

Breast Cancer UK

Project title: Evaluating the Endocrine Disruptive Properties of Glyphosate

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Establishment and validation of a cellular model system to measure estrogenic effects

We have established and validated a tissue culture cell model system to measure estrogenic effects using different methods:

- Three different types of human breast cancer cells (MCF7, T47D, MDA-MB-231) are used to determine if pollutants can substitute for the natural hormone (estrogen) in promoting cell growth.

- MCF7 cells are totally dependent on the presence of estrogen for growth; T47D cells are moderately hormone dependent for growth and MDA-MB-231 cells are hormone independent. Thus a combination of assays using these different cell types allows the evaluation of pollutants as to their ability to substitute for estrogen in promoting cell growth.

- A modified version of the T47D cell line (called T47D-Kluc) harbouring a stably integrated copy of a luciferase reporter gene under control of a promoter containing estrogen response elements, is used to see if any estrogenic effects observed act via the estrogen receptor.

These two methods have been validated using a natural hormone (17 β -estradiol) and synthetic chemicals with known estrogenic endocrine disrupting capability (bisphenol A).

Endocrine disruptive effects of glyphosate/Roundup

Three different glyphosate-based herbicide Roundup formulations, pure glyphosate, and one pesticide adjuvant mixture, have been tested in the cell culture systems described above. A tiered approach has been used to assess and validate endocrine disrupting effects of glyphosate/Roundup:

Tier 1: hormone-dependent proliferation

Using an MTT (color change assay based on activity of the enzyme succinate dehydrogenase), subtle cell proliferative effects have been detected with some of the Roundup formulations at non cytotoxic levels in MCF7 cells (Figure 1, upper panel; blue arrow). In addition, we observed a cell proliferative effect with glyphosate alone albeit at a higher concentration to that of Roundup in both MCF7 and to a lesser degree in T47D cells (Figure 1; upper and lower panels; red arrows).

The involvement of adjuvants and glyphosate in the proliferative effects provoked by glyphosate-based herbicide (Roundup) formulations was then measured in the three cell lines (Figure 2). While the proliferative effects provoked by glyphosate were confirmed (Figure 2, upper and middle panels; red arrows), no effects of adjuvants were measured.

Tier 2: Estrogen receptor activation

The investigation of direct estrogen receptor activation using the T47D-Kluc cells indicate that glyphosate alone but not Roundup is able to directly interact with estrogen receptors and stimulate luciferase gene activation suggesting an endocrine disrupting capability for this compound (Figure 3A; red arrows). Direct interaction of glyphosate with the estrogen receptor was confirmed by adding the potent estrogen receptor antagonist (ICI) into the mixture, which effectively blocked the stimulating effects of glyphosate (Figure 3B; red arrows).

Tier 3: Gene expression profiling

A transcriptome (gene expression) profiling has been performed using the estrogen dependent MCF7 cells to test the involvement of other hormonal signaling pathways (including other non-classical estrogenic pathways) and confirm any endocrine disruptive effects.

The transcriptome alterations induced by three concentrations of glyphosate, three concentrations of Roundup, and two concentrations of an adjuvant were compared to the effects of the natural hormone estradiol or to the known endocrine disrupting chemical bisphenol A. A large number of genes had their levels of expression altered by glyphosate, its commercial formulation or its main adjuvant (Figure 4).

Functional interpretation of gene expression pattern alterations are currently underway. A preliminary analysis of alterations in the expression of genes involved in cell cycle progression (Figure 5) confirms the effects of glyphosate (at 10 ppm) and of Roundup (at 100 ppb) at a high level of statistical significance.

The biological annotation "cell cycle" (GO:0007049) represents the progression of biochemical and morphological phases and events that occur in a cell during successive replication cycles or nuclear replication events. It reflects cellular proliferation and thus possible tumor promoting potential.

The remaining analysis to determine linkage of gene expression changes to other cellular functions is in progress.

