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ROLE OF LIPOSOLUBLE HORMONES AND MATERNAL mRNAs IN THE EPIGENETIC LONG-TERM MODULATION OF ZEBRAFISH DEVELOPMENT

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SUMMARY

ROLE OF LIPOSOLUBLE HORMONES AND MATERNAL mRNAs IN THE EPIGENETIC LONG-TERM MODULATION OF ZEBRAFISH DEVELOPMENT

PART I: Expression analysis of maternal and zygotic steroid hormone receptor mRNAs during zebrafish (*Danio rerio*) embryogenesis.

I have analyzed by qRT-PCR and/or RT-PCR the abundance and degradation rates of maternal mRNAs for nine steroid hormone receptors and their possible replacement by corresponding embryonic transcripts in both ovulated oocytes and embryos of zebrafish collected at 0, 1, 2, 4, 8, 12, 24 and 48 h post-fertilization (hpf). The mRNAs encoded the nuclear receptors for progesterone (Pr), androgen (Ar), estrogen (Erα, Erβ1 and Erβ2), glucocorticoids (Gr), mineralocorticoids (Mr) and the membrane progestin receptor-α and β (mPrα and β). *Gr* mRNA was the most abundant maternal transcript in oocytes and early embryos, followed by *erβ2* and *ar* mRNAs. They declined during the first 8 hpf, being replaced, thereafter, by the embryonic messengers. *Erβ1* and *mr* transcript levels were low until 8 hpf, but increased steadily during embryonic transcription from 24 to 48 hpf. *Pr* transcripts were detectable only in ovulated oocytes and at 24 and 48 hpf. At these stages, there was a slight increase of *erα* mRNA that initially was very low. *Mprα* and *β* mRNAs were expressed in ovulated oocytes and faintly persisted during the first 4 hpf. There was no subsequent embryonic expression of these transcripts. The possible involvement of maternal mRNAs for glucocorticoid and sex hormone receptors in the programming of early zebrafish development is intriguing, since they mainly occur at stages in which gene replication predominates over transcription.

PART II: Morpholino knockdown of glucocorticoid receptor in zebrafish during embryogenesis

The glucocorticoid receptor (Gr) is involved in various physiological processes, including growth, osmoregulation and reproduction in various species of vertebrates. In this study, I have investigated the function of Gr during zebrafish embryogenesis using antisense Morpholino (MO) technology approach in order to block *gr* mRNA translation. I observed that Gr-morphants were characterized by small head and eyes, short body, pericardial edema, disrupted melanophore patterning, failure to inflate the swim bladder, tail curling, and abnormal swimming behaviour. The effectiveness of Gr knockdown was further verified by Western blotting and *in vitro* transcription and translation system, which showed marked reduction of the Gr protein in morphant embryos. Microarray analysis revealed that Gr functions prevalently as a negative regulator of gene transcription. Three genes found to be up-regulated following MO knockdown by microarray analysis, including *caspase-8*, *igf2α* and *centaurin-1α*, were evaluated by semiquantitative RT-PCR throughout the embryonic development. Furthermore, whole mount *in situ* hybridizations indicated that, whereas marker expression pattern was mostly unchanged in morphant embryos, three markers involved in neurogenesis, *egr2b*, *emx1* and *six3.1*, were up-regulated.

In conclusion, the knockdown of Gr in zebrafish revealed a requirement of both maternal and zygotic *gr* in multiple developmental processes involved in neurogenesis and gut and accessory organs formation. There are indications that the maternal receptor is critical in early embryogenesis, but more work is needed to elucidate the functional transition from the maternal to the zygotic *gr* and whether they affect distinctive components of the genetic machinery.

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RIASSUNTO

RUOLO DI ORMONI LIPOSOLUBILI E mRNA DI ORIGINE MATERNA NELLA MODULAZIONE EPIGENETICA A LUNGO TERMINE DELLO SVILUPPO IN ZEBRAFISH

PARTE I: Analisi dell'espressione degli mRNA materni e zigotici per recettori di ormoni steroidei durante lo sviluppo nel pesce zebrato (*Danio rerio*).

Ho analizzato mediante qRT-PCR e/o RT-PCR la presenza ed il grado di degradazione degli mRNA materni codificanti per nove recettori di ormoni steroidei, e la loro possibile sostituzione da parte dei trascritti embrionali corrispondenti, sia negli ovociti ovulati che in embrioni di pesce zebrato raccolti a 0, 1, 2, 4, 8, 12, 24 e 48 ore dopo la fecondazione (hpf). Gli mRNA studiati sono quelli codificanti i recettori nucleari per il progesterone (Pr), gli androgeni (Ar), gli estrogeni (Erα, Erβ1 ed Erβ2), i glucocorticoidi (Gr), i mineralcorticoidi (Mr) ed i recettori di membrana α e β per i progestinici (mPrα e mPrβ). L'mRNA codificante la proteina Gr costituisce il trascritto materno maggiormente presente negli ovociti e nei primi stadi di sviluppo embrionale, seguito dagli mRNA codificanti i recettori Erβ2 ed Ar. Tutti questi trascritti diminuiscono durante le prime 8 ore di sviluppo, e vengono poi sostituiti dai rispettivi messaggeri embrionali. I livelli dei trascritti per *erβ1* e *mr* sono bassi prima delle 8 hpf, ed aumentano costantemente, dopo l'inizio della trascrizione embrionale, passando dalle 24 alle 48 hpf. L'mRNA codificante il recettore Pr è stato rilevato soltanto negli ovociti ovulati e nei campioni corrispondenti a 24 e 48 hpf. A questi stadi di sviluppo c'è anche un leggero aumento dell'mRNA codificante la proteina Erα. I trascritti per i recettori mPrα e mPrβ sono presenti negli ovociti ovulati e persistono, a basse concentrazioni, fino alle 4 hpf.

Non è stata evidenziata la presenza di trascritti embrionali codificanti per questi due geni. E' interessante il possibile coinvolgimento degli mRNA codificanti Gr e i recettori degli ormoni sessuali nella programmazione dello sviluppo embrionale precoce del pesce zebrato, dal momento che sono presenti principalmente negli stadi di sviluppo in cui la replicazione genica predomina sulla trascrizione.

PARTE II: Inattivazione genica del recettore dei glucocorticoidi di pesce zebrato mediante morfolino

Il recettore dei glucocorticoidi (Gr) è coinvolto in numerosi processi fisiologici, tra cui la crescita, l'osmoregolazione e la riproduzione in molte specie di vertebrati. In questo studio, ho analizzato la funzione del Gr durante l'embriogenesi di pesce zebrato utilizzando la tecnologia degli oligonucleotidi antisenso morfolino per inibire la traduzione dell'mRNA codificante Gr. Ho osservato che i morfanti di Gr presentano occhi e testa di dimensioni ridotte, corpo corto, edema pericardico, riduzione della pigmentazione, mancata insufflazione della vescica natatoria, coda incurvata e comportamento natatorio anormale. L'efficacia dell'inattivazione genica del Gr sono stati verificati mediante *Western blotting* ed utilizzando un sistema di trascrizione e traduzione *in vitro*, che hanno dimostrato una riduzione della traduzione del Gr negli embrioni morfanti. Analisi di *microarray* hanno messo in evidenza che questa proteina funziona prevalentemente come repressore della trascrizione genica. I geni *caspase-8, igf2α* e *centaurina-1α* risultati sovraespressi mediante *microarray* dopo trattamento con morfolino, sono stati analizzati mediante RT-PCR semiquantitativa durante tutto il corso dello sviluppo embrionale. Inoltre, esperimenti di ibridazione *in situ in toto* indicano che, mentre in generale il modello di espressione dei marcatori utilizzati non cambia nei morfanti, tre marcatori coinvolti nel processo di neurogenesi, e cioè *egr2b*, *emx1* e *six3.1*, sono risultati sovraespressi.

In conclusione, i risultati ottenuti dall'inattivazione genica del *gr* di pesce zebrato rivelano la necessità dei trascritti di origine materna in molteplici processi di sviluppo, quali neurogenesi e formazione di intestino ed organi accessori. Si dovrà comunque analizzare più in dettaglio la transizione funzionale dai trascritti materni a quelli zigotici e stabilire se essi influenzino componenti distinti dei meccanismi a livello genomico.

INTRODUCTION

1. Zebrafish as a model organism

The zebrafish species, *Danio rerio* (former name: *Brachydanio rerio*), belongs to the Superclass of the Gnathostomata, Class Actinopterygii, Division Teleostei, Subdivision Euteleostei, Superorder Ostariophysi, Order Cypriniformes, Family Cyprinidae, Subfamily Rasborinae, Genus Danio (Fig. 1). Zebrafish have originated in the Southeast Asia and their nowadays natural habitats are the tropical freshwaters of Asia and Africa (Dahm and Geisler 2006). For several years, the adult zebrafish has been employed for toxicological investigations. Recently, the zebrafish is a common and useful model organism for studies of genetics, developmental biology, neurophysiology and biomedicine (Alestrom *et al*., 2006; Dahm and Geisler 2006; Sumanas and Lin 2004).

As to the advantages of zebrafish reproduction and embryogenesis, they are easy to keep and breed all year round under laboratory conditions. One adult fish spawns hundreds of eggs once every 5 days (Dahm and Geisler 2006). Generation time is short, typically 3-4 months, making it suitable for selection experiments. Moreover, fertilization is external, thus live embryos are accessible to manipulation and can be monitored through all developmental stages under a dissecting microscope. Development is rapid, with all major organs developing within 36 h at 28.5°C (Kimmel *et al*., 1995).

Importantly, the embryo can be subjected to the microinjection of nucleic acids and expression of specific proteins can be suppressed throughout the developing embryo by the use of antisense agents; this enables monitoring the roles of specific proteins in development or in drug response. Moreover, the transparency of the developing embryo gives a unique quality to whole-mount *in situ* hybridization (Fjose *et al*., 1992) and antibody staining (Wilson *et al*., 1990).

In addition, advances in zebrafish gene mapping, phenotype analysis and extensive EST databases make zebrafish a convenient model organism for many biological fields. The zebrafish genome, as available in the zv7 assembly on the Ensembl website (http://www.ensembl.org/index.html), is virtually complete. Seventy percent of the genome has been sequenced with > 99.999% accuracy. For the rest of the genome, a so-called whole

genome shotgun approach has been used, which has coverage of 5.5 times (Schaaf *et al*., 2009).

Fig. 1. Adult female (top) and male (bottom) zebrafish. The characteristic horizontal stripes running along the body and fins gave this species its name. Females can be recognized by their larger belly accommodating the eggs. Males tend to be more yellowish in color than female zebrafish.

2. The development of the zebrafish

Since my thesis focuses on the roles of maternal mRNAs encoding steroid hormone receptors in zebrafish early embryogenesis, I would like briefly to review the important stages of embryonic development in this species which were previously described by Kimmel *et al*. (1995). Morphological staging information is based on embryonic development at 28.5°C. The process can be divided into seven periods: zygote (0 - ¾ hours post-fertilization or hpf), cleavage (0.7 - 2¼ hpf), blastula (2¼ - 5¼ hpf), gastrula (5¼ -10 hpf), segmentation (10 – 24 hpf), pharyngula (24 - 48 hpf) and hatching period (48 - 72 hpf). This work will be focusing mainly on stages from blastula to gastrula, when most patterning and cell fate determination processes take place. Figure 2 shows some stages of early embryonic development examined in my study.

Before fertilization, cytoplasm and yolk are intermixed in the egg, which is surrounded by a transparent chorion. At the one-cell-stage (**zygote period**), the fertilization activates yolk-free cytoplasm to accumulate at the animal pole under the micropyle, where the future embryo will form. The fertilized zygote contains all necessary information for its development in the zygotic genome and in maternally deposited mRNAs and proteins (Pelegri, 2003).

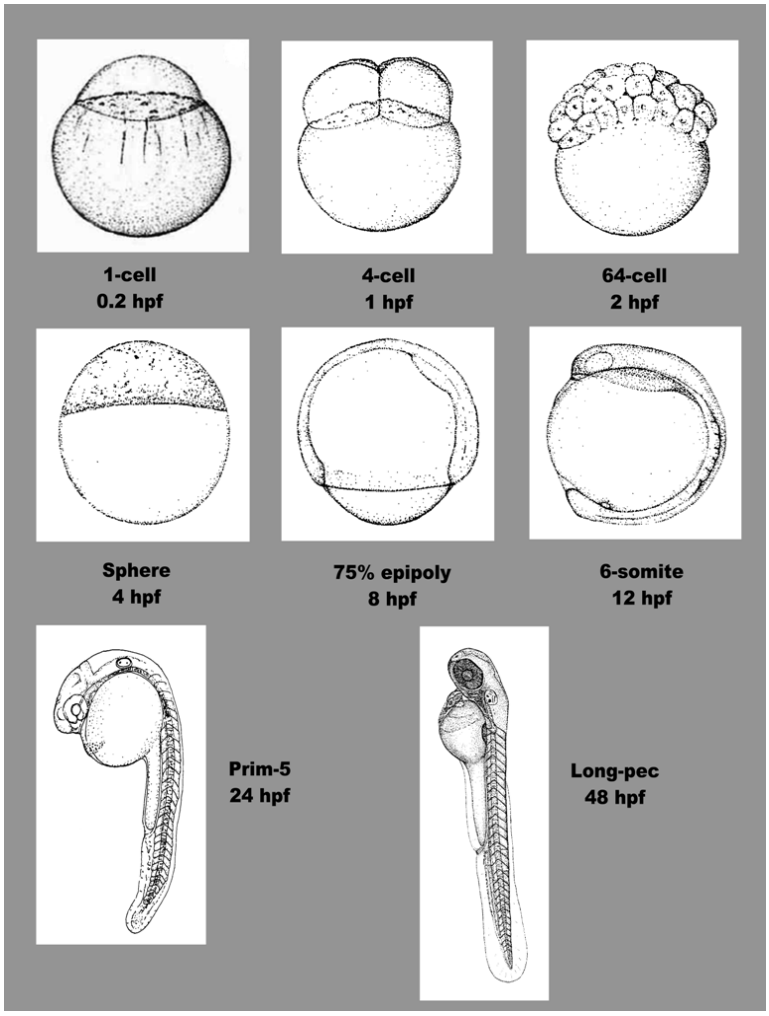


Fig. 2. Overview of early zebrafish development. hpf: hours post-fertilization. Images modified from Kimmel *et al*., 1995.

During the early **cleavage stages,** the zygote starts to divide in a discoidal-meroblastic manner, leaving newly formed blastomeres interconnected by cytoplasmic bridges. Blastomeres are cleaved radial-symmetrically without cell growth. With the fourth cleavage (16-cell-stage), the central cells become completely divided from the others. In contrast, marginal blastomeres remain cytoplasmically connected to the yolk cell. Already during the early cleavage stages, the embryo undergoes its first subdivision into two cell lineages: (a) germ-line cells, which inherit unique maternal transcripts demarcating them as primordial germ cells (PGCs), and (b) somatic cells forming the large bulk of the embryo.

In the **early blastula**, formal staging is by estimating the number of cells in the blastoderm. So, the term **"**early blastula**"** is meant for the 128-cell stage through the 2k-cell stage. Beginning at cycle 10, at 3 hpf, the cycle lengthens and cells lose local synchrony, defining the beginning of the midblastula transition (MBT), when the embryo begins the transition from maternal to zygotic control of development. Marginally, most cells collapse and release their cellular content, including nuclei, into the underlying cytoplasm of the yolk cell. The so-called "high blastula" refers to the time when the blastoderm is perched high on the yolk cell, where there is a visual line of yolk syncytial nuclei. At late blastula stages, distinct morphogenetic movements commence: epiboly starts with the doming of the yolk cell over which cells migrate and spread in an animal-to-vegetal direction, thereby thinning the blastoderm. This consists of a monolayer of flattened **enveloping layer** (EVL) cells covering more loosely organized **deep cells.** The single-celled yolk contains a syncytium of nuclei, the **yolk syncytial layer** (YSL), formed from cells at the margin of the blastoderm.

The **gastrula period** extends from 5¼ to about 10 hpf, the time when the germ layers begin to form. The developmental stages are indicated as 'percentages of epiboly'. This nomenclature refers to the extent to which the blastoderm has spread over the yolk cell along the animal-vegetal axis, or, in other words, the percentage of the entire distance between the animal and vegetal poles covered by the blastoderm. At 50% epiboly, at 6 hpf, the rim of the blastoderm thickens into a bilayered structure termed the germ-ring, consisting of an outer layer, the ***epiblast,*** and an inner layer, the ***hypoblast.*** As the radial intercalations of the late blastula become less frequent, it is possible to predict the future fate of regions of the embryo. Outer prospective ectodermal cells that interact directly with the environment are separated from inner cells affiliated with *e.g.* nutritive functions. Involution or deep cell movement at the blastoderm margin is more pronounced at the dorsal as compared to the ventral side. At this time, cells start convergence and move to the prospective dorsal side of the embryo, leading to the formation of a knob-like thickening at the dorsal margin, called the shield. When epiboly is completed at the end of gastrulation, the yolk is completely covered by the blastoderm, and the concerted cell movements have established the dorsal-ventral and anterior-posterior body axes. After gastrulation the embryo is further patterned and elongated in the course of the segmentation period along its axes. In particular, the tail bud extends away from the yolk cell to produce the embryonic tail region. The gut tube forms in close

apposition to the yolk surface and the notochord primordium separates from the adjacent somitic mesoderm, which is progressively subdivided into the segmentally arranged somites.

The **segmentation period** encompasses the remainder of the first day of development, from 10 to 24 hpf, when the embryo begins the subdivision of the body plan. Many additional features can be seen at this stage. The cephalic neuroectoderm thickens and, by the 18-somite stage, the segmentation of the brain is evident, both in broad subdivisions, such as forebrain, midbrain and hindbrain; and in finer subdivisions, such as the rhombomeres of the hindbrain. The eye primordium and the ear primordium appear. The cells of the notochord begin to expand and straighten out the tail of the embryo. At the completion of the first day of development, the embryo has between 8,000 and 10,000 cells. With notable major exceptions, such as the neural crest-derived structures of the jaw and endodermally derived structures of the gut tube, the development of the major systems of the embryo are laid out. The embryos are touch-sensitive and their hearts will start beating.

After 2 days post fertilization or dpf, the embryo hatches from the chorion and has completed most of its morphogenesis. At 5 dpf, larvae develop a swim-bladder and begin to swim and feed. Sexual maturity is reached within 3-4 months, and adults can live for 2.5 - 4 years.

