Expression Analysis of Glucocorticoid Hormone During Early Developmental Stage of Zebrafish (*Danio rerio*) its Therapeutic Implications

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Abstract

Glucocorticoid is prescribed for the treatment of many human diseases. The work was performed to investigate the role of glucocorticoid hormone on the gene expression during zebrafish (*Danio rerio*) embryogenesis. Microarray hybridization technique was used to analyse dys-regulation of different gene expression at 12 hours post fertilization (hpf) when single cell fertilized eggs were treated with glucocorticoid hormone. The expression of 149 genes were affected, with 143 up-regulated and 6 down-regulated. The results suggest that glucocorticoid may be also used in therapeutic approach to change genetic expression at early developmental stage.

Key words: Glucocorticoid hormone, Expression, Zebrafish.

Introduction

In fish, like other vertebrates, the cellular responses allied with cortisol signaling are thought to be mediated by a glucocorticoid receptor (GR). Cortisol is also known to play an important role in modulating a vast array of physiological processes, including organ development, protein and fat metabolism, carbohydrate homeostasis, immune response, neural activity, memory, behaviour and bone formation in both unstressed and stressed animals (Mommsen et al., 1999; Barnes, 2006; De Kloet et al., 2005; Wang, 2005; Migliaccio et al., 2007). Since cortisol exerts different effects on the various organs of the body, it is likely that the functional targets of GR are different in each tissue and these tissue-specific mechanisms need to be elucidated in order to understand the functional genomics of the stress response (Phuc Le et al., 2005; Vegiopoulos and Herzig, 2007).

Mammalian studies using microarrays and bioinformatics tools have identified that GR is responsible for gene regulatory networks in the liver of mice treated with dexamethasone. Some genes are directly linked to GR signaling, while other genes are regulated indirectly by interaction of GR with other transcription factors, including estrogen receptor (ER) and CCAAT/enhancer binding protein beta (C/EBPB) (Aluru and Vijayan, 2007). Due to their various actions, glucocorticoids have become fundamental therapeutic agent. As a class of compounds including synthetic analogues, they are among the most prescribed drugs in the world. Clinically, glucocorticoids are widely prescribed in the treatment of chronic autoimmune/inflammatory and allergic diseases, such as asthma, inflammatory bowel disease, rheumatoid arthritis, and skin disorders (Schaff *et al.*, 2009).

Studies on several fish species have shown that maternal steroid hormones may be involved in the early development of the offspring. For example, sex steroid hormones were found in the eggs of coho salmon (*Oncorhynchus kisutch*) (Feist *et al.*, 1990) and testosterone in medaka (*Oryzias latipes*) (Iwamatsu *et al.*, 2006). On the basis of these findings, the present study was undertaken to discover the effect of glucocorticoid hormone on the gene expression of zebrafish at 12 hours post-fertilization (hpf) embryos as well as to establish its therapeutic implications.

Materials and methods

Fertilized zebrafish eggs were pooled and incubated in cortisol treatment solution and control solution for 2 h for development and fixation at 12 hpf. The concentration of cortisol treatment solution was 13.8 μ M while the control solution contained 2 ml abs. ethanol/ 1 L fish water.

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After treatment, the eggs were gently washed at least 5-fold in water to eliminate any trace of cortisol and ethanol. Finally the eggs were frozen in liquid nitrogen at 12 hpf to perform molecular analyses.

RNA extraction: Total RNA extraction of cortisoltreated and control embryos using Trizol reagent was performed according to the manufacturer's instructions (Invitrogen, Milan, Italy). The extracted RNA samples were stored at -80°C until future use.

Extracted RNA samples, obtained from embryos were analyzed by agarose (Fisher Molecular Biology, USA) gel electrophoresis for quality check. The concentration of total RNA was quantified by NanoDrop Spectrophotometer (Celbio, Milan, Italy).