Summary

Our results to date demonstrate that glyphosate has estrogenic properties as it is able to:

- Stimulate proliferation of estrogen dependent human breast cancer cells, especially MCF7.
- Stimulate expression of a reporter gene under control of estrogen/estrogen receptors, which is blocked by the ICI estrogen antagonist.
- Estrogenic effects are at relatively high concentrations (ppm range) but below cytotoxic levels.
- Downstream effects induced by glyphosate and Roundup are causing disturbances in the levels of a high number of genes.
- Transcriptome profiles are reflective of endocrine disturbances provoked by glyphosate and Roundup.

Expected publication date of results in a peer reviewed journal: autumn 2016.

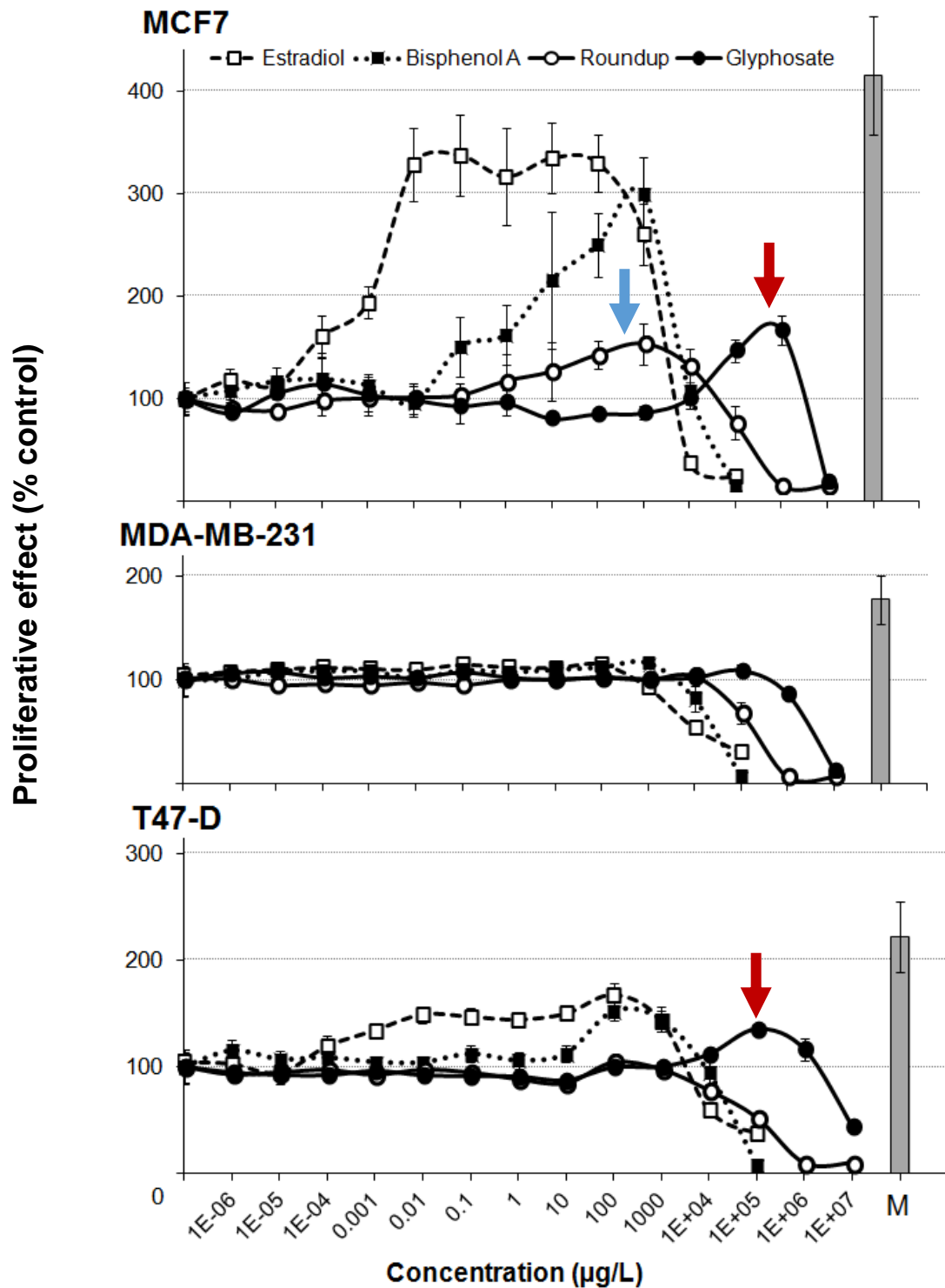


Figure 1. Proliferative effect of glyphosate and Roundup on mammary cells in the E-Screen Bioassay. After 24h of steroid hormone starvation cells were treated for 5 days with the test compounds. Numbers of cells was measured by an MTT color change assay based on the activity of the enzyme succinate dehydrogenase. Results are expressed as proliferative effect percentage relative to the proliferation of cells under hormone-free conditions. Data are the mean +/- SE of three independent experiments, each one performed in triplicate. Note proliferative effect of glyphosate in MCF7 and T47D cells (red arrows) and Roundup in MCF7 cells (blue arrow).