3. Maternal vs. zygotic receptor transcripts

The yolk of ovulated oocytes in teleost fish, as in other oviparous vertebrates, contains large amounts of liposoluble hormones, such as steroid, thyroid and retinoid hormones (McCormick, 1999; Irie and Seki, 2002), taken up from the maternal circulation or the follicular envelope. 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), cortisol in the eggs of Mozambique tilapia (*Oreochromis mossambicus*) (Shiraishi *et al*., 1999), and testosterone in medaka (*Oryzias latipes*) (Iwamatsu *et al*., 2006). In addition, dynamic changes in cortisol content profile were reported in tilapia (Hwang *et al*., 1992) and zebrafish (Alsop and Vijayan, 2008), showing a steady decline from fertilization until hatching in both species.

Experimental changes of steroid concentrations were shown to cause distinct effects on offspring development, thus demonstrating that these hormones are not only correlated with the maternal hormonal condition during oogenesis, but are also biologically active throughout the first embryogenic steps, as suggested by Brooks *et al*. (1997). Lower overall growth rates were observed in larvae derived from embryos supplemented with cortisol, the main stress hormone, both in the orange-spotted grouper, *Epinephelus coioides* (Tay *et al*., 1997) and in the ambon damsel, *Pomacentrus amboinensis* (McCormick, 1999). Moreover, the treatment of rainbow trout, *Oncorhynchus mykiss*, oocytes with cortisol before fertilization was correlated with depressed post-metamorphic body growth of the offspring, without alteration in premetamorphic growth (Belvedere *et al*., 1999). The same effect was obtained in zebrafish, *Danio rerio*, but in this case the hormonal treatment was performed just after fertilization (in preparation).

In order to induce such long-term epigenetic effects on gene expression by maternal hormonal programming during early embryonic stages, cognate receptors must be precociously translated from maternal mRNAs, before the activation of zygotic gene transcription, which mostly occurs from the midblastula transition (MBT) beginning at cycle 10 (Kimmel *et al*., 1995).

MBT is characterized by an initial phase of rapid and synchronous cell divisions, beginning just after fertilization and lasting into the blastula stage. At some point, cell division slows down and becomes asynchronous, a change commonly known as midblastula transition (MBT, 512-cell stage), as described before. The early embryo is transcriptionally largely silent, and the initial, rapid cell divisions are sustained by messenger RNAs that the mother deposited in the eggs. During the maternal-to-zygotic transition, oocytes-specific transcripts are degraded and maternal transcripts common to oocytes and early embryos are replaced by zygotic transcripts. It has been recently demonstrated that a large number of maternal mRNAs can be coordinately destroyed in this phase by the activity of a microRNA, miR430, that is transcribed at this transition point (Giraldez *et al*., 2006). Thus, the early development of embryos takes place largely in the context of stored maternal mRNAs and proteins that have critical roles in directing early developmental processes in teleosts.

The work by Mathavan *et al*. (2005) on the transcriptome of zebrafish embryos using microarray, shows a differential degradation of maternally-loaded gene transcripts, implying a

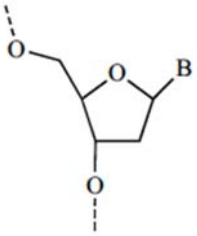
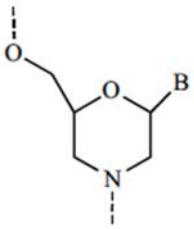
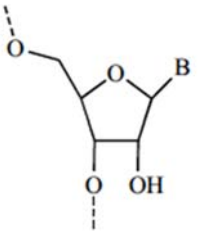
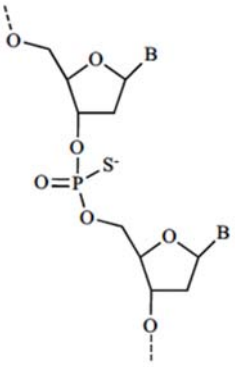
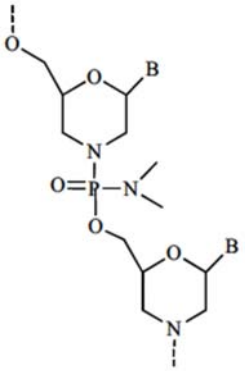
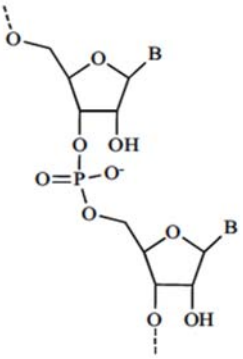
potential relevance of specific maternal genes at later zygotic stages. The authors also show a cohort of genes that actively increase transcription prior to the onset of MBT (from 64-128-cell stage onwards), a finding that has revised the earlier thinking that MBT marked the onset of all zygotic gene transcription. Moreover, it was demonstrated by semi-quantitative RT-PCR that zebrafish eggs contain maternal mRNA for cortisol receptor. This declines during the first 8 hpf, being replaced, thereafter, by the corresponding embryonic mRNA (Belvedere *et al*., 2005).

Data in the literature regarding hormone receptor transcripts present in zebrafish oocytes and deriving from cDNA microarray analysis or EST libraries, are limited. Some genes, and in particular genes related to cell communication, may be present in a very small proportion of oocyte transcription profiles and thus not detected by these methods; some specific genes could be absent from the microarray datasets; and finally, published papers are generally related only to the family of estrogen receptor genes (Tingaud-Sequeira *et al*., 2004; Lassiter *et al*., 2002), whereas no data exists regarding progestin, androgen or glucocorticoid steroid receptors. Thus, although many genes important for cell signaling or organogenesis have been studied in zebrafish, the global analysis of liposoluble hormone receptor gene expressions will probably provide useful information regarding cognate hormone functions during development.

4. Antisense technology in developmental biology

Agents which inhibit the expression of selected genes through a Watson/Crick base-pairing mechanism are commonly referred to as antisense or gene knockdown agents. Three major gene knockdown types have been devised with respect to off target effects and sequence specificities: 1) phosphorothioate-linked DNA (S-DNA); 2) short interfering RNA (siRNA); and 3) Morpholino (Table 1) (Summerton, 2007).

Table 1. Structure type of gene knockdown agent (Summerton, 2007)

Class	RNase H-dependent	Steric block	Interfering RNA
Type	***Phosphorothioate***	***Morpholino***	***Short Interfering RNA***
Subunit structure B= A, C, G, T(U)	 **Deoxyribose ring**	 **Morpholine ring**	 **Ribose ring**
Backbone structure	 **S-DNA**	 **Morpholino**	 **siRNA**
Principle site of action	**nucleus**	**Nucleus and cytosol**	**cytosol**

The RNA interference is a mechanism that has found a growing number of applications in genetic engineering thanks to the possibility of studying the function of genes by silencing their expression (loss of function): it induces the degradation of mRNAs and thus prevents the formation of the proteins that they code for. Since RNA interference may not totally abolish the expression of the gene, this technique is sometimes referred to as a "knockdown", to distinguish it from "knockout" procedures in which the expression of a gene is entirely eliminated.

Short interference RNAs are made up of small synthetic fragments of 21-23 bp with an overhang of 2 or 3 nucleotides at the 3-end that are normally the result of processing by DICER, an enzymatic complex that converts either long dsRNAs or small hairpin RNAs into siRNAs. The latter, synthetically produced, can also be exogenously introduced into cells by various transfection methods to knockdown a gene of interest. Once inside the cells, siRNAs do not need to be processed by DICER, but are equally incorporated into an RNA-induced silencing complex (RISC) (Agrawal *et al*., 2003). The antisense strand of the siRNA then guides the RISC to the complementary mRNA, which is consequently cleaved by the complex. This approach permits to analyse different siRNAs for each gene and to find the one able to determine a greater silencing (Fig. 3).

An alternative approach to RNA interference is the antisense Morpholino (MO) technology that has proven to be a powerful tool to knockdown specific targets in zebrafish. RNAi and Morpholino are emerging as the most effective tools in bringing about functional silencing of genes. Both of these molecules are complementary oligonucleotides that interfere with mRNA translation. This reduces protein levels and functionally silences the gene.

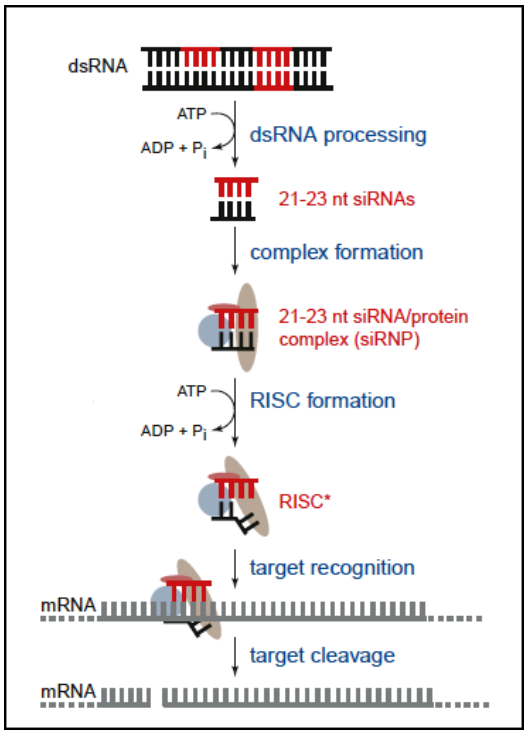


Fig. 3. The pathway of RNAi (Zamore, 2001).

5. Morpholino-mediated gene knockdown

Morpholinos are useful for developmental studies. The efficacy of MOs is clear in a variety of therapeutic and genomic applications and in a large number of model systems, including frog, fly, nematode, mouse, snail, and leech (Song *et al*., 2002; Tessmar-Raible and Arendt, 2003; Seitan *et al*., 2006). They are synthetic molecules, usually 25 bases in length, which are analogous to RNA or DNA (Summerton and Weller, 1997). They are different from DNA in two aspects. The phosphodiester linkage in DNA is replaced by a phosphorodiamidate linkage in Morpholino. Secondly, the five-membered deoxyribose ring in DNA is replaced by a six-membered morpholine ring (Ekker, 2000; Corey and Abrams, 2001). These modifications make them highly resistant to degradation by enzymes while, at the same time, they do not affect binding by Watson-Crick pairing (Ekker, 2000; Summerton, 2007). Morpholinos do not degrade their target RNA molecules, but act through an RNase-H-independent mechanism (steric block mechanism) by binding to mRNA and blocking translation initiation of the targeted transcript (Summerton, 1999). In this case, the knockdown of the gene can be analyzed by Western blotting, as the targeted protein must become undetectable. They can be delivered to zebrafish embryos by injection at one-cell stage for uniform distribution, and are stable for several days (Moulton and Yan, 2008). The effect of MOs is dose-dependent (Nasevicius and Ekker, 2000).

5.1. Types of Morpholinos

Translation-blocking Morpholinos (Fig. 4) are targeted against the 5'-untranslated region of an mRNA or the beginning of the open reading frame, which can efficiently abolish translation. Proof of principle Morpholino experiments showed that MO-mediated knockdown can phenocopy well characterized mutant phenotypes. For effective gene inactivation of mRNA targets, MOs are often designed to be complementary to the 25 nucleotides including the translation initiation site (AUG) and the following 22 nucleotides or any 25-mer sequences in the mRNA contained in the 5'-UTR upstream of that site. Target sequences are selected that are unique, have high nucleotide complexity, and have minimal secondary structures or potential self-complementation. They presumably acts by preventing ribosomal binding to the mRNA. In the absence of ribosomal binding, translation is blocked

and the target gene can be silenced (Summerton, 1999; Ekker and Larson, 2001). Morpholinos show a high affinity, low toxicity and very few non-specific side effects (Summerton, 1999; Summerton and Weller, 1997).

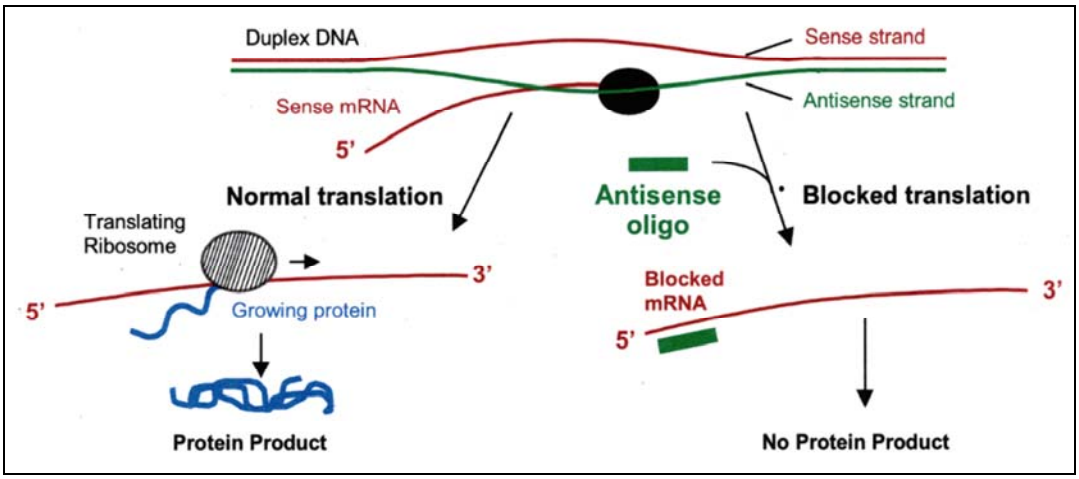


Fig. 4. Translation blocking by Morpholino (http://www.gene-tools.com/).

A different MO-based approach, which allows quantification of the knockdown efficiency, was developed to block pre-mRNA splicing instead of its translation. **Splice blockers** (Fig. 5) are complementary to splice acceptor (intron-exon boundary) or donor (exon-intron boundary) sites in the unspliced RNA, thereby competing with the splicing machinery for the binding site. MO binding leads to either erroneous exon excision or intron retention. PCR analysis of the RNA from MO-injected embryos (morphants) provides information about the efficiency of the gene knockdown (Draper *et al*., 2001).

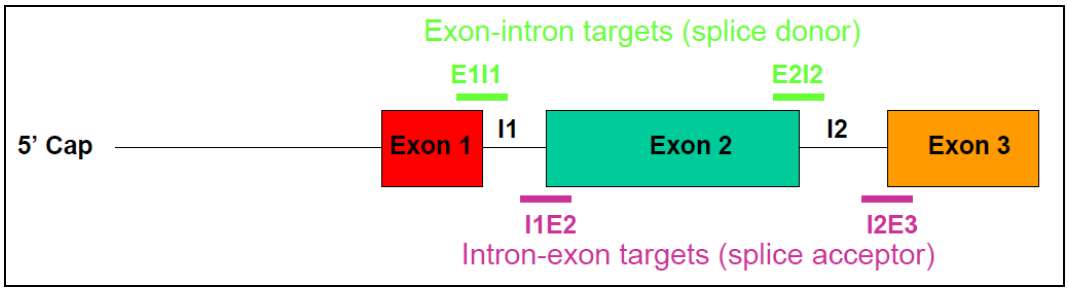


Fig. 5. Splice blocking by Morpholino (http://www.gene-tools.com/).

5.2. Controlling Morpholino experiments

As to limitations for use, MOs may sometimes confer toxicity to cells and induce off-target effects. Morpholino can cause stress-induced cell death in the central nervous system and somites. Although MOs act in a sequence specific manner, there is always a chance of blocking also other genes containing similar sequences (Eisen and Smith, 2008). In order to assess these components of the MO phenotype, one has to perform a variety of control experiments. To check MO efficacy, it is appropriate to check the protein loss on a Western Blot or in whole mount tissue staining. However, this is often limited due to unavailability of zebrafish specific antibodies. Additionally, one can control for unspecific effects by injecting a mismatch Morpholino - an oligonucleotide similar but different by at least 5 units to the working Morpholino. Such a modified agent should be unable to bind to its target and, hence, causes no gene knockdown phenotype (Eisen and Smith, 2008). Also, the specific MO phenotype is most convincing if it is possible to rescue through mRNA injection, or, if those experiments are inconclusive, by evidence that two Morpholinos complementary to different parts of the 5'-UTR have the same effect, or by evidence from other overexpression and/or underexpression studies (Hesman, 2002).

Delivery is typically accomplished by microinjection into one- to 8-cell stage zebrafish embryos. Calibration of injection volume is essential for reproducibility between experiments. The interpretation of MO-induced phenotypes is facilitated through the use of appropriate controls.

5.3. Morpholino in zebrafish

Morpholino oligonucleotides have recently emerged as a tool for gene-specific knockdown in the zebrafish, thus representing an alternative strategy to investigate the roles of various genes in developmental processes. MOs have been most successfully used to study gene function in zebrafish owing to its rapid external development, transparent embryos and the ease of delivery of MOs. The use of MOs in zebrafish has shown these compounds to be sequence specific and extremely potent in all cells during the first 50 hpf in zebrafish embryos as targeted gene knockdown chemicals (Nasevicius and Ekker, 2000). This time period in the zebrafish embryo includes the fundamental vertebrate processes of segmentation and

organogenesis. Many novel gene functions have been successfully analyzed in zebrafish using MOs, as shown in Table 2.

Table 2. List of some novel gene functions studied using MOs in zebrafish.

Gene name and function	Phenotype	Specific control for Morpholino	Reference
Receptors			
Receptor protein-tyrosine	Apoptosis in brain, undifferentiated	Absent	van der Sar *et al*., 2002
Frizzled-2 (fz2), wnt receptor	Undulating axial structures	2nd MO	Sumanas *et al*., 2001
Transcription factors			
Pdx-1	Pancreas defects, digestive organ	RNA rescue	Yee *et al*., 2001
Forebrain embryonic zinc	Ventral forebrain defects	RNA rescue	Yang *et al*., 2001
her1, hairy-related transcription factor	Perturbs somite formation	2nd MO	Holley *et al*., 2002
hhex, homeobox gene	Liver development, digestive organ chirality	RNA rescue	Wallace *et al*., 2001
skiA, Smad-interacting co-repressor	Ventralisation	2nd MO, RNA rescue	Cui *et al*., 2001
Secreted signaling molecules			
Antivin 1 (atv1), activin/nodal inhibitor	Expansion of hatching gland and notochord	RNA rescue	Agathon *et al*., 2001
Antivin 2 (atv2), lefty2	Expansion of hatching gland and notochord	RNA rescue	Agathon *et al*., 2001
Ets1-Related Protein	Complete absence of circulation in zebrafish embryos	RNA rescue	Sumanas and Lin 2006
wnt8	Defects in head patterning, trunk and tail formation	Phenocopy of a deficiency, RNA rescue	Lekven *et al*., 2001
fgf3	Reduction in nk2.1b expression	RNA rescue	Shinya *et al*., 2001
Glial cell line-derived neurotrophic factor (GDNF)	Defects in enteric neuron path-finding	RNA rescue	Shepherd *et al*., 2001

6. The nuclear receptor family

The glucocorticoid receptors belong to the superfamily of nuclear receptors. All nuclear receptor proteins exhibit a characteristic structure that consists of five to six domains of homology [A/B, C (DNA-binding), D (hinge region), and E (ligand-binding) domains] on the basis of regions of conserved sequence and function. The C and E domains are highly conserved across vertebrates (Kumar and Thompson, 1999) (Fig. 6).