LiCl precipitation: The key requirement for microarray hybridization is highly pure RNA, in which A_{260}/A_{230} must be higher than 2.2 and A_{260}/A_{280} should be higher than 2. Extracted RNA was purified by LiCl precipitation. For this purpose, RNA samples were heated at 40°C for 6 min and centrifuged at 12,000 *g* for 15 min at 4°C. Then, 9.5 µl of 4 M LiCl was added to each tube, which was left overnight at 4°C for precipitation. It was then centrifuged at 12,000 *g* for 20 min at 4°C and 200 µl of 75% EtOH were added to the pellet for washing. The mixture was again centrifuged at 12,000 *g* for 20 min at 4°C and the supernatant discarded. Finally, the RNA was re-quantified by NanoDrop Spectrophotometer.

RNA quality control: After LiCl precipitation, RNA quality assessment and quantification were performed by using Agilent 2100 bio-analyzer and RNA LabChip. The bio-analyzer software automatically generates the ratio of the 18S to 28S ribosomal subunits. This ratio plays an important role in determining the level of sample degradation in gel electrophoresis. The Agilent technologies have also introduced a new tool for RNA quality assessment through RNA Integrity Number (RIN). The RIN software algorithm allows the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact one. Only the samples having RIN value greater than 8 were used to perform microarray hybridization.

Two-color Microarray hybridization: Two-Color Microarray-based gene expression analysis (Agilent Technologies, Santa Clara, CA) was performed for transcript quantification in embryos treated with cortisol at 12 hpf. The analysis was performed at CRIBI, Italy, using Agilent Whole Zebrafish Genome Oligo Microarrays 4x44K slide. The experimental design is shown in Table 1.

Agilent's Two-Color Microarray-based Gene Expression Analysis used cyanine 3- and cyanine 5labeled targets to measure gene expression in control and treated samples.

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The software determines feature intensities and ratios (including background subtraction and normalization), rejects outliers and calculates statistical confidences (*P* values). Subsequently, the genes were classified into functional groups using gene ontology and analyzed individually at the site "Entrez Gene" (http://www.ncbi. nlm. nih.gov/gene).

SAM 3.0 (Significance Analysis of Microarrays) statistical program was used to analyse expression of upand down-regulated genes using 1% False Discovery Rate (FDR), which points out statistically significant up- and down-regulated genes from the set of a microarray experiment.

Results and Discussion

RNA quality control: RNA quality was confirmed by analysing RIN using Agilent 2100 bio-analyzer. RIN was assessed on the base of the presence or absence of degradation products in the entire electrophoretic trace of the RNA sample. In this way, interpretation of an electropherogram was facilitated, comparison of samples was enabled and repeatability of experiments was ensured. The results of single analyzed sample were described as an electropherogram and a virtual image of an internal standard agarose gel. Excellent quality of RNA was assessed when the bands of 28S and 18S rRNAs were well separated and their correspondent peaks were in good evidence. RNA samples used in this experiment obtained RIN values 9.6, 10 and 9.7 for control samples and 9.5, 10 and 8.6 for cortisol treated samples. From this result it is clear that RNA quality was good and could be used for further analysis because RIN value greater than 8 is acceptable for microarray hybridization(Schroeder et al., 2006; Thompson, et al., 2007).

cRNA quantification and incorporation of cyanine: The hybridyzation experiment was carried out according to the experimental design whereas the cRNA quantification and incorporation of Cy3 (green) and Cy5 (red) of each sample is shown in Table 2. Treated samples were labelled with Cy5 and the control with Cy3.

Table 1. The experimental design of microarray analysis.Cy3= Control and Cy5=Treated.

Experiments	Cy3	Cy5
Array 1_1	1.1 Control 12 hpf	1.1 Cortisol-treated 12 hpf
Array 1_2	1.2 Control 12 hpf	1.1 Cortisol-treated 12 hpf
Array 1_3	1.3 Control 12 hpf	1.1 Cortisol-treated 12 hpf

Scanning and microarray data analysis: Hybridization expression data were collected by scanning the signal intensities of the corresponding spots on the array by dedicated fluorescence Agilent's DNA microarray scanner. The spatial distribution of significantly up- and down-regulated features for array 1_1, array 1_2 and array 1_3 is shown in Figure 1, where red spot indicates that the fluorescence intensity of the Cy5 signal is higher than that of Cy3, which means that the corresponding gene is over-expressed. Green spots indicate that the fluorescence intensity is higher in the control sample than treatment sample, which means that the corresponding gene is down-regulated.

