MicroRNA expression changes in A549 alveolar epithelial cells after 7 days *in vitro* incubation

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Background and Aims

A549 cell line is a widely used *in vitro* model of human alveolar type II epithelial cells. Its applications include modelling airway function and disease, drug metabolism, pulmonary exposure to toxicants and viruses, and cancer research. Long-term cultures of A549 cells better mimic real human exposure, and understanding the intrinsic cellular changes that occurred during the experimentation can be important to assess research results. For this purpose, microRNA (miRNA) differential expression (DE) profiling allows a global view of the cellular pathways that are modified in response to external signals and over time. We used next-generation sequencing and three bioinformatics tools to identify A549 DE miRNAs after 7 days in culture, and their corresponding up- or down-regulated functional pathways.

Methods

Total RNA was isolated from A549 cells and used to generate indexed cDNA libraries for sequencing in the MiSeq (Illumina). Two sequencing runs were performed and the raw data was processed using the small RNA analysis workflow (Illumina). DE analysis of raw counts was conducted using DESeq2 v1.18.1, baySeq v2.12.0 and RNASeqGUI v1.1.2. The latter was used to perform DE with the EdgeR Exact Test. A false discovery rate (FDR) of 0.05 was set as the cut-off. The DIANA-miRPath v3.0 was used to identify miRNA target genes and regulated KEGG pathways. The complete set of experimentally-validated genes targeted by the DE miRNAs that were common to the 3 tools was used as input for KEGG analysis.

Results

A total of 51 and 50 unique miRNAs were up- and down-regulated (46 and 45 with a FDR < 0.05), respectively, in A549 cells between the 24 h and the 7 days incubation, but their number was depended on the bioinformatics tool. Of these, 24 and 26 miRNAs were commonly found to be up- and down-regulated in the three tools, respectively (Fig. 1). The prediction of the functional pathways enriched by the DE miRNAs resulted in 77 statistically significant (*p*-value < 0.05) KEGG functional pathways. After filtering these pathways for the current cell, tissue and organ type, the remaining 51 functional pathways (Fig. 2) were distributed according to 19 KEGG categories, which are depicted in Fig. 3. The top 10 KEGG predicted pathways are listed in Table 1.

Table 1. Top 10 KEGG functional pathways up- or down-regulated in A549 cells after 7 days.

KEGG PATHWAY	*p*-value	# target genes	# DE miRNAs
Proteoglycans in cancer	5.35208E-17	161	47
Adherens junction	3.67346E-12	66	47
Protein processing in endoplasmic reticulum	7.29602E-10	137	47
Ubiquitin mediated proteolysis	3.62636E-09	113	45
Spliceosome	3.62636E-09	108	47
Pathways in cancer	3.62636E-09	290	47
Hippo signaling pathway	1.61171E-08	114	46
Cell cycle	8.46108E-08	99	45
Viral carcinogenesis	1.3132E-07	148	47
RNA transport	6.57308E-07	132	46

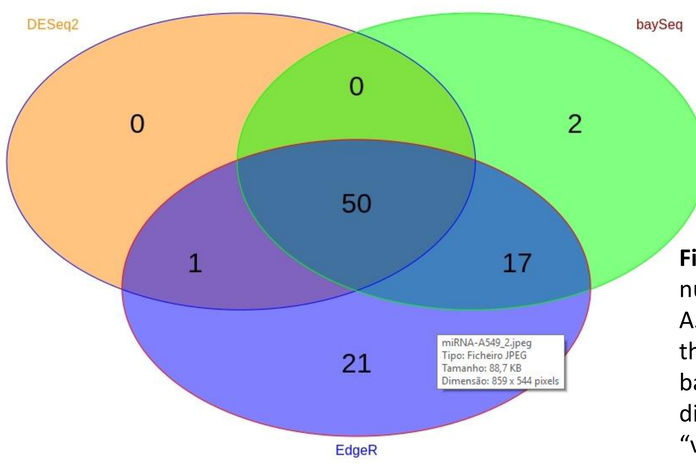

Figure 1. Venn diagram showing the number of differently expressed miRNAs in A549 cells after 7 days in culture, using three different DE tools: DESeq2 (orange), baySeq (green) and EdgeR (blue). The diagram was produced using the R package "venn diagram" according to Chen, 2017[1].