Nuclear receptors can be grouped into four classes according to their ligand binding, DNA binding, and dimerization properties: steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors. This family comprises 49 members which are divided into six evolutionary groups. Nuclear receptors function as transcription factors. Their actions can be genomic or non-genomic, ligand dependent or independent, and contribute to gene activation, gene repression, or release of gene repression. Interestingly, the human glucocorticoid receptor (GR, NR3C1) was one of the first nuclear receptor proteins to be cloned by Ron Evans and his colleagues together with the estrogen receptor (ER) (Germain *et al*., 2006). Two receptor subtypes can be distinguished: type 1 acts as homodimer, while type 2 acts as heterodimer after ligand binding.

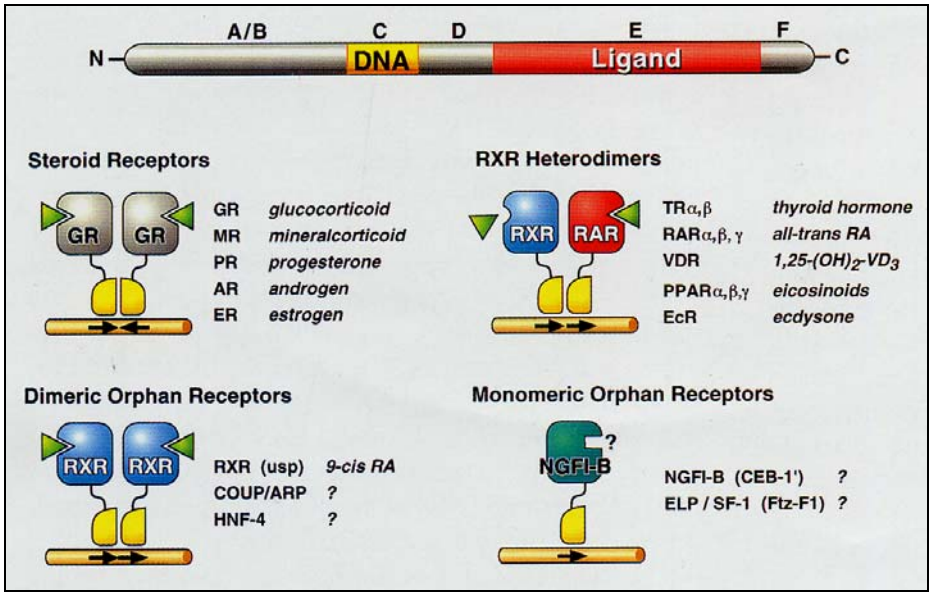


Fig. 6. Common structure of nuclear receptors and four classes according to their ligand binding (? refer to orphan receptors for which ligands are not known) (Germain *et al*., 2006).

Type 1 comprises the steroid receptors for estrogens [*e.g.* estradiol; ERα (NR3A1) and ERβ (NR3A2)], glucocorticoids [cortisol; GR (NR3C3)], mineralcorticoids [aldosterone; MR (NR3C2)], progestins [progesterone; PR (NR3C1)], and androgens [testosterone; AR (NR3C4)]. Non-steroid hormone receptors, such as vitamin D receptor [VDR (NR1I1)], thyroid hormone receptor [TR (NR1A)], retinoic acid receptor [RARα (NR1B1), RARβ (NR1B2), RARγ (NR1B3)], peroxisome proliferator-activated receptor [PPAR (NR1C)] act predominantly as heterodimers with the retinoic X receptor [RXR (NR2B)]. For a number of nuclear receptors, so-called orphan receptors, the natural ligands have not yet been identified or may not exist (Mangelsdorf *et al*., 1995).

7. Stress axis in teleost fish

Cortisol is the main corticosteroid hormone in teleosts secreted in response to stressor exposure and plays a key role in osmoregulation, growth and reproduction (Varsamos *et al*., 2005; Yada *et al*., 2005). The biosynthesis of cortisol in fish is similar to that in mammals and involves a microsomal enzymatic pathway. Cytochrome P450c17 catalyses reactions that diverts intermediates form corticosterone synthesis into the cortisol biosynthesis pathway. Moreover, fish possess the mitochondrial inner membrane monooxygenase P450scc and the P450c11 catalysing the 11ß-hydroxylation of deoxycortisol/deoxycorticosterone (Fig. 7) (Mommsen *et al*., 1999).

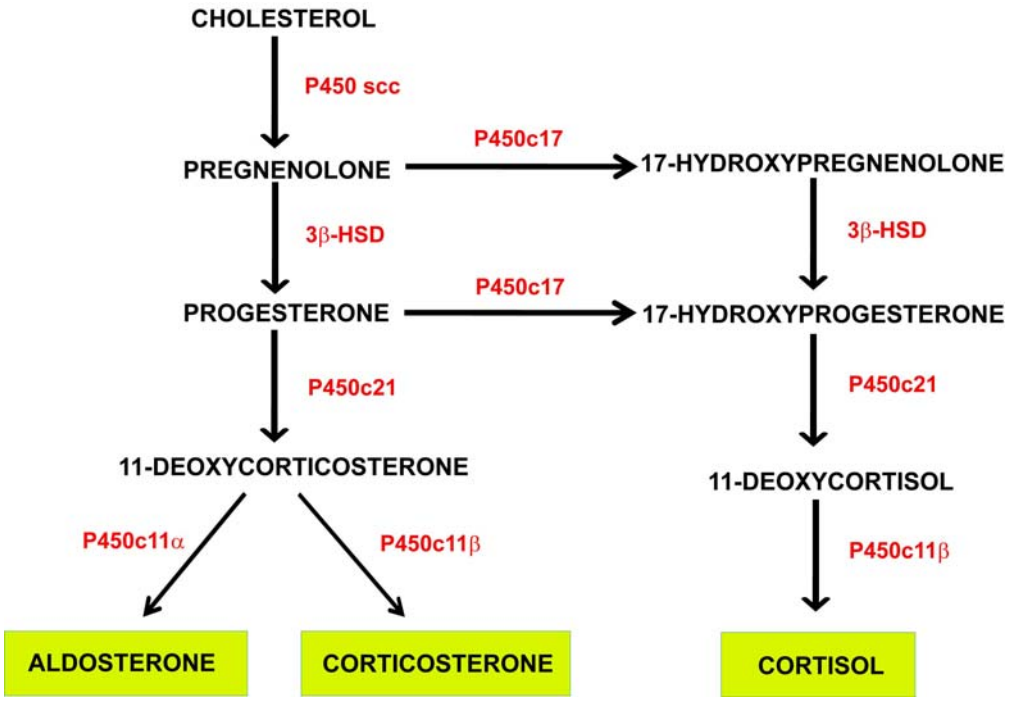


Fig. 7. Biosynthesis pathways of corticosteroids (modified from Luu-The *et al*., 2008).

In many fish, cortisol is found in both unfertilized oocytes and fertilized eggs, but rapidly decreases in the earliest stages of embryogenesis until hatching, when the hypothalamic-pituitary-interrenal axis (HPI axis), the neuroendocrine system regulating corticoid secretion, has been shown to be functionally established (Hwang *et al*., 1993).

Briefly, the activation of the HPI axis begins at the hypothalamus (Fig. 8), which receives inputs transmitted from central and peripheral nervous systems. Several factors, including hormones, stress and negative feedback of cortisol at the level of the hypothalamus and the pituitary can modulate the hypothalamic neurosecretory nuclei in the preoptic area to secrete corticotropin-releasing hormone (CRH). The nerve endings of the CRH-ergic neurons terminate in fish near the corticotropic cells of the anterior pituitary, inducing the cleavage of adrenocorticotropic hormone (ACTH) from the larger proopiomelanocortin (POMC) protein precursor. In response to CRH, the corticotrops release ACTH into the bloodstream, where it circulates to the interrenal cells dispersed throughout the head kidney (homologous in fish to the adrenal gland in mammals). ACTH binds to the melanocortin 2 receptor (MC2R) on the interrenals and activates cortisol biosynthesis and release (Aluru and Vijayan, 2008). Cortisol acts on target tissues by binding to the glucocorticoid receptor (GR), a ligand-activated

nuclear transcription factor. GR binds to glucocorticoid response elements (GREs) in the DNA upstream of target genes involved in glucose metabolism, osmoregulation, immune function and behaviour (Mommsen *et al*., 1999; Bury and Sturm, 2007). Cortisol also binds to MR, but the contribution of GR and MR signaling to cortisol action in target tissues is unclear in fish (Vijayan *et al*., 2005; Bury and Sturm, 2007).

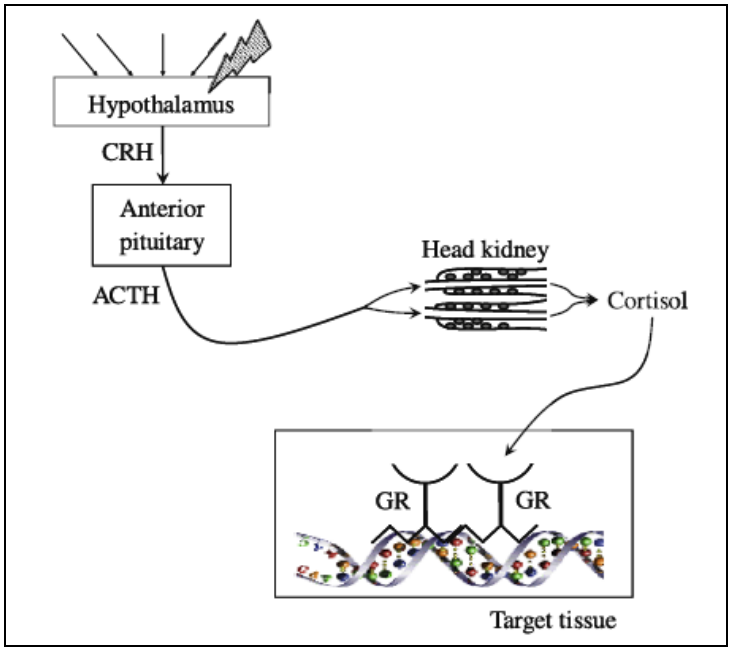


Fig. 8. A brief overview of the HPI axis. Upon exposure to a stressor, the hypothalamus secretes CRH in the area of the corticotropic cells of the anterior pituitary. In response, the corticotrops secrete ACTH into the circulation, which stimulates cortisol synthesis and secretion from the interrenal cells of the head kidney in fish. Cortisol binds to the GR and regulates the expression of target genes. Two GRs have been identified in all teleosts examined except zebrafish.

The occurrence of cortisol in eggs suggests maternal deposition of this hormone in eggs from the circulation during oogenesis (Barry *et al*., 1995a; Hwang *et al*., 1992; Stouthart *et al*., 1998). Ontogeny of zygote steroid biosynthesis has been reported in many fish, but mostly after hatching, such as tilapia (*Oreochromis mossambicus*) after 1 day (Hwang and Wu 1993; Hwang *et al*., 1992), and milkfish *(Chanos chanos)* after 3 days (Hwang *et al*., 1992). However, the capability of the interrenal tissue to produce corticoids does not necessarily mean that the HPI axis as a whole is integrated and capable of responding to stress with an increase in cortisol production. The interrenal tissue of rainbow trout produced significant

cortisol in response to adrenocorticotropin ($ACTH^{1-24}$) before and after hatching *in vitro* (Barry *et al*., 1995a) and endogenous cortisol was observed already 1 week after hatching *in vivo* (Barry *et al*., 1995b). However, only until 2 weeks after hatching the HPI axis was found to be functionally integrated and responsive to stress *in vivo* (Barry *et al*., 1995b). In common carp, whole body cortisol levels are increased by stressors (mechanical pressure) only from 50 hpf onwards, indicating that the HPI axis is fully functional at the time of hatching. Thus, it is suggested that the functional integration of the HPI axis occurs concomitantly with the maturation of the hypothalamic or sensory components of the system, rather than at the level of interrenal cells, as also suggested by studies in mammals (Stouthart *et al*., 1998). On the other hand, the development of the corticoid system during the early life stages of vertebrates is not well understood. Specifically, little is known about the precise timing of the activation of cortisol synthesis or the molecular mechanisms involved.

8. The glucocorticoid receptor in zebrafish

In humans, two glucocorticoid receptor (GR) splice variants occur: hGRα and hGRβ, which are identical between amino acids 1-727 and then diverge. hGRα is the canonical GR that contains 50 specific C-terminal amino acid residues (to a total of 777 aa) and form two helical structures. hGRb contains 15 specific C-terminal amino acid and lacks hGRα's transactivational activity. Thus, hGRα plays an important role in ligand binding, while hGRβ acts as a dominant-negative inhibitor of hGRα (Schaaf *et al*., 2008). Interestingly, a GRβ-isoform has been detected not only in humans, but also in zebrafish, as described below.

In fish, Ducouret and colleagues have isolated the first full-length cDNA of a piscine glucocorticoid receptor (rtGr1) from rainbow trout (*Oncorhynchus mykiss*) in 1995 (Bury *et al*., 2003). The discovery of a second trout Gr isoform (rtGr2), which showed significant sequence disparity in the A/B domain of the gene, indicated that the two isoforms (Gr1 and Gr2) are encoded by two distinct genes, both of which are sensitive to cortisol (Bury *et al*., 2003). Until now, two *gr* genes have been found in most teleostean fish, including the cichlid Burton's mouthbrooder (*Haplochromis burtoni*) (*Hbgr1* and *Hbgr2*; Greenwood *et al*., 2003), and the European sea bass (*Dicentrarchus labrax*, L.) (*gr1*, Terova *et al*., 2005 and *gr2*, Vizzini *et al*., 2007). In the latter species, the two different receptors encoded by these genes

display high amino acid sequence identity, especially in the part of the gene coding for the C-terminal part. Differential regulation of the expression of Gr1 and Gr2 has been observed after stress and immune challenges. It has been showed that Gr2 is active at low basal cortisol levels, whereas Gr1 is the 'stress receptor' that becomes active at higher circulating cortisol concentrations (Stolte *et al*., 2008a)

Conversely, only one *gr* gene has been found in some fish species, such as the Japanese flounder (*Paralichthys olivaceus*), brown trout (*Salmo trutta*) (Stolte *et al*., 2006) and zebrafish (Stolte *et al*., 2006; Schaaf *et al*., 2008; Alsop and Vijayan, 2008). The analysis of the syntenic regions of the fish *gr* genes shows that the genomic region surrounding the zebrafish *gr* gene is well conserved and is highly similar to the region surrounding the *gr2* gene of fugu, tetraodon, medaka, rainbow trout and stickleback in a phylogenetic tree (Fig. 9). The loss of the *gr1* gene copy in zebrafish has happened relatively late in the evolution of the species (Stolte *et al*., 2008b).

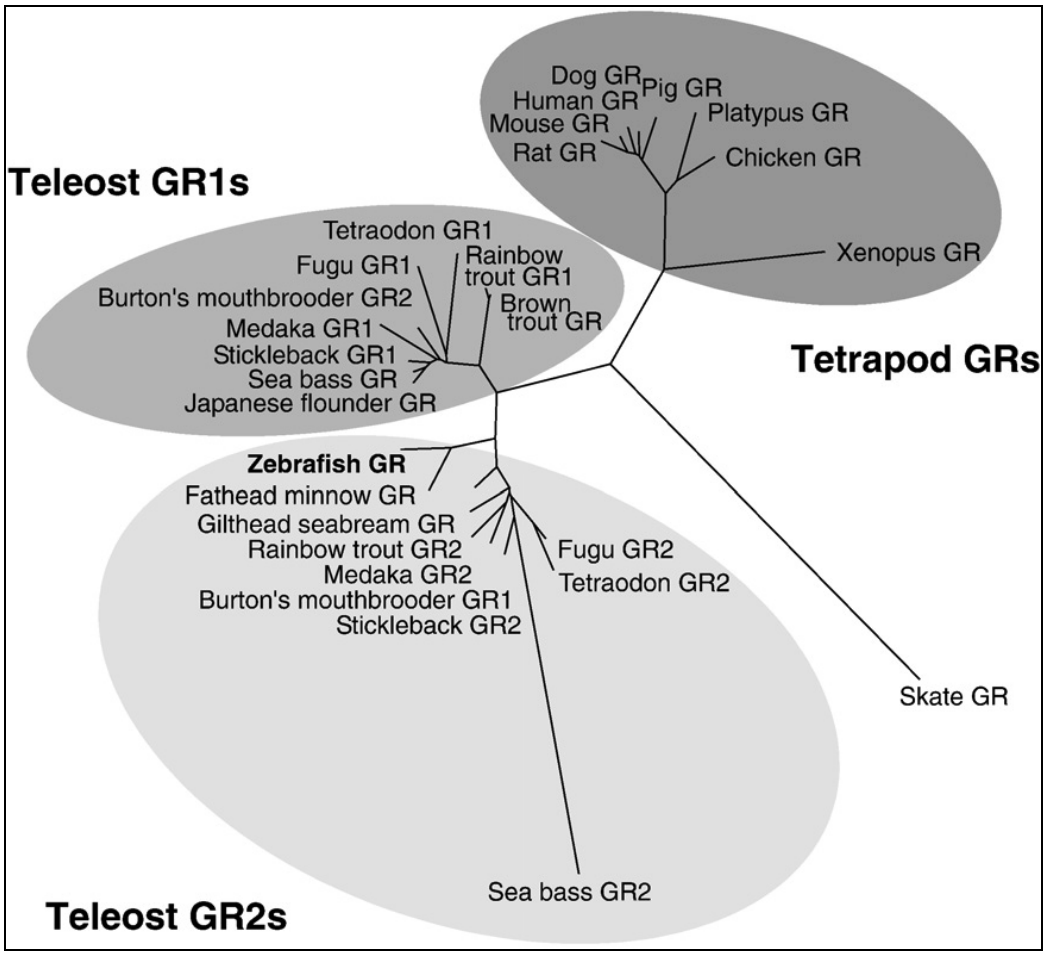


Fig. 9. Phylogenetic tree of the teleost fish and tetrapod GRs. The zebrafish GR clusters within the GR2 clade of teleostean GRs (Schaaf *et al*., 2009)

The structure of *gr1* and *gr2* genes in fish is highly similar to the structure of the human *GR* gene. At the protein level, fish Grs display a high level of similarity to the human GR as well. In the ligand-binding domain, between 85-95% of the amino acids of fish Grs are similar to those in the human GR and in the DNA binding domain this number exceeds 98% for most fish Grs studied (Fig. 10).

