosmotic microenvironment, NP cell may regulate transmembrane Ca^{2+} flux to elevate pH as progesterone-mediated oocyte maturation process, in which Ca^{2+}-activated chloride channel ClC-0 can mediate pH changes[23].

In human, alpha-linoleic acid (ALA) could be converse into longer-chain polyunsaturated fatty acids (PUFAs) through de-saturation and elongation pathway, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are important for optimal tissue function[24]. Some studies have indicated that hypotonic swelling could activate ALA metabolism related enzyme(s) to produce PUFAs and then open transient receptor potential vanilloid 4 (TRPV4) in mammalian cells[25]. TRPV4 is a Ca^{2+}-permeable cation channel within the vanilloid receptor subgroup of the transient receptor potential (TRP) family[26], and it also has been implicated in Ca^{2+}-dependent signal transduction in hypo-osmotic IVD[7].

In addition, high osmolality strongly inhibits NP cells' proliferation by activating the G2 and G1 cell cycle checkpoints[27] and provoking DNA damage in the form of double strand breaks in NP cells. Such damage could be removed by nucleotide excision repair[28]. Within a hyperosmotic environment, NP cells preserve their ability to sense DNA damage and nucleotide excision repair, as confirmed by the reactivation of p53 by ionizing radiation, retain the MRN (Mre11–Rad50–Nbs1) complex in the nucleus, and phosphorylate H2A.X (H2A histone family, member X). DNA repair efficiency of NP cells was found to be significantly increased under hyperosmotic pressure[29].

Conclusions

In this study, a PPI-based approach was used to analyze the significance among IVD related pathways. New significant pathways are found and analyzed using the PPI datasets and expression profiles. The results are inconsistent with our prior knowledge of IVD. The new significant pathways present new alternative insights for IVD pathology. Our work indicates that comprehensive and system-wide analysis provides evidence for IVD and complements the traditional component-based approaches.

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Conflict of Interest

None to declare.

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