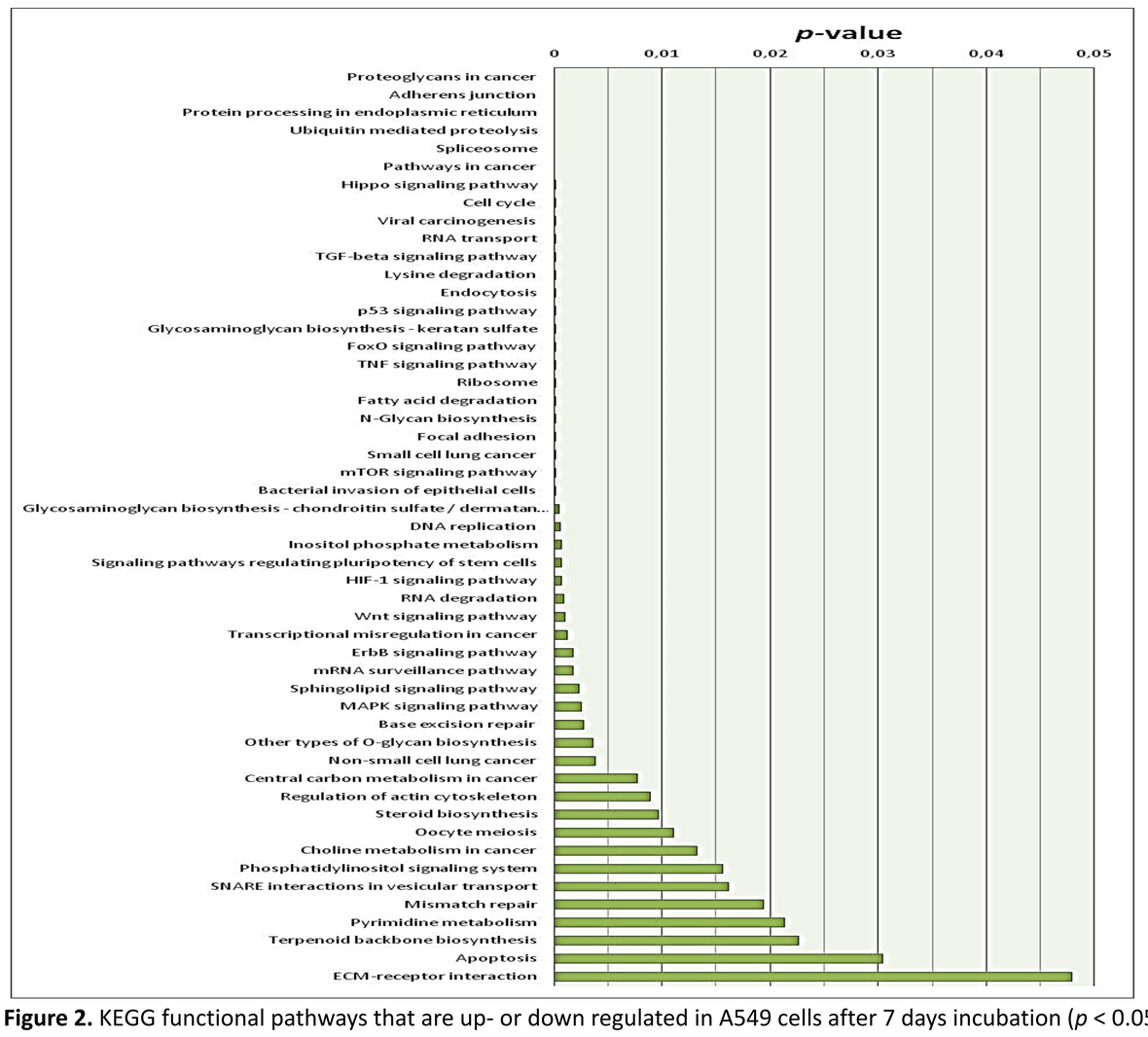

Figure 2. KEGG functional pathways that are up- or down regulated in A549 cells after 7 days incubation ($p < 0.05$).