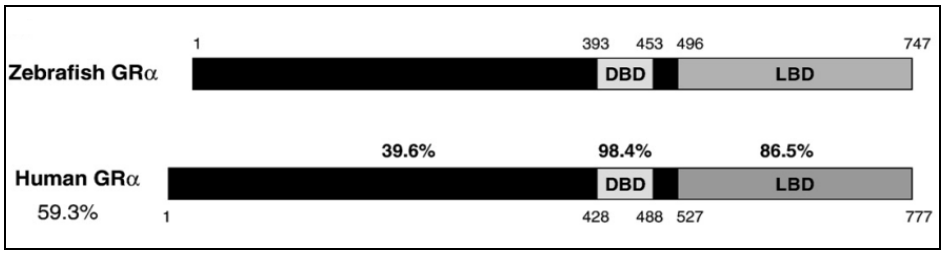


Fig. 10. Comparison between the human and zebrafish GR α-isoforms. GR possess the four functional domains common to nuclear hormone receptors: a large N-terminal domain, involved in transcriptional activation, a DNA-binding domain (DBD) which contains two zinc-fingers and a ligand-binding domain (LBD). Percentages indicate the fraction of amino acids similar between human and zebrafish per domain. The overall level of similarity is 59.3% (Schaaf *et al*., 2009).

The human and zebrafish *GR* genes are composed of 9 exons, of which the first is entirely noncoding and the ninth contains the 3'UTR (Fig. 11). Another remarkable characteristic of the zebrafish *gr* gene is the possibility of alternative splicing, which results in a Gr isoform that is identical to the canonical Gr in the N-terminal domain, the DNA binding domain and most of the ligand binding domain, but contains a different amino acid sequence at its C-terminus. This isoform is called the zebrafish Grβ, since it highly resembles the human GRβ isoform. The zebrafish Grβ is similar to its human equivalent in structure, function and expression level. The zebrafish Grα and β isoforms are identical between amino acids 1 and 696. Sequence alignment of the human and zebrafish GRs show that the divergence point between the α and β isoforms is identical in humans and zebrafish.

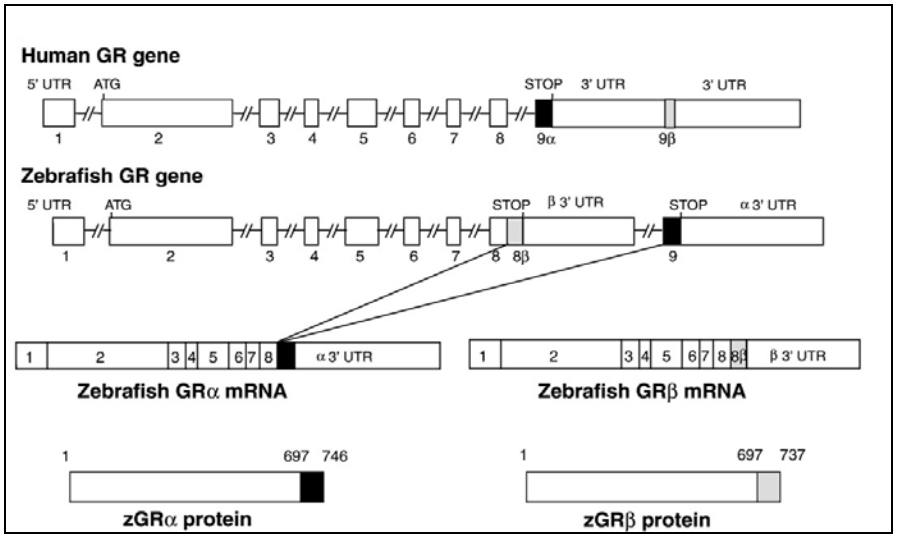


Fig. 11. Comparison between the human and zebrafish GR genes. Both genes contain 9 exons. A remarkable difference is the location of exon 9β in human and exon 8β in zebrafish.

9. The expression level of *z-grα* and *z-grβ* mRNAs

Schaaf and his colleagues (2008) demonstrated that the expression levels of *z-grα* mRNA in adult tissue (Fig. 12A) and embryos at different developmental stages (Fig. 12B) were significantly higher than those of *z-grβ* mRNA. Interestingly, the relatively low expression levels of *z-grβ* were similar to those observed in human cells tissues (Oakley *et al*., 1996; Dahia *et al*., 1997), in which significantly lower levels of *z-grβ* mRNA are found compared with *z-grα*. However, the low level of *hGRβ* mRNA expression is not reflected at the protein level, probably due to the greater stability of the *hGRβ* protein.

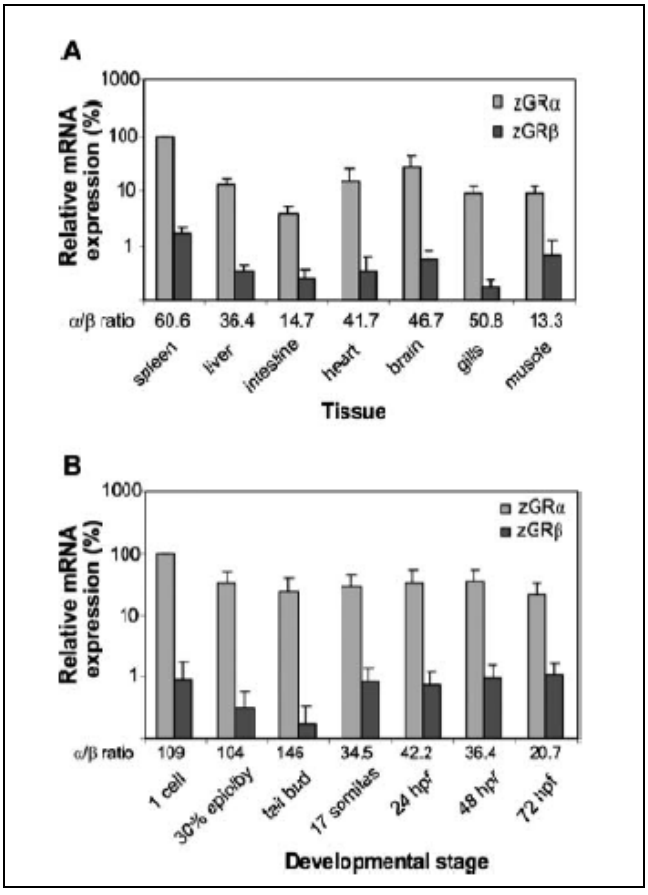


Fig. 12. The expression of *z-grα* and *z-grβ* mRNAs (A: zebrafish adult tissues; B: different developmental stages of zebrafish embryo) (Schaff *et al*., 2008).

10. Aim of the study

10.1. *Experimental Part I*

The objective of the first experiment was to determine the dynamics of maternal liposoluble hormone receptor mRNAs for progesterone (*pr*), androgen (*ar*), estrogen (*erα*, *erβ1* and *erβ2*), glucocorticoids (*gr*), mineralocorticoids (*mr*) and membrane progestin receptor-α and β (*mprα* and *β*) in zebrafish ovulated oocytes and embryos sampled at 8 time points from fertilization up to hatching (48 hpf). The experiment was performed by qRT-PCR and/or RT-PCR.

10.2. *Experimental Part II*

In this study, I intended to apply antisense technology against a steroid hormone receptor to evince its roles on embryonic development. Thus, the subsequent study has been focused on zebrafish Gr using antisense morpholino oligonucleotides (MO) to disrupt targeted *in vivo* *gr* mRNA translation, in order to examine the effects of MO-treatment on gene transcription and gross morphology following mRNA knockdown. Gene expression analysis was performed by a specifically designed cDNA microarray containing a set of target transcripts that were up- or down-regulated with respect to the embryos obtained from control groups. Furthermore, macroscopical changes due to the receptor mRNA inactivation were analyzed using morphological analysis.

MATERIALS AND METHODS

11. Zebrafish (*Danio rerio)* maintenance

Zebrafish of wild-type strain, purchased from local pet shops, were used and maintained as described in Westerfield (1995) and Lawrence (2007). Oocytes were obtained by stripping and embryos by natural mating with subsequent culture in an incubator at 28.5°C with a photoperiod of 12 h light/12 h dark.

To analyze the abundance and degradation rate of maternal mRNAs for nine steroid hormone receptors and their possible replacement by corresponding embryonic transcripts (for the list see result's section 31), three replicate samples were taken at each of 9 time points comprising unfertilized eggs and embryos collected at 0, 1, 2, 4, 8, 12, 24, and 48 hpf. Such time points correspond to the following 7 developmental stages, according to Kimmel and co-workers (1995): maternal stage (unfertilized eggs), zygote stage (0 hpf), cleavage stage (1 and 2 hpf), blastula stage (4 hpf), gastrula stage (8 hpf), segmentation stage (12 hpf), and pharyngula stage (24 and 48 hpf), when embryonic development terminates and hatching occurs (Mathavan *et al*., 2005).

For the other analyses performed in this thesis, time points of the samplings are reported in the corresponding result's section.

12. Total RNA extraction

Total RNA was extracted from pools of 20-60 embryos at the desired stages of development using TRIzol reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. The number of embryos was chosen according to the size of embryos at each time point.

Briefly, each sample is homogenized in 1 ml of TRIzol reagent and incubated at RT for 5 min to permit the complete dissociation of nucleoprotein complexes. The homogenates are then transferred into new 1.5-ml tubes and added with 200 µl of chloroform. The tubes are shaken vigorously by hand for 15 s, incubated for 2-3 min at RT and centrifuged at 12000 *g* for 15 min at 4°C. Following centrifugation, the mixture is separated into 3 phases: a lower

red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the upper aqueous phase. The aqueous phase is transferred to a new tube and 500 µl of isopropanol is added to each tube. Tubes are incubated at -20°C for at least 3 h. After incubation, the samples are centrifuged at 12000 *g* for 15 min at 4°C. The supernatants are carefully discarded and RNA pellets are washed twice by adding 1 ml of 70% ethanol and centrifuging at 7500 *g* for 15 min at 4°C. The RNA pellets are dried under chemical hood about 5-10 min and re-suspended in 20-30 µl of nuclease-free H_2O. RNA samples are stored at -80°C.

13. Reverse transcription of total RNA

Reverse transcription of total RNAs was performed using ThermoScript™ RT-PCR system (Invitrogen) according to the manufacturer's instructions.

Briefly, 2 µg of the purified total RNA are mixed with Mix I reaction, incubated at 65°C for 5 min and then placed on ice. After quick chilling, 8 µl of Mix II are added to each sample. The samples are incubated at 25°C for 10 min, 55°C for 45 min and 85°C for 5 min. Then, 1 µl of Rnase H (2 U/µl) is added and the mix is incubated at 37°C for 20 min more. The reactions are diluted with water to 40 µl and stored at -20°C. In a standard PCR protocol, 2 µl of reverse transcription product are used as template.

Mix I	
Component	**Amount**
Random hexamers	1 µl
Total RNA (2 µg)	X µl
dNTPs mix (10mM)	2 µl
DEPC-treated water	to 12 µl

Mix II	
Component	**Amount**
5X cDNA synthesis buffer	4 µl
DTT (0.1M)	1 µl
RNase OUT (40U/ µl)	1 µl
RT thermoscript (15 units/ µl)	1 µl
DEPC H_2O	1 µl

14. Amplification of DNA by PCR (polymerase chain reaction)

PCR is the standard method for amplifying DNA with known flanking sequences. Usually the reaction was carried out in a 0.2 ml PCR reaction tube. Biotherm *Taq* polymerase (Società Italiana Chimici, SIC, Rome, Italy) was routinely used for the PCR amplifications.

Generally, total cDNA (usually corresponding to 100 ng of total RNA) was amplified by PCR containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5-2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of the respective primers, 1.25 U of Biotherm *Taq* DNA polymerase (SIC) and ultrapure water to a final volume of 25 μl. Touch-down amplification was performed with annealing temperatures decreasing from 62-60°C to 52-50°C over 10 cycles, while the subsequent 30 cycles were maintained at the lower temperature. For each PCR, a negative control was prepared by replacing the cDNA solution with sterile water to check for cross-contamination. PCR products for each gene of interest were sequenced to confirm amplicon identity.

At the end of the PCR reaction, samples were kept at -20°C. After the PCR reaction , 6-8 μl of each product were analyzed by agarose gel electrophoresis. For cloning of amplified PCR products, DNA fragments of interest were excised form the gel and isolated as described.

Table 3. List of primers used.

Primers	Sequence	Position	Product size (bp)	Accession number
pr-F	5′-GCAGATGATCAGCATCCTG- 3′	+ 375 → + 393	359	DQ017620
pr-R	5′-GGACAAACTGCATAGCCA- 3′	+ 716 → + 733		
ar-F	5′-GCTGCTCTTCTCACCAG- 3′	+ 2298 → + 2314	323	EF440290
ar-R	5′-CGAGAGATGCTTCATCTG- 3′	+ 2562 → + 2579		
erα-F	5′-CTGTCTGCTCACGACAG- 3′	+ 1067 → + 1084	323	AF349412
erα-R	5′-AGCCACAGTTGCTAAGAG- 3′	+ 1373 → + 1390		
erβ1-F	5′-GTGCCATGACTACGCTTCTG- 3′	+ 580 → + 599	275	AJ414566
erβ1-R	5′-CGCCAGAGCTGTCTCTG- 3′	+ 838 → + 854		
erβ2-F	5′-CAGACCTCTGTCTCAGCA- 3′	+ 1421 → + 1438	224	AF516874
erβ2-R	5′-CAGCAGACACAGCAGCTT- 3′	+ 1624 → + 1641		
gr-F	5′-GACAGCACTATACCAGACAC- 3′	+ 1807 → + 1826	300	EF567112
gr-R	5′-CTTCAACATCTGTTCACAC- 3′	+ 2088 → + 2103		
mr-F	5′-AGAAGTGGTGTTCGCTG- 3′	+ 2532 → + 2548	341	NM_001100403
mr-R	5′-GAACAAACTCCTGGCTCA- 3′	+ 2588 → + 2872		
mprα-F	5′-CATGGCTTCCGGTTAATTGTC- 3′	+ 10 → + 30	205	AY149121
mprα-R	5′-GCCCAATCTGCTCCATCA - 3′	+ 197 → + 215		
mprβ-F	5′-CTGGCATGCTCAGCGCTG - 3′	+ 21 → + 38	240	AY149120
mprβ-R	5′-GACCCAGCTGCTGCAGAC - 3′	+ 243 → + 261		
ef1α-F	5′-GACAAGAGAACCATCGAG- 3′	+ 169 → + 186	271	NM_131263
ef1α-R	5′-CCTCAAACTCACCGACAC- 3′	+ 430 → + 447		
z-*gr*-F	5′-GACAGCACTATACCAGACAC-3′	+ 1951 → + 1934	263	EF436284
z-*gr*-R	5′-CTTCAACATCTGTTCACAC-3′	+ 219 6→ + 2214		
z-*cent*-F	5′-GAGAGGCAGGAGTTCACG-3′	+ 401 → + 418	743	NM_001002715
z-*cent*-R	5′-GCTCATAGGTGTGCAGATGAG-3′	+ 1124 → + 1144		
z-*casp8*-F	5′-GGGCAAAGCTGGGAAGATC- 3′	+ 377 → + 395	569	AF273220
z-*casp8*-R	5′-CTCCGTGTGAGAGAATACAGC- 3′	+ 926 → + 946		
z-*igf-2a*-F1	5′-CTTTCCACAAACCGCCTGC- 3′	+ 92 → + 110	683	BC085623
z-*igf-2a*-R1	5′-GAAAAAGTGCCTCTACTGACC- 3′	+ 755 → + 775		
glu-F1	5′-ATGTCAGTGGAATGCAGC- 3′	+ 1394 → + 1411	-	EF436284.1
t-*gr*2-F1	5′-GTGTTCAGCCTGTATCCTC- 3′	+ 1372 → + 1390	-	NM_001124482
z-*casp-8*-R1	5′-CTTCTTCTAGAGGAAGTCTGC- 3′	+ 557 → + 577	-	AF273220
z-*cent*-R2	5′-CATCTCTGCTCTCGTCCTC- 3′	+ 443 → + 461	-	NM_001002715

15. Quantitative reverse-transcription and PCR (qRT-PCR)

Optimizations of reagent concentrations and amplification conditions were performed by conventional PCR. Reverse transcriptase reactions were carried out with 2 μg of total RNA combined with specific reverse primers for *erβ1* and *erβ2*, *ar*, *gr*, *mr* and *ef1α* (elongation factor 1α) mRNAs and SuperScript® VILO™ RT-PCR Synthesis Kit (Invitrogen). Reactions were performed at 25ºC for 10 min, 45ºC for 60 min and 85ºC for 5 min. The final cDNAs were kept at -20ºC before use.