Table 2. Quantification of cRNA and incorporation rates of cyanine Cy3 and Cy5 for control and treatment samples, respectively.

Samples	Labeling	Concentration cRNA (ng/µl)	Volume (µl)	Dye (picomol/µl)	Incorporation rate (pmol/µg)
1.1 Control 12 hpf	СуЗ	411	30	7.3	17.8
1.2 Control 12 hpf	СуЗ	362	30	3.2	8.8
1.3 Control 12 hpf	СуЗ	293	30	4.7	16.4
1.1 Cortisol-treated 12 hpf	Cy5	248	30	5.1	12.5
1.1 Cortisol- treated 12 hpf	Cy5	407	30	4.7	13.4
1.1 Cortisol- treated 12 hpf	Cy5	350	30	2.5	8.3



Figure 1 Spatial distribution of significantly up- and down-regulated features. A: Array1_1, B: Array1_2, C: Array1_3.

Image processing was performed using Agilent's Feature Extraction Software (FES) and the normalization of data was performed automatically. This software offers, among other features, the possibility to visualize the results of the data analysis in a log ratio versus log processed signal scatter plot (Figure 2).

Finally, SAM 3.0 (Significance Analysis of Microarrays) statistical program was used to analyze the up- and down-regulated genes due to cortisol treatment. Statistical analysis indicates the up-regulation of 143

genes down-regulation of 6 genes. The analysis was performed from public databases (National Centre of Biotechnology Information; NCBI, USA) for specific gene description. The annotations used were derived from Gene Ontology (GO), which provides information on molecular function, as well as from various pathway resources for information on involvement in biological signaling pathways. A descriptions of up- and down-regulated genes at 12 hpf with their functional groups and not annotated genes are shown in Tables 3 and 4, respectively.



Figure 2 Scatter plot of log ratio versus log processed signal intensities. Red spot: significantly up-regulated genes (P < 0.05). Green spot: significantly down-regulated genes (P < 0.05). Yellow spot: Not differentially expressed genes. Blue spot: Genes used to normalize. A: Array1_1, B: Array 1_2, C: Array 1_3.

of cortisol, Bioactivity either maternal or exogenous, is expected in zebrafish embryos because the mRNA encoding its main cognate receptor, GR, is the most abundant among maternal transcripts encoding nuclear and membrane steroid receptors inovulated oocytes (Pikulkaew et al., 2010). Its translation into protein to act as GR-cortisol complex on gene targets is to be assumed in the embryo, because the activation of the GR signaling pathway is essential for mesoderm formation in zebrafish (Nesan et al., 2012). At this stage, cortisol appears to influence the rates of zygotic transcription with a prevalent up-regulation and a minor down-regulation. By knocking down glucocorticoid receptor mRNA, 114 and 37 transcripts were up- and down-regulated respectively which is the agreement to our result (Pikulkaew et al., 2011). In addition, intelectin, TLR-5M and TLR-5S mRNA transcripts were poorly and highly expressed at different embryonic stages when oocytes immersed 3 h prior to fertilization in cortisol-enriched ovarian fluid at either 100 ng/ml or 1000 ng/ml (Li *et al.*, 2011).