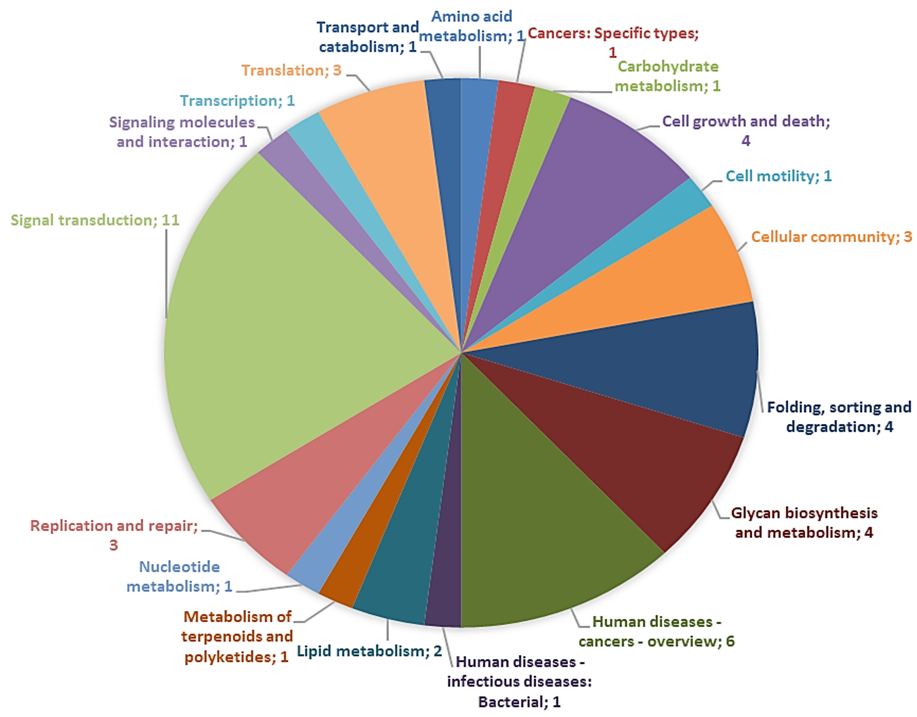

Figure 3. Number of enriched molecular pathways distributed according to their KEGG category

Discussion

In this study, DE miRNA in A549 cells after 7 days in culture showed that:

- Cell cycle is one affected pathway (associated to the altered DNA replication pathway).
- Ten pathways were related to aminoacid, carbohydrate, glycan, lipid, terpenoids and polyketides, and nucleotide metabolism, indicating an overall cellular metabolic adaptation to deprived conditions.
- Pathways related to control of cell growth, proliferation, differentiation and survival (TGF-β, Wnt, mTOR, and FoxO pathways) and related to cellular response to stress (MAPK and HIF-1 pathways) were enriched.
- Deregulation of many of these molecular pathways is related to malignancy and tumorigenesis, e.g., the p53, Wnt or ErB. Accordingly, pathways in cancer is altered in the category Human Diseases, and proteoglycans in cancer is the major enriched pathway.
- The altered Hippo pathway is a complex network that modulate cell proliferation, differentiation, and migration[2]. It is regulated by cell-cell contact, cell polarity and actin cytoskeleton, among other signals[3]. Many upstream regulators for this pathway are components of tight junctions and adherens junctions.
- Another clue that cells are slowing their metabolic activity is the enrichment of pathways linked to DNA transcription and translation, and protein folding, sorting and degradation.
- The affected ubiquitin proteolytic system has influence on the regulation of cell cycle, differentiation and development, response to extracellular effectors and stress, modulation of cell surface receptors and ion channels, DNA repair, regulation of the immune and inflammatory responses and organelle biogenesis[4].

Conclusion

The interconnections observed between the cellular functional pathways that were enriched with the DE miRNAs, providing evidence of a consistent higher order pathway network, indicates that miRNA DE analysis can be a reliable tool for investigating real time cellular molecular and biological events. Our results indicate that, after 7 days in culture, A549 cells are adapting to the external modifications in culture medium and to cell-cell communication through global changes in cell cycle, metabolism and activation or silencing of signalling pathways that are determinant of cell proliferation, differentiation and fate. Many of these functional pathways are biological targets in respiratory and cancer research. Moreover, it is very likely that A549 cells, already with the shortcoming of being a continuous cancer cell line, will be a far distant model of a normal lung epithelium with time, making the extrapolation of *in vitro* results to the *in vivo* situation even more difficult.

Acknowledgments

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1- Chen et al. 2017 VennDiagram: Generate High-Resolution Venn and Euler Plots. R package version 1.6.18. https://CRAN.R-project.org/package=VennDiagram
2- Meng et al., 2016 Genes & Development 30:1-17
3- Zhao et al. 2007 Genes & Development 21: 2747-2761
4- Ciechanover et al. 2000 BioEssays 22(5): 442-451