To create the standard curves for qRT-PCR, sequenced PCR products were cloned into the pGEM-T Easy vector (see material and methods section 19) (Promega, Milan, Italy). The copy numbers of the plasmid DNA templates were calculated according to the plasmid molecular weight and then converted into copy numbers on the basis of Avogadro's number. Serial dilutions of 10^8–10^2 plasmids/μl were used to generate the calibration curves.

qRT-PCR analysis was performed on the same RNA samples used for RT-PCR. All samples were analyzed in triplicate in 20-μl volumes using DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Euroclone, Milan, Italy). PCR reactions were performed with the Applied Biosystems 7300/7500 Real Time PCR System (Applied Biosystems, Monza, Italy). Real-time PCR conditions were optimized after trying various times and temperatures for each cycling step as well as by establishing the concentrations of $MgCl_2$ (2.5 mM for receptor transcripts and 4 mM for *ef1α* mRNA), and forward and reverse primers (0.4 μM). The reaction conditions were as follows: enzyme activation at 95°C for 3 min followed by 40 cycles at 95°C for 1 min (denaturation) and 55ºC for 45 s (annealing and elongation). Threshold values for threshold cycle (Ct) determination were generated automatically by the Applied Biosystems software. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product. The results were reported as expression, after normalization, of the transcript amount with respect to the reference gene (*ef1α*). To correct for the increase of *ef1α* expression from early to late stages, the level of expression of *ef1α* within each group of samples was normalized to a randomly selected "control" group (UF), according to Billiau and co-workers (2001), Essex-Fraser and co-workers (2005) and Ings and Van der Kraak (2006), as follows:

$$\text{levels of expression of } ef1\alpha = \frac{\text{individual value within a stage}}{\text{mean value within a stage / mean value of control group}}$$

16. Agarose gel electrophoresis

DNA samples, obtained from PCR reactions, miniprep, gel extraction or enzymatic digestion, were analyzed by agarose gel electrophoresis. According to their presumptive size, samples were loaded on 0.8-1.5% agarose gel in TAE 1X containing ethidium bromide or GelRed™ (Biotium, SIC) both diluted 1:20000. The resulting bands were photographed in a UV chamber supplemented with a camera.

17. DNA purification

17.1. Phenol/chloroform extraction and precipitation of DNA

Phenol/chloroform extraction is an easy way to remove proteins from nucleic acid samples. Nucleic acids remain in the aqueous phase and proteins separate into the organic phase or lie at the phase interface. This protocol was applied for cleaning linearized plasmidic DNA used as template for the *in vitro* transcription of antisense RNA probes for whole mouth *in situ* hybridization or for RNA rescue experiments.

Briefly 50 µl of sterile water, 50 µl of chloroform and 50 µl of phenol are added to 50 µl of digestion product; the reaction mix is vortexed vigorously to mix the phases and centrifuged at 12000 *g* for 1 min. After centrifugation, the supernatant (aqueous phase) is transferred to a new tube and mixed with 50 µl of chloroform and 50 µl of phenol. The mix is vortexed and centrifuged at 12000 *g* for 1 min. Again, the supernatant is transferred into a new tube, mixed with 100 µl chloroform and centrifuged at 12000 *g* for 1 min. After transferring the supernatant into a new tube, 10 µl of 4 M NaCl and 250 µl of 100% ethanol are added and gently mixed. The sample is chilled at -80°C for at least 1 h. The sample is then centrifuged at 12000 *g* at 4°C for 20 min. The pellet obtained is washed with 70% ethanol, dried and resuspended in 10-15 µl of nuclease-free water H_2O.

17.2. Purification of nucleic acids by different kits

For purifying nucleic acids different commercial kits were used (PCR purification, Jet Quick, Euroclone; DNA clean and concentrator^{TM}-5, Zymo Research Corporation, SIC). The purification procedure was carried out as described in the manufacturer's manual. DNA was eluted from the column with nuclease-free water, typically in a volume of 8-30 µl.

18. Enzimatic digestion of plasmid DNA

Enzymatic digestion of plasmid DNA was used for analysis of cloning products and to linearize plasmids for antisense probe synthesis for *in situ* hybridization. In the latter case, restriction enzymes were chosen depending on the orientation of the insert in the vector DNA. All restriction enzymes used were from Promega.

The reaction mixes usually contain, in a total volume of 20-50 µl, between 5 and 15 U of restriction endonuclease, depending on the amount of DNA to be cut. After enzymatic digestion the fragments were gel-purified.

The reaction was set-up as follows:

Component	**Amount**
Restriction enzyme 10X buffer	1X
Acetylated BSA (10ug/ µl) 100X	1X
DNA	X µl (1-5 µg)
Restriction enzyme (10 U/ µl)	5-15 U
Nuclease-free H_2O to a final volume of	20-50 µl

The incubation temperature depends on the restriction enzyme used. The restriction enzymes employed in this work are listed in the table below.

Restriction Enzime	Recognition sequence
*Apa*I	GGGCC▼C
*Bgl*II	A▼GATCT
*Eco*RI	G▼AATTC
*Sal*I	G▼TCGAC
*Spe*I	A▼CTAGT
*Xba*I	T▼CTAGA
*Bam*HI	G▼GATCC
*Xho*I	C▼TCGAG
*Pst*I	CTGCA▼G

18.1. Gel purification of restriction fragments

The DNA was run on 1% agarose gel and the desired fragments were cut out from the gel under UV light. Then, the gel slices were purified with the commercial kit Wizard SV Gel and PCR Clean-Up System® (Promega), as described in the manufacturer's manual. The DNA samples were eluted with 15-30 µl of nuclease-free water and kept at -20ºC.

19. Cloning

19.1. Ligation

DNA fragments were ligated into pGEM®-T Easy vector using the pGEM®-T Easy vector system (Promega), according to the manufacturer´s instruction manual. To calculate the appropriate amount of DNA fragments to include in the ligation reaction, we used the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert} \times \text{insert : vector molar ratio}}{\text{kb size of vector}} = \text{ng of insert}$$

The reaction is usually settled as follow:

Component	Amount
2X rapid ligation buffer, T4 DNA ligase	5 µl
pGEM®-T Easy vector (50 ng)	1 µl
PCR product	X µl
T4 DNA ligase (3 U/ µl)	1 µl
Nuclease-free water	to 10 µl

The reaction is incubated at 4ºC overnight, and then used to transform chemically-competent bacteria.

19.2. Bacterial transformation

To obtain transgenic bacteria, DNA plasmids from the ligation step were transferred into competent *E. coli* cells (JM109 from Promega or Match 1 from Invitrogen) by heat shock induced transformation.

Briefly, competent cells are thawed on ice, mixed with 2 µl of the ligation reaction and incubated on ice for approximately 30 min. The suspension is then heated to 42°C for 30-40 s. Subsequently the suspension is incubated on ice for 1 min; 250 µl of SOC medium are added and the sample is shaken at 37°C for 1 h. The Ampicillin LB-Agar plates are prepared with 80 µl of X-Gal (20 mg/ml) and 100 µl of the inductor IPTG (0.1 M) and equilibrate at RT. Fifty µl and 100 µl of the transformation culture are subsequently plated on LB-plates that are incubated at 37°C overnight. Bacteria carrying a plasmid without insert possess a functional ß-galactosidase gene and produce blue colonies due to the X-Gal reaction. In contrast, bacteria containing a plasmid with the insert of interest present disrupted β-galactosidase gene and produce white colonies.

19.3. Identification of positive colonies by PCR reaction

Single white colonies were picked from the plates with pipette tips. The single colony presents on the tip was then both streaked onto a fresh replicate LB-plate as well as added to the PCR Mix I. The PCR tubes were incubated at 98°C for 10 min for destroying the bacterial cells. Then the PCR mix II, containing the *Taq* polymerase, was added.

The reaction was done as follow:

PCR Mix I	
Component	**Amount**
10X buffer reaction	2 µl
MgCl2 (50 µM)	1 µl
dNTPs (10mM)	0.6 µl
Primer Forward (10µM)	0,6 µl
Primer Reverse (10µM)	0,6 µl
Nuclease-free water	to 20 µl

PCR Mix II	
Component	**Amount**
10X buffer reaction	2 µl
Biotherm *Taq* (1.25 U)	0.2 µl
Nuclease-free water	to 10 µl

The program used is as follow:

Temperature	Time	Cycles
95ºC	2 min	1
95ºC	45 s	35
55ºC	1.10 min	
72ºC	2 min	
72ºC	10 min	1
20ºC	2 min	1

19.4. Purification of plasmidic DNA (miniprep)

Plasmidic DNA that contains the insert of interest was purified by plasmid mini-preparation using High pure plasmid isolation kit (Roche, Milan, Italy) according to the manufacturer's instructions. Briefly, single cell colonies are cultured in 5 ml of LB-medium supplemented with 50 µg/ml ampicillin, and incubated on a shaker (200 rpm) at 37°C for 16 h. The bacteria are separated from the medium by centrifugation of 5 ml of each culture in 2 ml tubes at maximum speed for 1 min. The pellets are chemically treated to separate bacterial chromosomal DNA and proteins from the plasmid DNA. This is eluted with 30 µl of nuclease-free water and then further characterized by restriction digestion and/or sequencing.

20. DNA sequencing

DNA sequencing was performed by BMR Genomics (Padova, Italy). For this purpose a mix containing the specific primer (3.2 pmol) and the required amount of purified DNA was prepared. The mix was dried at 65ºC and then delivered to BMR for sequencing.

The purified DNA was prepared by gel extraction or, when a single band is visualized on agarose gel, it can be purified by the following enzymatic reaction:

- PCR product (volume variable with the quantity of PCR product)
- Exonuclease I (5 U)
- Phosphatase (1 U)

The reaction was carried out as follow:

37 ºC for 15 min

80 ºC for 15 min

4 ºC for 10 min.

21. Knockdown experiments

Knockdown experiments were performed by microinjection of antisense oligonucleotides called Morpholino. MO oligonucleotides were designed and synthesized by Gene Tools, Philomath, OR, USA (www.gene-tools.com). The sequences of MOs used in this work are as follows:

*gr*MO-ATG-1: 5'- CATTCTCCAGTCCTCCTTGATCCAT- 3'

*gr*MO-ATG-2: 5'- CGTTCCTGTTTTGATTTGTTGCATC- 3'

*gr*MO-SP: 5'- GCCAGAGATATATATGGAATACCTTCA- 3'

gr-5mmMO: 5'- CATT**g**TCCA**c**TCCT**g**CTT**c**ATC**g**AT- 3'

*gr*MO-ATG-1 and *gr*MO-ATG-2 were designed around the start codon (ATG) of zebrafish glucocorticoid receptor coding sequence (Accession number EF567112). *gr*MO-SP is an exon-skipping morpholino: the use of this morpholino determine the skipping of exon 3 and the loss of the translation reading frame.

Moreover, the mutated morpholino *gr*-5mmMO is the specific negative control of *gr*MO-ATG-1.

Morpholino oligos were delivered as a prequantitated, sterile, salt-free, lyophilized solid in a glass vial. The stock solutions (8 mg/ml) was prepared with nuclease-free water, as advised by Genetool's protocols, and kept at RT temperature. Morpholino working solutions were prepared diluting stock solutions in Danieau 1X (see buffer composition), and adding Phenol Red (Sigma, Milan, Italy) to make the solutions visible during microinjection. Different MO concentrations were tested in a range between 0.5 and 2 μg/ml.

21.1. Needle preparation

Injection needles were pulled from 1.2 mm thin walled glass capillaries (World Precision Instruments, WPI, Germay) with a pipette puller (Flaming/Brown p-97 Micropipette Puller, Setter Instruments, Crisel Instruments, Rome Italy) and the parameters were set as follows: heat = 395, velocity = 60, time = 50, pull = 60. Before injection, embryos were collected and placed aligned against a clean glass slide in the lid of a Petri dish. Injections were performed on a dissecting microscope using a micromanipulator attached to a microinjector (Leica Microsystems, Milan, Italy) controlled by a pedal to inject the desired amount of reagent. The samples (MO and mRNAs) were loaded with GELoader tips 0.5-20 μl (Eppendorf, Milan, Italy) into the injection needles. Then, the tip of injection needles was broken with dissection forceps. The samples (MO and mRNA) were injected into the yolk sac of 1 or 2-cell stage embryos. After injection, embryos were incubated in 1X fish water (see buffer composition) and kept at 28.5ºC in an incubator. Morpholino- and/or mRNA-injected-embryos were raised to the desired stages for observations or collected for further analysis.

22. Histological methods

22.1. Embryo preparation

The embryos were embedded and oriented in the desired position in 1% low melting temperature agarose (LMT) (Promega) modifying a protocol gently offered by Dr. Nicola Modena. The agarose embedded embryos were cut as small cubes and were subsequently transferred to 15 ml falcon tube and kept on ice. The agarose embedded embryos were subsequently dehydrated as follow:

- 25 % ethanol for 30 min

- 50 % ethanol for 30 min
- 75 % ethanol for 30 min
- 80 % ethanol for 30 min
- 90 % ethanol for 30 min
- 100 % ethanol for 2 x 15 min
- 50 % ethanol and 50 % buthanol for 20 min
- 100 % buthanol for 2 x 20 min
- 50 % butanol and 50 % paraffin for 2 x 20 min
- 100 % paraffin for 2 x 20 min

Agarose blocks containing embryos were oriented and embedded in Paramat (BDH, VWR, Milan, Italy). Then embryos were sectioned at 10 μm using a rotary microtome (Rotary-One LKB, Australia). The slides were subsequently rehydrated as following:

- Xylene for 5 min
- Absolute ethanol for 3 min
- 80 % ethanol for 3 min
- 80 % ethanol for 3 min
- Distilled water for 3 min

22.2. Staining

The slides were stained with Hematoxylin and Eosin following the standard protocol.

- Hematoxylin of Mayer for 3 min
- Rinse with distilled water
- Tap water *(to allow stain to develop)*
- Rinse with distilled water
- Eosin for 30 s
- 80 % ethanol for 3 min
- 95 % ethanol for 3 min
- 100 % ethanol for 3 min
- Xylene for 5 min

23. Whole mount *in situ* hybridization

23.1. Probe preparation by *in vitro* transcription

gr-MO- and *gr*5mmMO-injected embryos were analysed by whole mount *in situ* hybridization for potential patterning defects using several developmental markers. The markers are listed in the table below.

Table 4. List of markers used in the whole mount *in situ* hybridization.

Gene	Vector	Endonuclease	RNA polymerase	Reference
chordin	pBluescriptKS(+)	*Spe*I	T7	Miller-Bertoglio *et al*., 1997
egr2b	pBluescriptKS(+)	*Pst*I	T3	Oxtoby and Jowell, 1993
pax2a	pGEM-3	*Bam*HI	T7	Krauss *et al*., 1991
emx1	pCS2	*Bam*HI	T7	Kawahara *et al*., 2002
six3a	pT7 Blue (R)	*Xho*I	T7	Wargelius *et al*., 2002
myod1	pBluescriptKS(+)	*Bam*HI	T7	Weinberg *et al*., 1996
z-gr	pCS2	*Bam*HI	T7	

Five µg of plasmidic DNA were linearized with the appropriate restriction enzyme. Then phenol/chloroform extraction and precipitation were performed to clean linearized plasmid, as described before (see paragraph 17.1.). Linearized DNA was checked on agarose gel to ensure that the plasmid was completely linearized.

Transcription of probes for *in situ* hybridization was performed using the SP6/T7 Transcription Kit (Roche).

Component	**Amount**
10X Buffer	2.0 µl
DTT 0,1 M	1.0 µl
Dig RNA labeling mix 10X	2.0 µl
RNAseOUT (10 U/µl)	1.0 µl
Linearized DNA	X µl (1 µg)
T7/Sp6 RNA polimerase (15-20 U/µl)	1 µl
DEPC H_2O	to 20 µl

After the addition of all reagents the sample was incubated at 37°C, for 2 h. The DNA template was then digested by adding 2 µl of DNAse, RNAse-free, and incubating the sample at 37°C for 15 min. The DNAse digestion was then stopped by adding 1 µl of 0.5 M EDTA.

The newly transcribed RNA was precipitated by adding 2.5 µl 4 M LiCl and 75 µl Et-OH (100%) for at least one h (or overnight) at -80°C. After precipitation, the RNA was pelleted by a 20 min centrifugation at 12000 *g* at 4°C, washed with 70% ethanol and re-suspended in 20 µl of RNase-free water.

23.2. Fixation and storage of embryo

Embryos were fixed in 4% PFA (Paraformaldehyde) overnight at 4ºC (or 2 h at RT) at the desired stage of development. For embryos older than 24 hpf, pigmentation was suppressed by raising embryos in 0.03% PTU (1-phenyl-2-thiourea, Sigma) in fish water. Afterwards, the embryos were washed in PBT (PBS plus 0.1% Tween 20, Sigma) and dechorionated by using forceps. Subsequently they were transferred in 100% methanol and stored at -20ºC for at least 1 h for dehydration. Embryos could be stored in this way for several months. All *in situ* hybridization washes should be done in approximately 500 µl of solution.

23.3. Day one: pre-hybridization treatments and hybridization

Embryos were rehydrated through a methanol series:

- 75% methanol in PBS for 5 min;
- 50% methanol in PBS for 5 min;
- 25% methanol in PBS for 5 min;
- 1X PBT for 5 min.

To obtain better permeability of the tissues for RNA probes and antibodies, fixed embryos were treated with Proteinase K (10 µg/ml in PBT) at RT. The length of the digestion was adjusted according to the developmental stage of the embryos (see table 5). Digestion was stopped by post-fixing embryos in 4 % PFA for 20 min. After post-fixing, samples were washed in PBT 1X for 5 min.

Table 5. Time of proteinase K digestion according to the developmental stages.

Developmental stages	Time of Proteinase K digestion
Blastula and gastrula	30 s
Early somitogenesis stages	1 min
Late somite stages (14-22 somite stages)	5 min
24 hpf embryos	15 min
48 hpf embryos	30 min
72 hpf embryos	45 min

After the washes in PBT, embryos were incubated in hybridization mix (HM: 50% formamide, 5X SSC, 500 µg/ml tRNA, 50 µg/ml heparin and 0.1% Tween 20) in a water bath for at least 2 h at 65ºC. Hybridization mix was then replaced with 200 µl of new hybridization mix containing 100-200 ng of DIG-labeled RNA probes.

23.4. Day two: post-hybridization washes

The embryos were washed in 500 µl of the following solutions at 65°C:

- 100% HM, (very brief wash);
- 75% HM /25% 2X SSC for 15 min;
- 50% HM /50% 2X SSC for 15 min;
- 25% HM /75% 2X SSC for 15 min;
- 2XSSC for 15 min;
- 0.2X SSC for 30 min.

Afterwards, the embryos were washed in 500 µl of the following solutions for 10 min each at RT:

- 75% 0.2X SSC/25% PBT;
- 50% 0.2X SSC/50% PBT;
- 25% 0.2 X SSC/75% PBT;
- 100% PBT.

After that, embryos were incubated at RT for several h with PBT containing 2% of sheep serum and 2 mg /ml of BSA.

Subsequently the embryos were incubated with Anti-Digoxigenin-AP antibody, conjugated with alkaline phosphatase (Roche), diluted 1:3000 in PBT containing 2% of sheep serum and 2 mg /ml of BSA and incubated at 4°C overnight.