Glucocorticoids are also used for the treatment of certain cancer like Hodgkin's lymphoma, multiple myeloma and acute lymphoblastic leukemia. Glucocorticoids can be moderately engaged to glucocorticoid-induced apoptosis due to its anti-inflammatory and antineoplastic actions (Rhen and Cidlowski, 2005). After binding with glucocorticoid receptor, this hormone can upregulate proapoptotic genes and downregulate antiapoptotic genes (Mok *et al.*, 1999). So maternal glucocorticoid dosage should be regarded as the fundamental integral sensor of the

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ankfy1 ankyrin repeat and FYVE domain containing 1 crfb8 cytokine receptor family member b8 rgs5b regulator of G-protein signaling 5b oxidative-stress responsive 1b adck1 aarF domain containing kinase 1 spag1b sperm associated antigen 1b tk1 TANK-binding kinase 1 zinc finger, DHHC-type containing 5a trima5.24 trimartite motif containing 35.24 adck1 cytic finger, 0 <licytic 0<="" finger,="" li=""></licytic>	slit3	slit (Drosophila) homolog 3	T ↑	_
crfb8 cytokine receptor family member b8 - rgs5b regulator of G-protein signaling 5b oxidative-stress responsive 1b adck1 aarF domain containing kinase 1 - spag1b sperm associated antigen 1b - - - - - tk1 TANK-binding kinase 1 - - zdhhc5a zinc finger, DHHC-type containing 5a -	ankfv1	ankyrin repeat and FYVE domain containing 1	r ↑	-
rgs5b regulator of G-protein signaling 5b - oxidative-stress responsive 1b adck1 aarF domain containing kinase 1 - spag1b sperm associated antigen 1b - - - - tk1 TANK-binding kinase 1 - - zdhhc5a zinc finger, DHHC-type containing 5a - - trima5-24 tripartite motif containing 35-24	crfb8	cytokine receptor family member b8	I ↑	-
oxsr1b oxidative-stress responsive 1b - adck1 aarF domain containing kinase 1 + - - - - spag1b sperm associated antigen 1b - -<td>rgs5h</td><td>regulator of G-protein signaling 5b</td><td>I ↑</td><td>-</td>	rgs5h	regulator of G-protein signaling 5b	I ↑	-
adck1 aarF domain containing kinase 1	over1h	oxidative-stress responsive 1h	I ↑	_
spag1b sperm associated antigen 1b 1 - tbk1 TANK-binding kinase 1 1 - zdhhc5a zinc finger, DHHC-type containing 5a 1 - trim35-24 tripartite motif containing 35-24 1 -	adek1	aarF domain containing kinase 1	 ↑	-
tbk1 TANK-binding kinase 1 - zdhhc5a zinc finger, DHHC-type containing 5a - trim35-24 tripartite motif containing 35-24	snag1h	sperm associated antigen 1b	 ↑	-
zdhc5a zinc finger, DHHC-type containing 5a 1 - - -	thk1	TANK-hinding kinase 1	 ↑	-
trim35_24 tripartite motif containing 35_24	zdbhc5e	zinc finger DHHC-type containing 5a	 ↑	-
	trim35_24	tripartite motif containing 35-24	 ↑	-

Table 3. Up- (\uparrow) and down-regulated genes at 12 hpf with their specific functions and descriptions.

stressor load on the mother's life during oogenesis while the cortisol deposit would act as an additional clinical factor in the maternal programming of embryo development.

Table 4. Not	annotated	up	and	down-regulated	genes	at	12
hpf.							

Name of genes	12 hpf			
	Up	Down		
zgc:114123	1	-		
zgc:66337	↑	-		
si:dkey-165a24.4	↑	-		
zgc:114175	↑	-		
si:dkey-11e23.5	↑	-		
im:6903943	↑	-		
si:dke-21k24.2	↑	-		
zgc:77816	↑	-		
zgc:112992	-	\downarrow		
zgc:171485	↑	-		
zgc:153845	↑	-		
si:dkey-7111.1	↑	-		
zgc:153893	1	-		
si:dkeyp-22b2.2	↑	-		
zgc:64022	1	-		
zgc:73144	1	-		
zgc:122979	1	-		
zgc:73359	↑	-		
zgc:153031	↑	-		
si:dkey-21k24.2	↑	-		
zgc:165519	↑	-		
zgc:110655	↑	-		
zgc:136758	↑	-		
zgc:113983	↑	-		

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