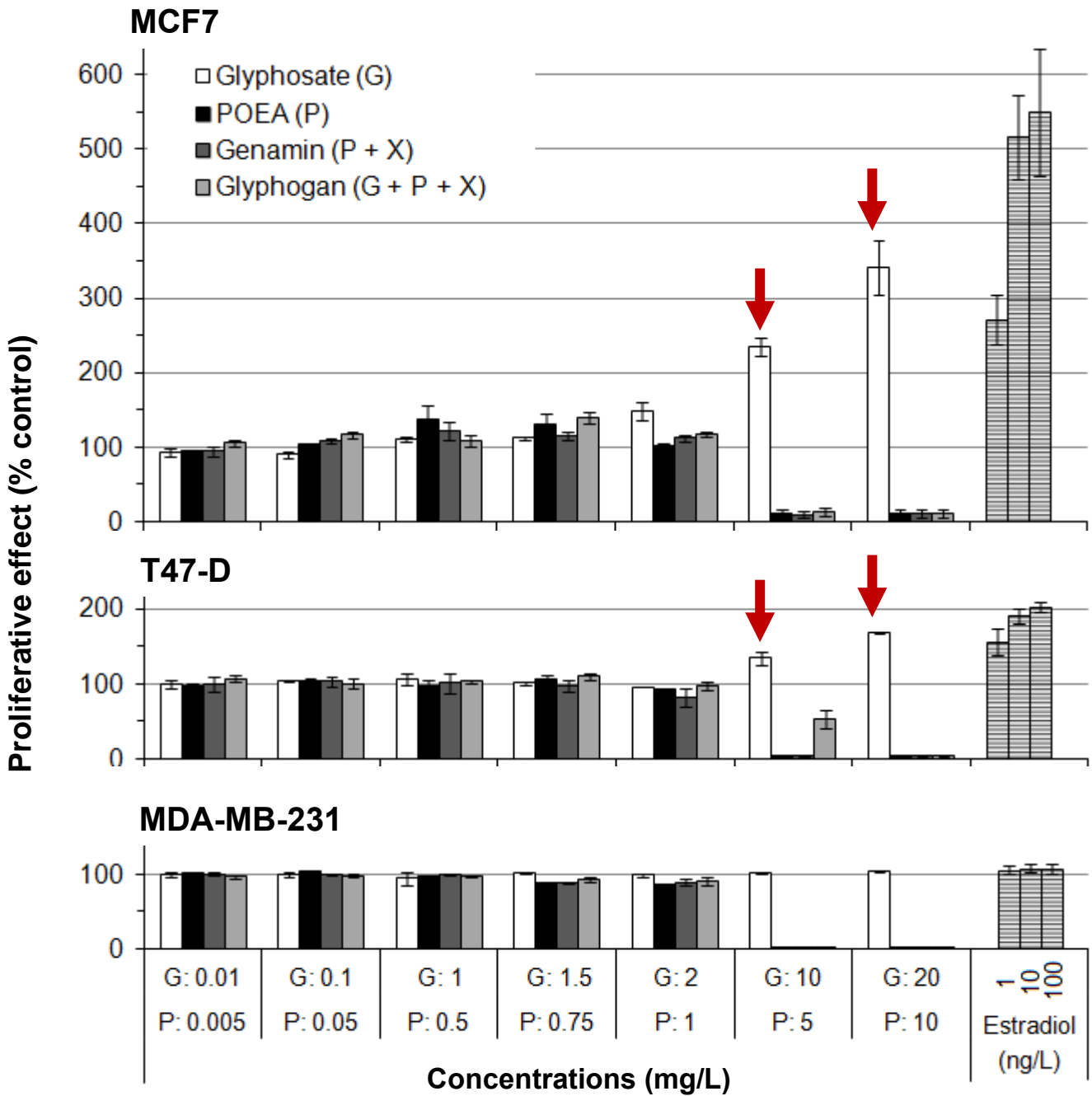


Figure 2. Measurement of proliferative effects of glyphosate-based herbicide adjuvants on mammary cells in the E-Screen Bioassay. After 24h of steroid hormone starvation cells were treated for 5 days with the test compounds. Numbers of cells was measured by an MTT color change assay based on the activity of the enzyme succinate dehydrogenase. Results are expressed as proliferative effect percentage relative to the proliferation of cells under hormone-free conditions. Data are the mean +/- SE of three independent experiments, each one performed in triplicate. Note proliferative effect of glyphosate in MCF7 and T47D cells (red arrows).