23.5. Day three: staining

The day after, the antibody solution was collected and the samples were washed 6 times for 15 min in PBT, and 3 times for 5 min in NBT-BCIP staining buffer. The samples were then transferred into 24 well-plates and staining buffer was replaced with staining solution containing NBT and BCIP (see buffer composition). The reaction was developed in the dark at RT, until the proper staining intensity was reached. The staining was stopped by two washes in PBT (2 x 5 min) and fixed in 4% PFA at RT for 2 h.

23.6. Imaging

Whole-mount *in situ* hybridization-stained embryos were mounted in glycerol 80% in PBT, observed under a microscope Leica DMR and photographed with a digital camera Leica DC500. The images were processed using the program Adobe Photoshop 6.0. Live and fluorescent embryos were photographed using the digital camera Leica DC500 connected to a dissecting microscope MZFLIII (Leica).

24. Preparation of *t-gr2* RNA for microinjection (mRNArescue)

Total RNA was extracted from a sample of trout liver (*Oncorhynchus mykiss*) and used for the synthesis of *t-gr2* cDNA template by using Trizol reagent (Invitrogen) and ThermoScript™ RT-PCR system (Invitrogen), respectively, as described before. Full-length cDNA was amplified using primers containing overhangs with restriction sites for further specific subcloning into the expression vector pCS2+.

The primers used for the amplification of *t-gr2* cDNA are the following:

t-gr2-F: ***CGGGATCCCG***CCAGCAGACCTACATGTTCCT (restriction site of *Bam*HI is shown as bold italic letters)

t-gr2-R: ***GCTCTAGAGC***CACACAGTCATTTCTGCTGGA (restriction site of *Xba*I is shown as bold italic letters).

Full-length *t-gr2* cDNA was cloned into pGEM-T Easy (Promega,) and subcloned into pCS2+ expression vector between its *Bam*HI and *Xba*I sites. One positive clone, after complete sequencing to check for nucleotide changes that could alter the amino acidic sequence or interrupt the translation reading frame, was linearized with *Xba*I. Efficiency of linearization was checked on agarose gel electrophoresis and the linear DNA was purified using phenol/chloroform extraction (see paragraph 17.1.). Full length mRNA was *in vitro* transcribed and 5'-capped with the SP6 polimerase using the kit mMessage mMachine according to manufacturer's protocol (Ambion, Milan, Italy).

The reaction was the following :

Component	Amount
2X NTP/CAP	10 µl
10X Reaction Buffer	2 µl
Linear template DNA (1 µg)	X µl
Enzyme Mix	2 µl
Nuclease-free Water	to 20 µl

The reaction was incubated at 37°C for 2 h. The template DNA was digested at the end of the reaction with DNAse I, RNAse-free. The RNA was then precipitated in LiCl and subsequently dissolved in nuclease-free water. The RNA concentration was determined using

a NanoDrop Spectrophotometer (Thermo Scientific, Euroclone). The *in vitro* transcribed mRNA was stored at -80°C. For rescue experiments, 200 pg to 800 pg RNA were injected per embryo.

25. Western blotting

25.1. Sample preparation

Embryos were collected in 1.5 ml microfuge tubes (80-100 embryos) at the desired stages. Embryos were de-yolked as described by Link and co-workers (2006). Briefly, de-yolking buffer (see buffer composition)was added to embryos and the mixture was homogenized by pipetting up and down. Embryos were shaken for 5 min at 100 *g* to dissolve the yolk. Embryos were pelleted at 300 *g* for 30 s and the supernatant discarded. They were resuspended in protein buffer (see buffer composition) with protease inhibitor cocktail (see buffer composition) and shaken at 4°C for 30 min. Embryos were centrifuged for 20 min at ~12000 *g* at 4°C. Supernatant was moved to a fresh 1.5 ml microfuge tube.

Protein concentration was determined using BCA protein assay kit (Pierce, Euroclone) according to the manufacturer's instructions. Concentration of proteins was determined via measuring the optical density using a GeneQuant1300 UV/Visible Spectrophotometers (GE Healthcare Life Sciences, Milan, Italy) at the wavelength of 562 nm. The samples were then stored at -80ºC.

25.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was performed using the X-Cell *SureLock*™ Mini-cell system (Invitrogen). Pre-cast 9-10 % NuPAGE® Novex Bis-Tris Gels (Invitrogen) were used according to the manufacturer's instructions. Protein samples (30-40 µg) were mixed as follows:

Component	Amount
NuPAGE® LDS Sample buffer (4X)	2.5 µl
NuPAGE® reducing reagent (10X)	1 µl
Sample	X µl (30-40 µg)
Sterile water	To final volume 10 µl

Then, the samples were denatured at 70°C for 10 min and directly loaded onto the gels. 1X SDS running buffer was prepared from 20X NuPAGE® MOPS SDS running buffer and NuPAGE® antioxidant was added to reduce samples. Electrophoresis was performed at 150 V for 2 h at 4°C. The molecular weight of proteins was estimated by running pre-stained or non-pre-stained marker proteins (BenchMark™ pre-stained protein ladder and BenchMark™ protein ladder, Invitrogen).

25.3. Blotting and detection

After separation by SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Roche) using the XCell II ™ block module system (Invitrogen) at 35 V for 90 min. For transfer, gels were sandwiched between gel-sized-Whatman 3 MM papers soaked in 1X NuPAGE® transfer buffer:

- 2 pieces of Blotting pad;
- 1 piece of Whatman paper;
- PVDF western blotting membranes;
- SDS-polyacrylamide gel;
- 1 piece of Whatman paper;
- 2 pieces of Blotting pad.

After transfer, the membrane was dried at RT and could be kept at 4°C for several weeks. For detection, the membrane was activated in methanol for 1 min, sterile water for 2 min and TBS-T 1X for 5 min and then incubated in blocking solution (BSA 5% and 1% of γ-globulin in TBS-T 1X) at 4°C overnight. Primary antibodies used were anti-GR diluted 1:800 (P-20, sc-1002, Santa Cruz Biotechnology, DBA, Segrate, Milan, Italy) and anti-α tubulin, diluted 1:1000 (H-300, sc-5546, Santa Cruz Biotechnology). Primary antibodies were diluted in TBS-T 1X and incubated with membrane-bound proteins either for 2 h at RT or overnight at 4°C. Membranes were washed 4 times in TBS-T 1X for 10 min at RT. They were then incubated for 1 h at RT with the secondary horseradish peroxidase-conjugated antibody (goat anti-Rabbit IgG HRP, Immunopure®; Pierce) diluted at 1:20000. Subsequently, the membranes were washed 4 times in TBS-T 1X for 10 min at RT. The protein-antibody complexes were

detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions and exposed on CL-XPosure X-ray film (Pierce). Film exposure varied from 30 s to 10 min.

26. *In vitro* transcription and translation experiment

26.1. Preparation of *z-gr* RNA

The full-length *z-gr* cDNA was amplified using primers containing overhangs with restriction sites for further specific subcloning into the vector pCS2+.

- *z-gr*-F: ***CGGGATCCCG***GCAAAATGGATCAAGGAGGA (restriction site of *Bam*HI is shown as bold italic letters)
- *z-gr*-R: ***GCTCTAGAGC***CTGCTGTTGGGAGGAGATTC (restriction site of *Xba*I is shown as bold italic letters)

Full-length *z-gr* cDNA was cloned into pGEM-T Easy (Promega) and subcloned into pCS2+ expression vector using *Bam*HI and *Xba*I. One positive clone, after complete sequencing to check for nucleotide changes that could alter the aminoacidic sequence or interrupt the translation reading frame, was linearized with *Xba*I. Efficiency of linearization was checked on agarose gel electrophoresis and the linear DNA was purified using the specific kit, as described before (see paragraph 18.1).

26.2. *In vitro* transcription and translation of *z-gr* RNA

In vitro transcription and translation was carried out using the kit Sp6 TNT® quick coupled transcription/translation system (Promega) following the manufacturer's protocol. Briefly, 20 µl master mix were combined with 1 mM methionine, 1 µg DNA templates (for the rationale of the experiment see results, section 35.1.2.), 1 µl Transcend™ Biotin-lysyl-tRNA (Promega) and nuclease-free water to final volume of 25 µl. The reactions were incubated for 90 min at 30°C. After incubation the reaction mix was stored at -80°C until use. Three microliter of this reaction mixture was resolved on 9-10 % NuPAGE® Novex Bis-Tris Gels (Invitrogen). The translated proteins were analyzed by non-radioactive method using Transcend ™ colorimetric non-radioactive translation detection systems (Promega) following the manufacturer's protocol.

27. Microarray experiments

Agilent Whole Zebrafish Genome Oligo Microarrays (two-color; Agilent) was performed to analyze the gene expression of embryos treated with *gr*MO-ATG-1 after 5 and 10 hpf. RNA was isolated using Trizol reagent as described before. The quality control of RNA samples was performed using Agilent 2100 Bioanalyzer platform (Agilent Technologies, Cernusco, Milan, Italy) and analyzed using the Agilent 2100 Bioanalyzer expert software. The software generate a RNA Integrity Number (RIN) that represents integrity and overall quality of total RNA samples.

The hybridization procedure was performed by Milteny Biotech (Germany), according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 825 ng of the corresponding Cy3- and Cy5-labeled fragmented cRNA were combined and hybridized overnight (17 h, 65°C) to Agilent Whole Zebrafish Genome Oligo Microarrays 4x44K using Agilent's recommended hybridization chamber and oven. Finally, the microarrays were washed once with 6X SSPE buffer containing 0.005% N-lauroylsarcosine for 1 min at RT followed by a second wash with pre-heated 0.06X SSPE buffer (37°C) containing 0.005% N-lauroylsarcosine for 1 min. The last washing step was performed with acetonitrile for 30 s.

27.1. Microarray data analysis

Fluorescence signals of the hybridized Agilent Oligo Microarrays were detected using Agilent's DNA microarray scanner (Agilent). 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). For determination of differential gene expression, FES-derived output data files were further analyzed using the Rosetta Resolverâ gene expression data analysis system (Rosetta Biosoftware, Agilent).

28. Fluorescent staining with acridine orange

A stock of acridine orange (5 mg/ml) was diluted in fish water at 1:500. Dechorionated embryos at 24 hpf were dipped into this solution and incubated at 28.5°C for 20 min in the dark. The embryos were washed twice for 10 min in fish water and then photographed.

29. Bioinformatic tools

Bioinformatic tools used are listed in the table 6.

Table 6. List of bioinformatic tools

Entrez PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
GenBank	http://www.ncbi.nlm.nih.gov/Genbank/
NCBI Blast	http://www.ncbi.nih.gov/BLAST/
ClustalW	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html
BMR Genomic	http://bmr.cribi.unipd.it/
Primer 3	http://frodo.wi.mit.edu/primer3/
David	http://david.abcc.ncifcrf.gov/
Kegg	http://www.genome.jp/kegg/

30. Statistical analysis

Data of Real time PCR were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variances (Bartlett's test). As data were not homogeneously distributed, a Kruskal-Wallis ANOVA was performed, and the non-parametric Mann-Whitney *U*-test was used for pairwise comparisons. The Statistica 5.5 software package (StatSoft, Tulsa, OK, USA) was used for statistical analyses.

For the anatomical analysis of morphant phenotypes, the significance of the mean differences between various experimental groups was determined by one-way ANOVA followed by Post Hoc test analyses. A P value < 0.05 was considered as statistically significant.

31. Abbreviations

bp	base pairs
BCIP	5-bromo-4-chloro-3-indolyphosphate, toluidine salt
BSA	bovine serum albumine
°C	celsius
cDNA	complementary DNA
Cy3	cyanine-3 (green)
Cy5	cyanine-5 (red)
DAPI	4',6-diamidino-2-phenylindole
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpf	days post-fertilization
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
Fig.	figure
g	gram
g	gravity force
h	hours
HM	hybridization mix
hpf	hours post-fertilization
IPTG	isopropyl ß-D-1-thiogalactopyranoside
KCL	potassium chloride
kDa	kiloDalton
l	liter
MBT	midblastular transition
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mmol	millimole
MO	morpholino oligonucleotides
mRNA	messenger ribonucleic acid
NaCl	sodium chloride

NaOH	sodium hydroxide
NBT	4-nitro blue tetrazolium chloride
ng	nanogram
NGS	normal goat serum
nl	nanoliter
OD	optical density
PCR	polymerase chain reaction
PFA	paraformaldehyde
pg	picogram
pH	potential of hydrogen
PTU	1-phenyl-2-thiourea
RNA	ribonucleic acid
RNase	ribonuclease
RIN	RNA integrity number
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription and polymerase chain reaction
s	second
SD	standard deviation
SDS	sodium dodecyl sulfate
TAE	tris-Acetate-EDTA
Taq	*Thermos aquaticus*
TBS	tris-buffered saline
Tris	trishydroxymethylaminomethane
U	unit
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
WT	wild type
X-Gal	(5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

32. Buffer composition

- Agarose gel loading dye (6x): 30% glycerol, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol.
- Anti-DIG-AP: anti-DIG-AP (Roche) diluted 1:3000 in PBT with 2% sheep serum and 2 mg/ml BSA
- Danieau solution (1X): 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES pH 7.6
- Deyolking buffer: 55 mM NaCl, 1.8 mM KCl, 1.25 mM $NaHCO_3$
- Fish Water Stock (50X): 25g Istant Ocean, 39.25 g $CaSO_4$, 5 g $NaHCO_3$ for 1 l
- Fish Water (1X): 20 ml Fish Water Stock (50x), 200 µL of stock solution (0.1% w/v) of methylene blue in 1 l
- Paraformaldehyde 4% (PFA 4%): 4% paraformaldehyde in 1X PBS
- Hybridisation buffer: 60% formamide (deionised), 5xSSC, 6 mM citric acid pH 6, 0.1% Tween-20, 50 ug/mL yeast RNA, 50 µg/mL heparin
- NBT-BCIP staining buffer: 100mM TRIS HCL pH 9.5, 50 mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20
- NBT-BCIP staining solution: NBT-BCIP staining buffer, 4.5 µl NBT (100 mg/ml), 3.5 µl BCIP (50 mg/ml)
- NuPAGE® MOPS SDS Running buffer (20X): MOPS 50 mM pH 7.7, Tris base 50 mM, SDS 0.1%, EDTA 1 mM
- NuPAGE® LDS Sample buffer (4X) pH 8.5: Glycerol 10%, Tris base 141 mM, Tris HCl 106 mM, LDS 2%, EDTA 0.51 mM, SERVA® blue G250 0.22 mM
- PBS: 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145 mM NaCl.
- PBT: 1X PBS, 0.1% Tween 20.
- Protein buffer: 40 mM K_2HPO_4, 10 mM KH_2PO_4, 65 mM KCl, 1 mM EDTA, pH 8

- Protease inhibitor cocktail: 1mM DTT, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 2 μg/ml aprotinin
- SDS runing buffer (1X): 50 ml MOPS SDS Running buffer 20X, 950 ml of distilled H_2O
- SSC (20x): NaCl 175 g, Na citric acid 88.2 g
- TAE-buffer (10X): 400mM Tris-Acetate, 11.4 ml glacial acetic acid, 10Mm EDTA
- TBE (20x): 1.75% (w/v) NaCl, 0.88% (w/v) sodium citrate, pH 7.0, 1.8 M Tris, 1.8 M boric acid, 0.05 M EDTA, pH 8.3
- TBS-T 1X: 100 ml TBS 10X , *Tween* 20 0,05 % for 1 l

RESULTS

33. Expression analysis of steroid hormone receptor mRNAs during zebrafish embryogenesis

33.1. RT-PCR

This protocol was used for a preliminary analysis of all steroid receptor transcripts considered in this work. Then, five transcripts (*erβ1* and *2*, *ar*, *gr* and *mr*) showing high expression levels or specific expression patterns, were selected for qRT-PCR. The following steroid receptor transcripts were analysed only by RT-PCR: *erα*, *pr*, *mprα* and *β*. The results obtained are presented in Fig. 13.

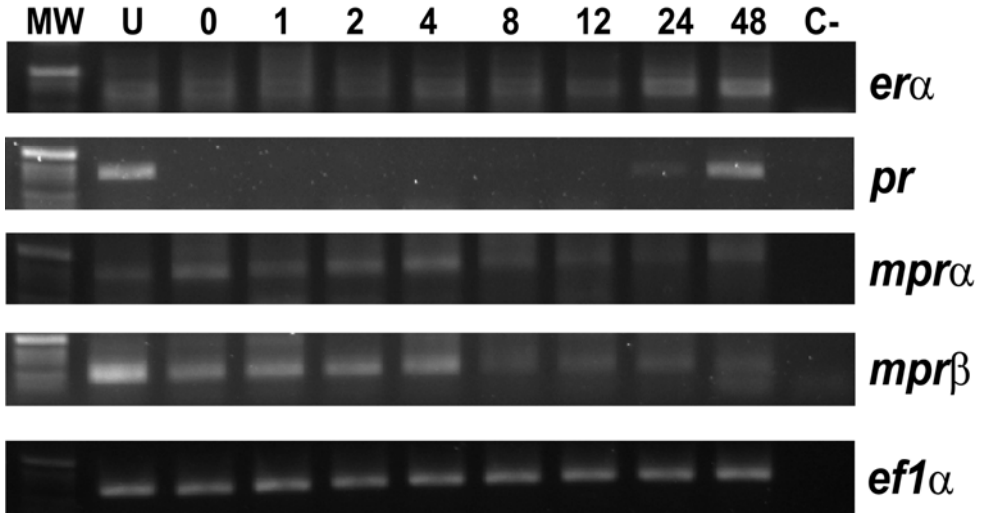


Fig. 13. Expression of *erα, pr, mprα* and *mprβ* transcripts during zebrafish development, shown by RT-PCR performed on RNA extracted at different developmental stages, using *ef1α* as a loading control. MW= DNA ladder; C- = negative control (water); U = ovulated oocytes; 0, 1, 2, 4, 8, 12, 24, 48 hpf.

Erα transcripts were initially very low and increased slightly at 24 and 48 hpf with the progression of embryonic gene transcription. *Pr* transcripts were evident only in ovulated oocytes and at 24 and 48 hpf. Transcripts for *mpr-α* and *β* mRNAs were expressed in ovulated oocytes and mainly in the first 4 hpf. There was no subsequent embryonic expression of these transcripts (Fig. 13).

33.2. qRT-PCR

33.2.1. Optimization and validation of qRT-PCR

The specificity of the primer pairs for *erβ1* and *2*, *ar*, *gr* and *mr* transcripts as well as for *ef1α* mRNA, used as an internal control, was established by RT-PCR and sequencing of the cDNA fragments.

Melt graphs were checked for the presence of primer dimers or spurious products, which were always absent when the specific transcript was detected. Specificity of real-time amplicons was determined by routine analysis of amplification profiles and melting curves. Random samples were also analyzed on agarose gel.

33.2.2. Quantification

Erβ1 transcript levels were stably low from 0 to 8 hpf, but steadily increased during embryonic transcription from 24 hpf to a maximum at 48 hpf, which were significantly different ($p<0.001$) compared to the expression at 8 hpf (Fig. 14A, Table 7).

Conversely, *erβ2* transcripts were initially present at high levels as maternal mRNAs, but declined sharply from 4 to 8 hpf ($p<0.001$, with respect to 2 hpf), remaining similarly low thereafter (Fig. 14B, Table 7).

Ar transcripts of maternal origin were initially moderately high, and decreased sharply from 4 to 8 hpf ($p<0.01$, compared to 4 hpf), being subsequently replaced at a low level by the corresponding embryonic mRNAs ($p<0,01$ from 8 hpf) (Fig. 14C, Table 7).

Gr transcripts were highly expressed as maternally transmitted mRNAs and declined throughout the first 8 hpf ($p<0.01$, from 0 hpf), being replaced, thereafter, by the corresponding embryonic mRNAs to a maximum at 48 hpf ($p<0,001$ from 8 hpf). These were the most abundant mRNAs analyzed. The expressions in ovulated oocytes and at 0 hpf were not significantly different from that at 48 hpf (Fig. 14D, Table 7).

Mr transcripts were very low in ovulated oocytes and during the first 8 hpf, showing a steep increase from 12 hpf until hatching (48 hpf) ($p<0.001$ from 8 hpf) (Fig. 14E, Table 7).

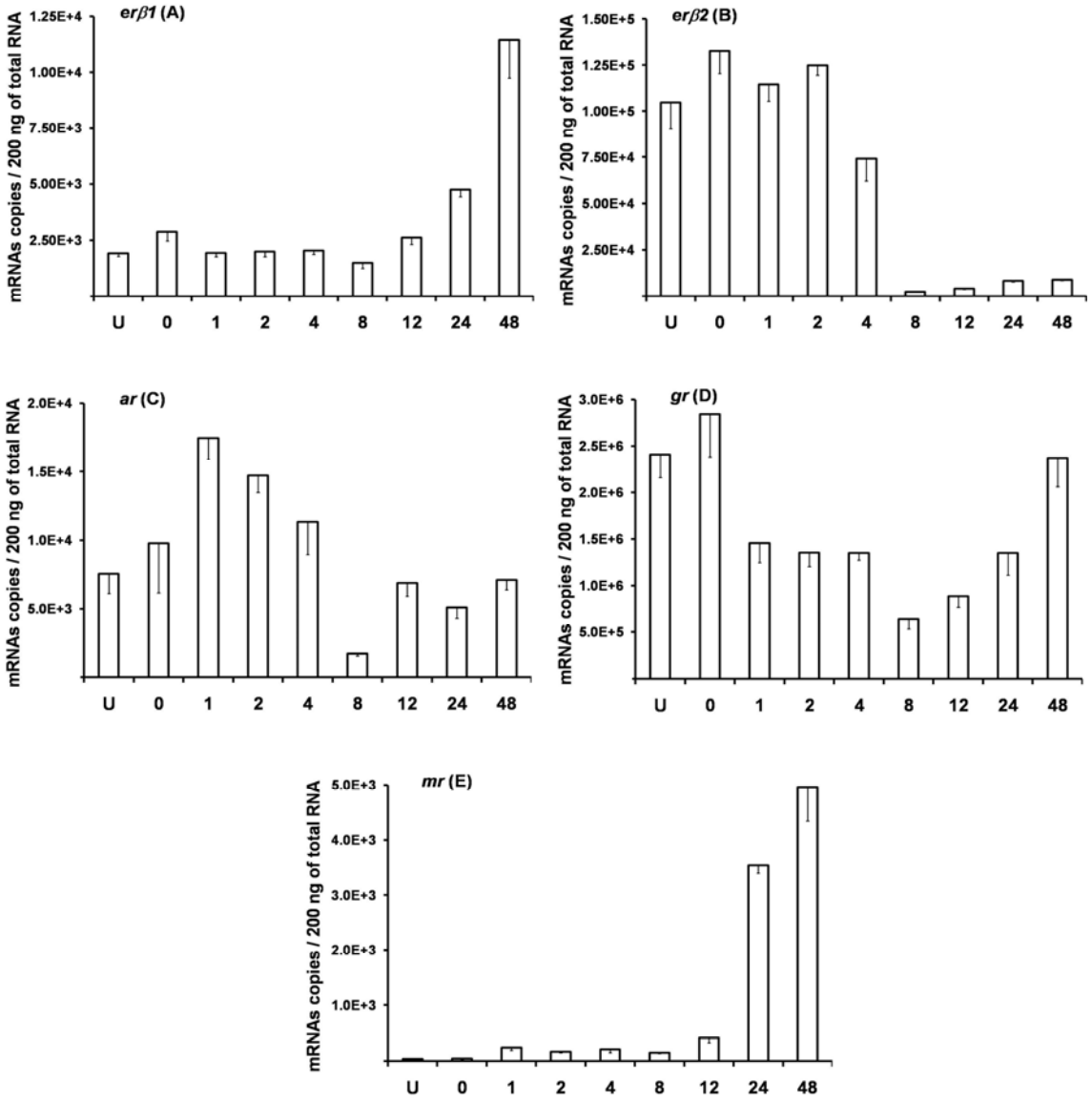


Fig. 14. Copy numbers of steroid receptor transcripts after normalization with *ef1α* mRNA as an internal control. In the abscissa: U = ovulated oocytes; 0, 1, 2, 4, 8, 12, 24, 48 hpf. Values represent the means ± SEM (n = 9) of expressions, as determined by qPCR. (A) *erβ1*; (B) *erβ2*; (C) *ar*; (D) *gr*; and (E) *mr* mRNAs.

Table 7. Results of the pairwise comparisons done by non-parametric Mann–Whitney U-test at specified p-values.

	0	1	2	4	8	12	24	48
Estrogen receptor β1 (erβ1)								
U	NS	NS	NS	NS	NS	NS	**	**
0	–	NS	NS	NS	*	NS	**	**
1		–	NS	NS	NS	*	***	***
2			–	NS	NS	NS	***	***
4				–	NS	NS	***	***
8					–	**	***	***
12						–	**	***
24							–	***
Estrogen receptor β2 (erβ2)								
U	NS	NS	NS	NS	**	**	**	**
0	–	NS	NS	NS	**	**	**	**
1		–	NS	*	***	***	***	***
2			–	**	***	***	***	***
4				–		**	**	**
8					–	**	***	***
12						–	***	***
24							–	NS
Androgen receptor (ar)								
U	NS	**	**	NS	**	NS	NS	NS
0	–	NS	NS	NS	**	NS	NS	NS
1		–	NS	NS	**	***	***	***
2			–	NS	**	**	**	**
4				–	**	NS	*	NS
8					–	**	**	**
12						–	NS	NS
24							–	NS
Glucocorticoids receptor (gr)								
U	NS	**	**	*	**	**	**	NS
0	–	*	*	*	**	**	**	NS
1		–	NS	NS	**	*	NS	*
2			–	NS	**	*	NS	*
4				–	**	*	NS	*
8					–	NS	*	***
12						–	NS	***
24							–	*
Mineralocorticoids receptor (mr)								
U	NS	**	**	**	**	**	**	**
0	–	**	**	**	**	**	**	**
1		–	NS	NS	NS	*	***	***
2			–	NS	NS	**	***	***
4				–	NS	*	***	***
8					–	***	***	***
12						–	***	***
24							–	NS

U = ovulated oocytes; 0, 1, 2, 4, 8, 12, 24, 48 hpf. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; NS = not significant

34. Effect of glucocorticoid receptor Morpholinos on zebrafish development

34.1. Selection of morpholinos for zebrafish glucocorticoid receptor

From the first analyses, I have demonstrated that glucocorticoid receptor (*gr*) expression is initially maternally supplied to the embryo and then supplemented with zygotic expression at MBT stage. To further clarify the role of *gr* function during development in zebrafish, Morpholino antisense oligonucleotide technology was used to knockdown the expression of Gr protein in zebrafish. Two non-overlapping MOs (*gr*MO-ATG-1 and *gr*MO-ATG-2) antisense to the zebrafish *gr* 5'-untranslated region and translation initiation codon (Fig. 15) were used. In addition, a five-nucleotide-mismatch *gr*-5mmMO and mRNA rescue protocols were also used to confirm the specificity of the MO.

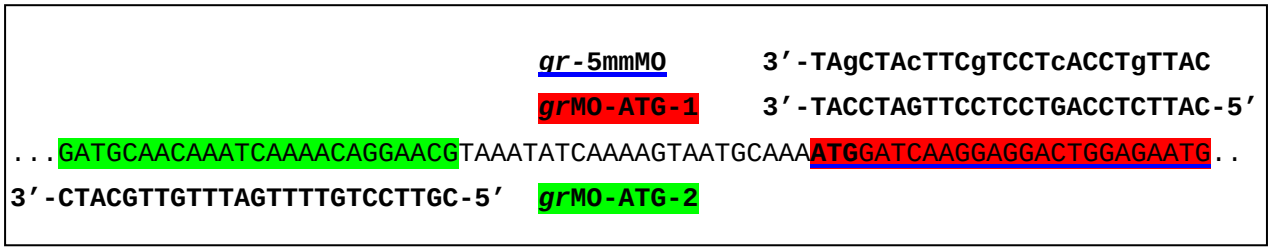


Fig. 15. Positions of MOs within the zebrafish glucocorticoid receptor α mRNA (EF567112) sequence targeted by antisense Morpholinos *gr*MO-ATG-1 (red); *gr*MO-ATG-2 (green) and *gr*-5mmMO (blue underlined). The ATG start codon is indicated in bold.

The exon-skipping MO (5'-GCCAGAGATATATGGAATACCTTCA) was designed to target exon/intron boundary between exon 3 and intron 3 of the *gr* mRNA. The in-frame translation process will be interrupted and the protein eventually produced will lack both DBD and LBD domains.

34.2. Optimization of MO injection

The MO's instructions from Gene Tools suggest injecting of 1 to 10 nL of 1 mM MO into the 1-cell zebrafish's embryo to give 1 to 10 μM final concentrations in the oocyte. In this study, I have used an injection volume of ~10 nL. *Gr*MO-ATG-1 was injected at

concentrations of 0.5, 0.8, 1 and 2 µg/µl, corresponding to approximately 5.2, 8.2, 10.3 and 20.6 ng of MO per embryo, respectively. The experiments were repeated three times. As expected, the results showed that the most heavily deformed morphant phenotypes and the highest number of dead embryos were obtained using the highest dose of *gr*MO-ATG-1 (20.6 ng). On the other hand, the lowest dose of *gr*MO-ATG-1 (5.2 ng) showed the least number of abnormal morphant phenotypes (Table 8).

Table 8. Phenotypes of embryos injected with *gr*MO-ATG-1

***gr*MO-ATG-1 (ng per embryo)**	**n.**	**Dead (%) (Means ± SD)**	**n. surviving**	**Normal (%) (Means ± SD)**	**Abnormal (%) (Means ± SD)**
5.2	253	26 ± 4	187	78 ± 3	22 ± 3
8.2	393	18 ± 1	322	28 ± 5	72 ± 5
10.3	460	12 ± 3	405	20 ± 2	80 ± 2
20.3	286	42 ± 1	166	26 ± 3	74 ± 3

The % of abnormal and normal embryos was calculated from the number of surviving embryos. Data was pooled from 3 experiments.

Five-nucleotide mismatch control MO (*gr*-5mmMO) was designed by altering five nucleotides in the sequence of *gr*MO-ATG-1 (see Fig. 3). It was determined that this MO failed to recognize any target sequence in the zebrafish genome and was therefore acceptable as a mismatch control MO (http://www.gene-tools.com/). *Gr*-5mmMO was injected individually into embryos at the different concentrations of 0.8 and 1 µg/µl, corresponding to 8.3 and 10.4 ng of MO per embryo, respectively. The number of abnormal embryos was high at the concentration of 10.4 ng [5% of mortality rate (10/200); 18% of abnormality rate (34/190)], suggesting that this control MO has toxic effects at the higher concentration. Instead, injection of *gr*-5mmMO at 8.3 ng could be used as a successful control for *gr*MO-ATG-1 because it determines a much lower number of abnormal embryos [5 % mortality rate (13/244) ; 4 % abnormality rate (10/231)].

After the optimization of MO injection, it appeared that 8.2 and 10.3 ng per embryo of *gr*MO-ATG-1 could be the best concentrations to use for characterization of the consequences of Gr protein deficiency. Nevertheless, the result with *gr*-5mmMO control MO shows that at

10.3 ng the embryos were more severely affected by toxicity than 8.2 ng; thus, using the higher dose of *gr*-5mmMO could increase the risk of encountering non-specific effects. So, in this study, I decided to use both *gr*MO-ATG-1 and *gr*-5mmMO control at the same concentration (8.2 ng).

Similarly, *gr*MO-ATG-2 and *gr*MO-SP were injected at concentrations of 0.8, 1 and 2 µg/µl, corresponding to approximately 8.2, 10.3 and 20.6 ng of MO per embryo, respectively. The optimization of *gr*MO-ATG-2 (Table 9) and *gr*MO-SP (Table 10) injection concentration gave results equivalent to those obtained with *gr*MO-ATG-1. Thus, 8.2 ng of *gr*MO-ATG-2 and *gr*MO-SP were used too. Finally, I concluded that the injection of all MO samples generates dosage-dependent knockdown effects and yields more severe defects with increasing concentrations.

Table 9. Phenotypes of embryos injected with *gr*MO-ATG-2.

***gr*MO-ATG-2 (ng per embryo)**	**n.**	**Dead (%) (Means ± SD)**	**n. surviving**	**Normal (%) (Means ± SD)**	**Abnormal (%) (Means ± SD)**
8.2	383	28 ± 3	276	28 ± 5	72 ± 5
10.3	376	44 ± 1	211	26 ± 3	74 ± 3
20.3	453	71 ± 7	131	48 ± 9	52 ± 9

The % of abnormal and normal embryos was calculated from the number of surviving embryos. Data was pooled from 3 experiments.

Table 10. Phenotypes of embryos injected with *gr*MO-SP.

***gr*MO-SP (ng per embryo)**	**n.**	**Dead (%) (Means ± SD)**	**n. surviving**	**Normal (%) (Means ± SD)**	**Abnormal (%) (Means ± SD)**
8.2	347	6 ± 3	326	94 ± 1	6 ± 1
10.3	422	10 ± 2	380	94 ± 1	6 ± 1
20.3	335	18 ± 22	275	87 ± 5	13 ± 5

The % of abnormal and normal embryos was calculated from the number of surviving embryos. Data was pooled from 3 experiments.

34.3. Evaluation of morphological defects in injected embryos

For the initial phenotypic characterization of the *gr*MO-injected embryos, 8.2 ng of *gr*MO-ATG-1, *gr*MO-ATG-2, *gr*MO-SP and *gr*-5mmMO were injected into 1-cell zebrafish embryos. The embryos were then examined throughout early development in comparison to age-matched controls (non-injected embryos - WT, and *gr*-5mmMO-injected). Several morphological parameters were used as a guideline for evaluating embryo phenotypes (Table 11). Morphant embryos were classified into two graded phenotypes (Class I and Class II), depending on the severity of defects compared to controls (Fig. 16.). The percentage of morphant phenotypes (class I and class II) and normal phenotypes at various concentrations of *gr*MO-ATG-1 and -2 were shown in Fig. 17 and Fig. 18, respectively.

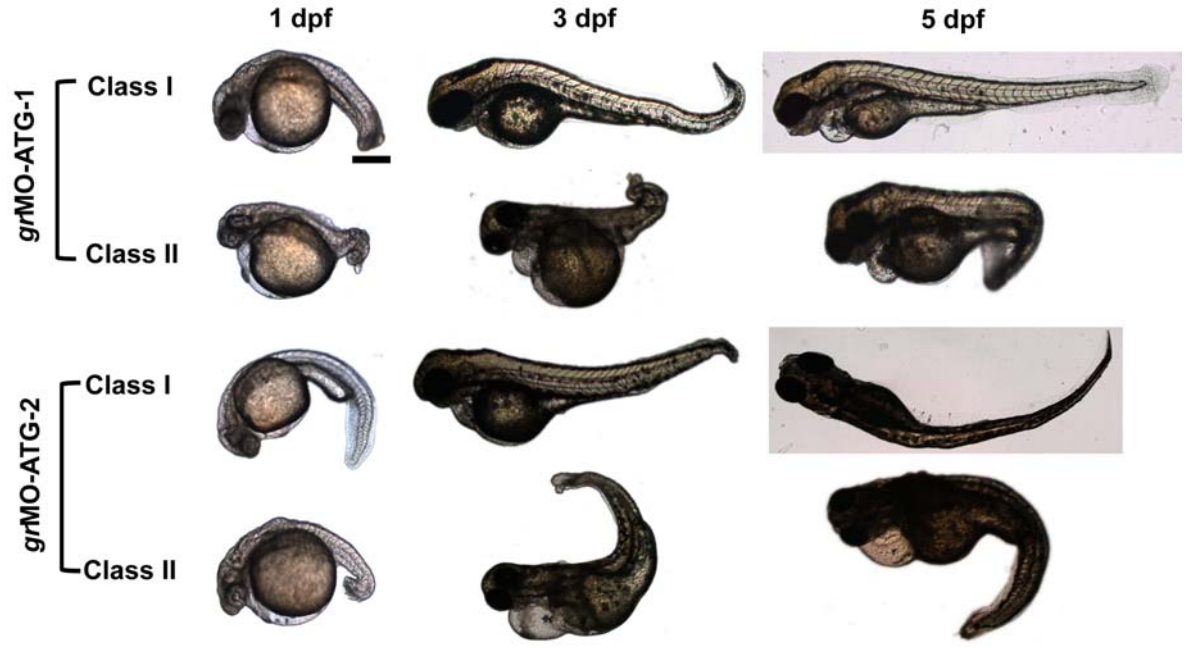


Fig. 16. Affected morphant embryos of *gr*MO-ATG-1 and -2 were classified into two graded phenotypes. Class I: displays mildly affected phenotypes: moderately shortened body, slightly curved tail and modest pericardial edema. Class II: displays severely affected phenotypes: markedly shortened body and spiral tail, and acute pericardial edema. Bar = 500 μm.