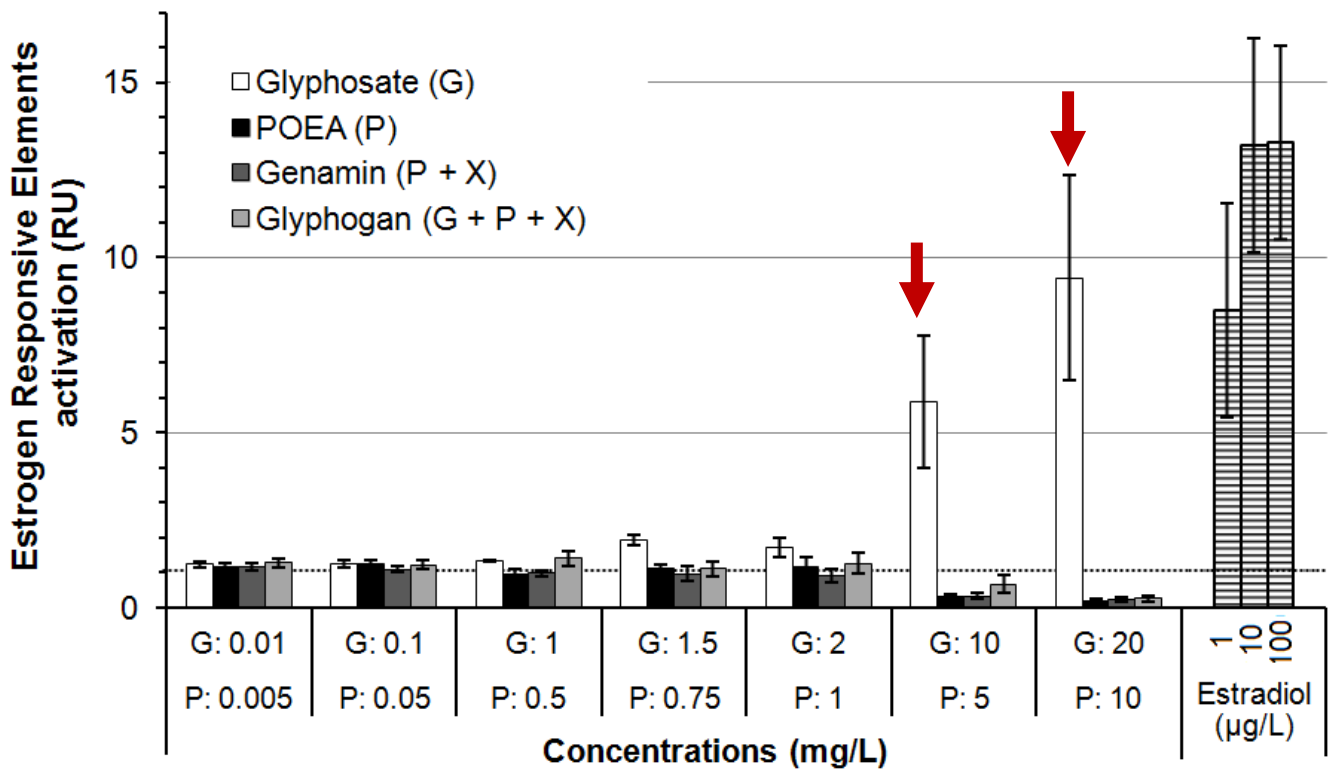
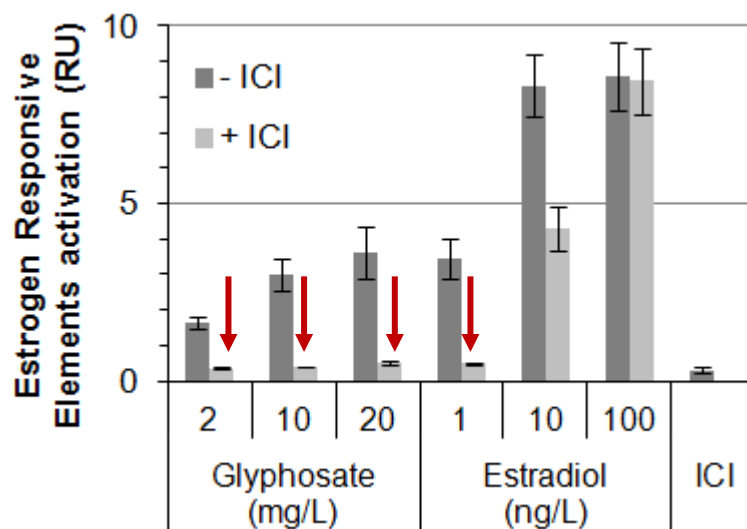
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Figure 3. Glyphosate stimulates luciferase reporter gene expression in T47D-Kluc cells. After 24 hours of steroid hormone starvation cells were treated with the test compounds for an additional 24 hours. Cells were then lysed and subjected to a bioluminescence luciferase reporter gene assay **(A)** Assays of cells treated with glyphosate alone (G), glyphosate formulation (Glyphogan), adjuvant mixture (Genamin) or POEA adjuvant. **(B)** Luciferase assays of T47D-Kluc cells treated with glyphosate in the absence or presence of the ICI estrogen antagonist. Note potent antagonistic effect of the ICI compound on glyphosate stimulation (red arrows).

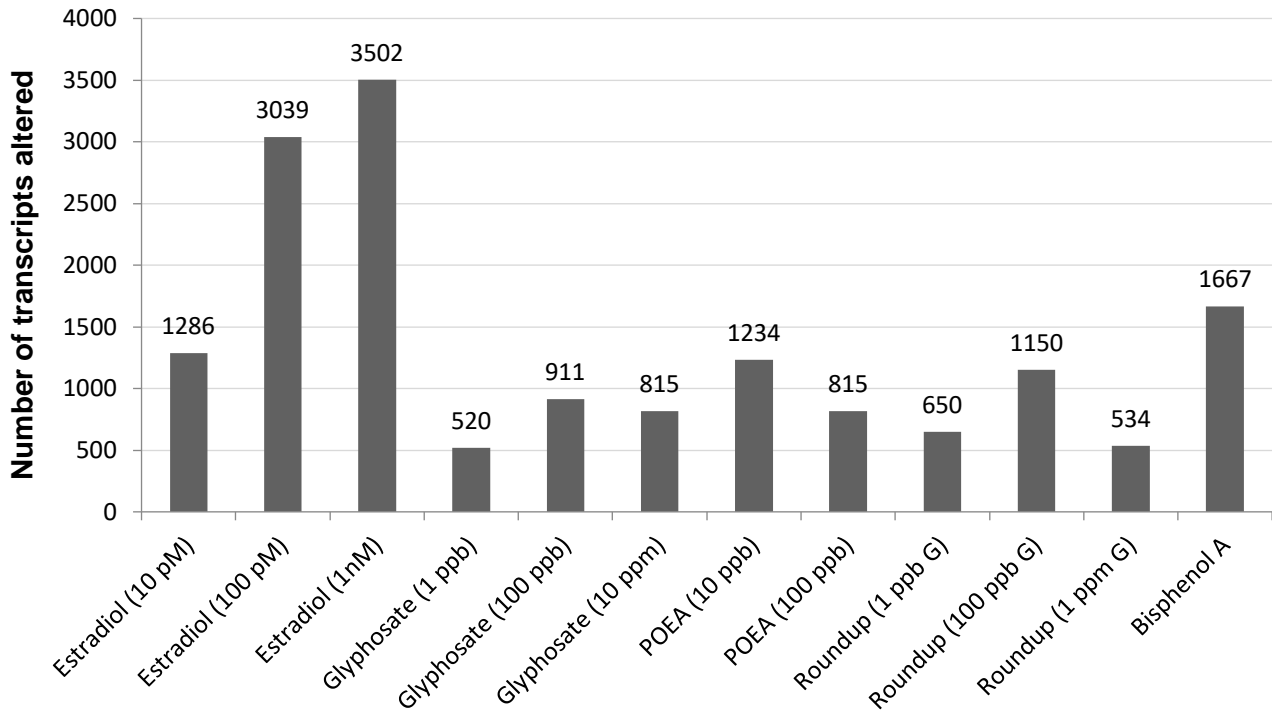


Figure 4. Number of transcripts whose expression is disturbed by the glyphosate/Roundup stimulation in the MCF7 cells. After 24 hours of steroid hormone starvation cells were treated with the test compounds for an additional 48 hours. Cells were then lysed, RNAs were extracted and subjected to a gene expression profiling using the Affymetrix Human Transcriptome Array 2.0. Statistical significance was determined using a two-sample t-test ($p < 0.05$; fold changes > 1.2).

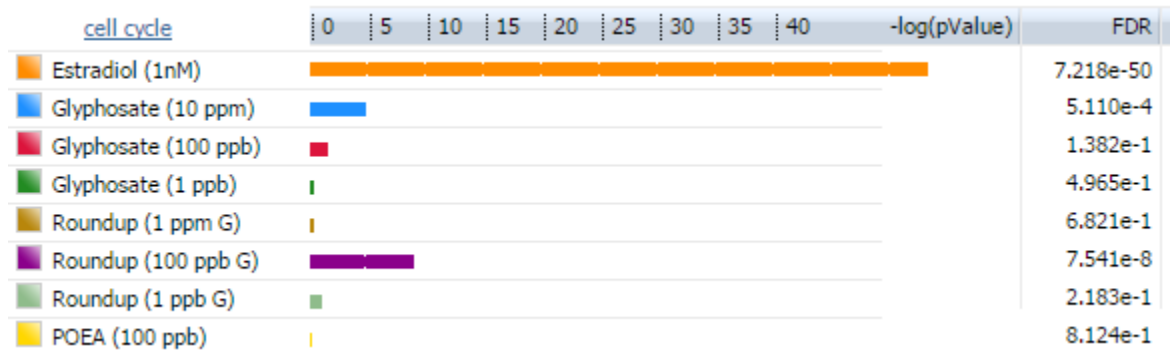


Figure 5. Transcriptome profiles reflecting cell cycle disturbances induced by glyphosate/Roundup in the MCF7 cells. MCF7 cells treated by glyphosate (1 ppb, 100 ppb, 10 ppm), Roundup (1 ppb of glyphosate, 100 ppb of glyphosate, 1 ppm of glyphosate), by the adjuvant POEA or by the positive control estradiol were subjected to a full transcriptome microarray analysis. Enrichment of transcriptome profiles in altered genes involved in cell cycle progression was studied. The p-values are determined by hypergeometric calculation and corrected for multiple comparisons (FDR).