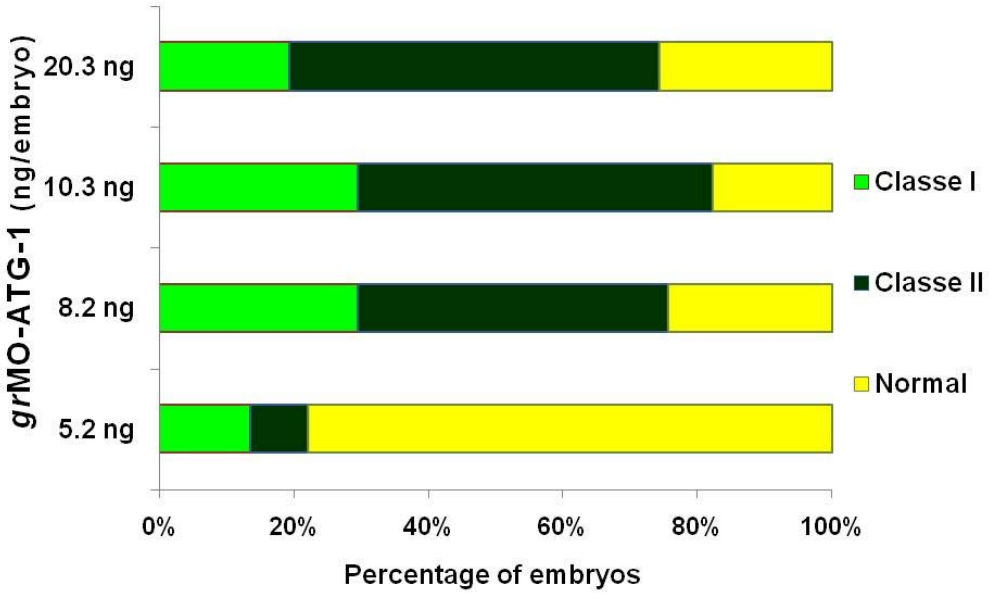


Fig. 17. Chart displaying the percentages of normal and abnormal phenotypes in Class I and Class II embryos after injection with *gr*MO-ATG-1.

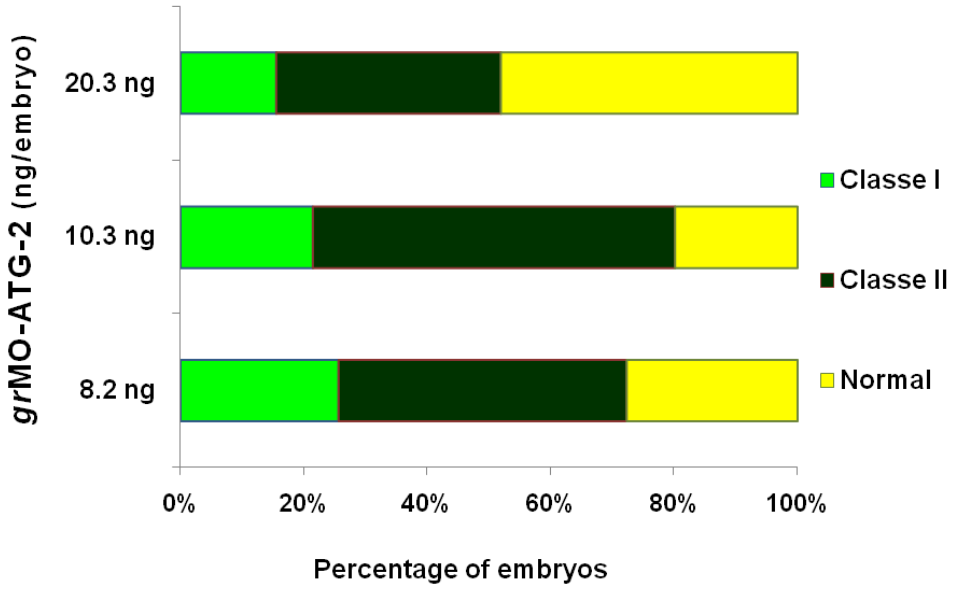


Fig. 18. Chart displaying the percentages of normal and abnormal phenotypes in Class I and Class II embryos after injection with *gr*MO-ATG-2.

Table 11. The morphological phenotypes during embryogenesis in zebrafish (http://www.gene-tools.com/)

Parameter	Developmental Stage
Body length & curvature	2 - 5 dpf
Brain morphology and head	1 dpf
Circulation & hemorrhaging	3 - 5 dpf
Ear morphology	2 - 5 dpf
Edema	2 - 5 dpf
Eye morphology	2 - 5 dpf
Fin morphology	5 dpf
Heart morphology	2 - 5 dpf
Liver and intestinal morphology	5 dpf
Jaw morphology	5 dpf
Movement	1 - 5 dpf
Notochord and floorplate morphology	1 - 2 dpf
Pigmentation	2 - 5 dpf
Somite morphology	1 - 5 dpf
Swim bladder	5 dpf
Yolk morphology	1 - 5 dpf

At 5 dpf, the embryos were laterally photographed and the lengths of their body, head, eye, and swim bladder measured in μm (Fig. 19).

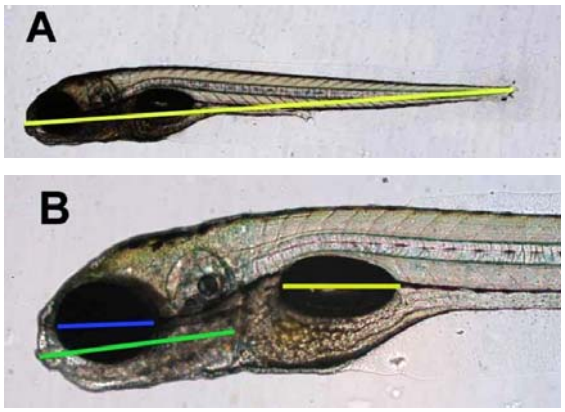


Fig. 19. The measurement of body length (yellow) (A), head (green), eye (blue) and swim bladder (yellow) (B)

34.3.1. Morphological phenotypes (Fig. 20)

In the early period of development (cleavage period), *gr*MO-ATG injected embryos (*gr*MO-ATG-1 and *gr*MO-ATG-2) appeared similar to controls (WT and *gr*-5mmMO-injected embryos) and *gr*MO-SP embryos. By **4 hpf** (sphere stage), however, *gr*MO-ATG injected embryos often exhibited a slight growth delay. **At 1 dpf**, the first morphological defect observed was a notable decrease in body length. Moreover, *gr*MO-ATG injected embryos had smaller head and eyes than controls and delayed pigmentation. They also displayed pericardial edema and reduced yolk extension (YE). In the more severely affected embryos (class II), the tail was often short, twisted and/or truncated, YE totally disappeared and yolk-sac edema was observed.

At 3 dpf, *gr*MO-ATG injected embryos still displayed a shorter and poorly developed body with respect to control and *gr*MO-SP embryos. Small head and eyes and pericardial fluid accumulation were still present at this stage. *gr*MO-ATG injected embryos were also less responsive to touch stimuli and their melanophore pattern along the body was disrupted.

At 5 dpf, the severity of developmental defects in *gr*MO-ATG injected embryos was further aggravated: very short body, small head, eyes and otic vesicles, pericardial edema and disrupted pigmentation along the horizontal myoseptum were found as compared to control and *gr*MO-SP embryos.

Initial swim bladder inflation normally occurs at approximately 96 hpf in the zebrafish, but most *gr*MO-ATG injected embryos had not inflated their swim bladder at this time and were lying on their side. Additionally, they exhibited a whirling movement when touched with a small needle. In control and *gr*MO-SP groups, the pectoral fins had normally developed and they were spending more time upright than on their sides. The *gr*MO-ATG injected embryos without a swim bladder at 5 dpf, were still in this condition at 9-10 dpf , suggesting that this knockdown effect was not due to delayed development. This impairment compromised proper feeding leading to starvation. Thus, only a few *gr*MO-ATG injected embryos survived throughout larval development.

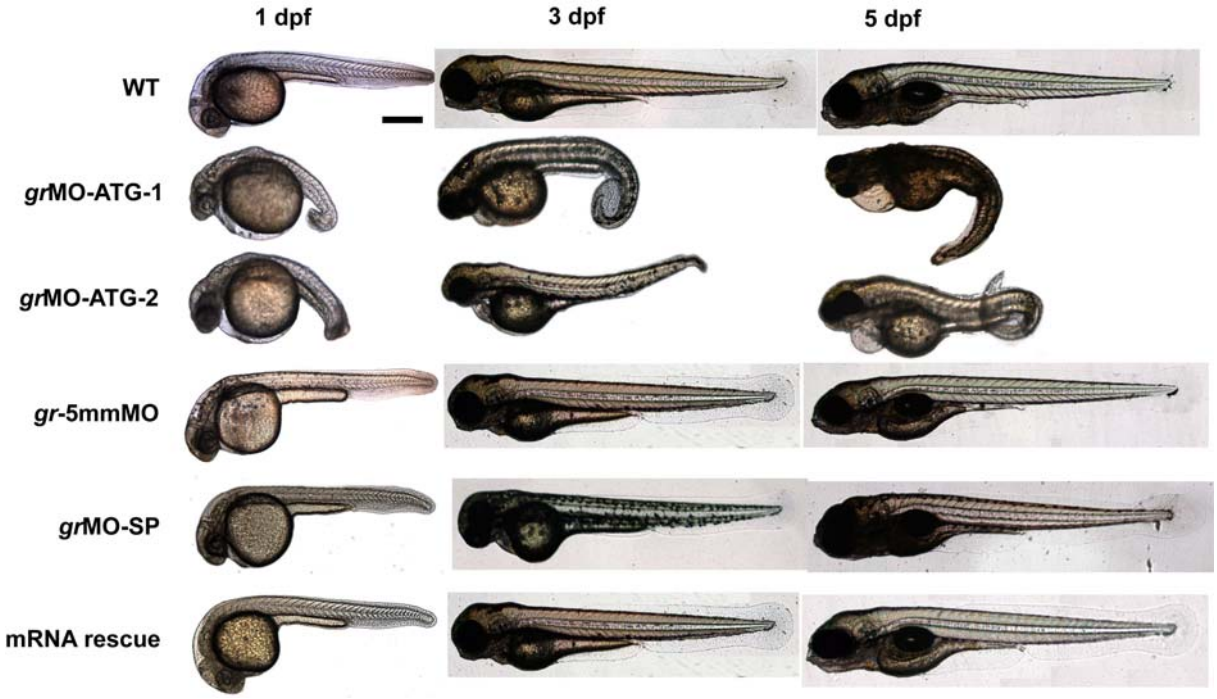


Fig. 20. Morphological appearance of MO-GR-injected embryos and control group at 1, 3 and 5 dpf. Bar = 500 μm.

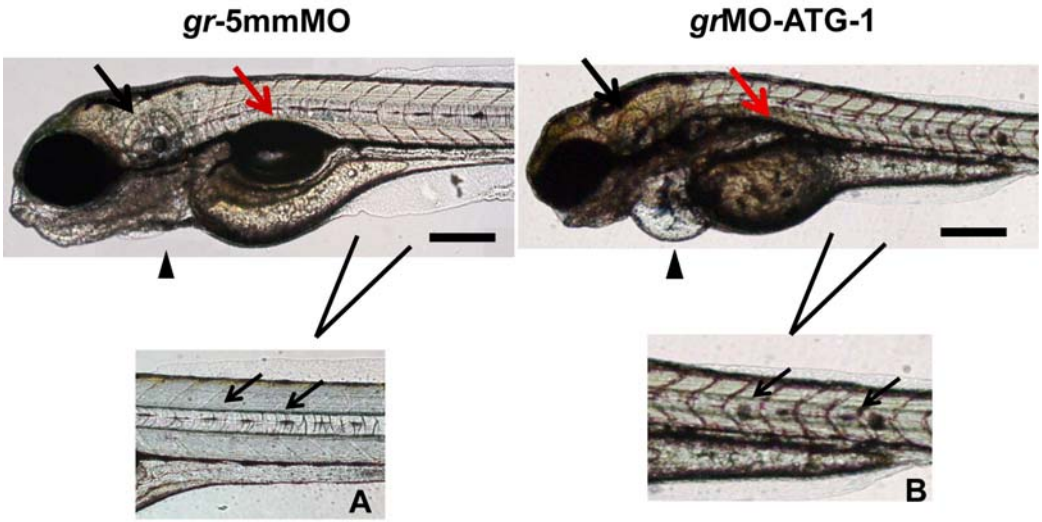


Fig. 21. *Gr*MO-ATG-1-injected embryos failed to inflate the swim bladder (red arrow) and displayed small otic vesicles (black arrow) and pericardial edema (arrow head) compared to control (*gr*-5mmMO). Bar = 300 μm. The alignment of melanophores along the horizontal septum was normal in control (*gr*-5mmMO) (A), but disrupted in morphant embryos (B).

34.3.2. Anatomical analysis of morphant phenotypes

In Gr-knockdown embryos, the body and head lengths and eye diameters were significantly shorter than those of WT, controls and *gr*MO-SP embryos at 5 dpf ($P<0.05$), as shown in Table 12.

Table 12. Effects of Gr-knockdown on body and head length and eye diameter at 5 dpf of zebrafish larvae. Results showed as average (means ± SD).

Experimental	Body length (µm±SD)	Head length (µm±SD)	Eye diameter (µm±SD)	Swim bladder length (µm±SD)
WT	3296 ± 39 [a]	206 ± 8 [a]	95 ± 4 [a]	113 ± 6
*gr*MO-ATG-1	2554 ± 473 [b]	154 ± 14 [b]	74 ± 7 [b]	9 ± 28
*gr*MO-ATG-2	2745 ± 473 [b]	162 ± 16 [b]	72 ± 16 [b]	10 ± 29
gr-5mmMO	3268 ± 80 [a]	188 ± 4 [a]	89 ± 2 [a]	108 ± 6
*gr*MO-SP	3167 ± 123 [a]	194 ± 7 [a]	92 ± 4 [a]	106 ± 8
mRNA rescue	3175 ± 80 [a]	194 ± 9 [a]	91 ± 4 [a]	107 ± 7

Means with different letters in each column indicate statistically significant differences from one another ($P< 0.05$).

34.3.3. Histological analysis of morphant phenotypes

To examine the histological changes induced by MO, *gr*MO-ATG-1, *gr*-5mmMO and WT embryos at 7 dpf were embedded in 1.5 % low-melting agarose before inclusion in paraffin in order to correctly orient specimens, a difficult step due to their small size (total length ~ 5 mm at 7 dpf).

Besides smaller head and eyes, the injection of *gr*MO-ATG-1 affected several organs. The intestinal epithelium of WT embryos was highly folded, whereas in morphant embryos it was flattened with signs of degeneration. Only morphant embryos failed to inflate their swim bladder, but had a normal layer of circular smooth muscles around it. The persistence of the yolk vacuole indicates that these embryos failed to reabsorb their yolk (Fig. 22.).

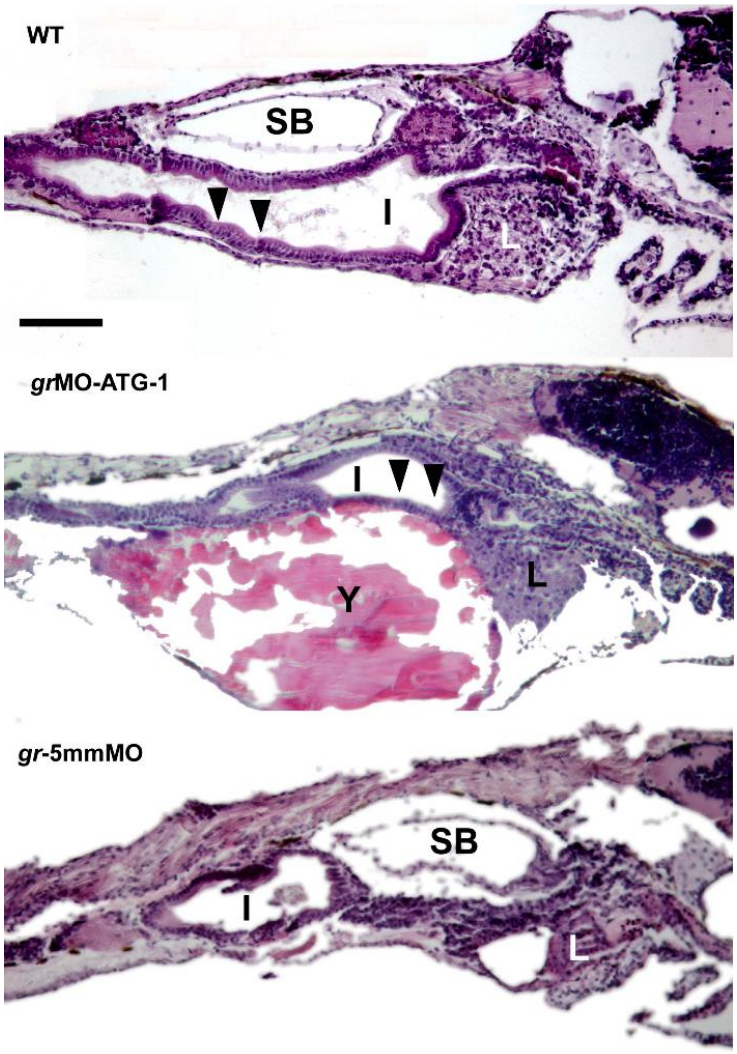


Fig. 22. Histological changes of embryos at 7 dpf; the embryos were laterally sectioned and stained with hematoxylin–eosin. The arrows indicate the intestinal epithelium. Y= yolk, I = intestine, SB = swim bladder, L = liver. Scale bar = 250 μm (20X).

35. Assessing the effectiveness of glucocorticoid Morpholino

35.1. Knockdown of Gr protein expression

35.1.1. Western blot analysis

To verify the effectiveness of the injected MO, rabbit antibodies against human GR were used to confirm a reduction in Gr protein level by Western blot analysis. Protein samples were extracted from embryos at 48 hpf, which had been: 1) injected at the 1-cell stage with 8.2 ng of *gr*MO-ATG-1; 2) co-injected with 8.2 ng of *gr*MO-ATG-1 and *t-gr2* mRNA rescue; 3)

injected with 8.2 ng of *gr*-5mmMO; and 4) WT embryos. α-Tubulin protein was used as a control of the quantity of loaded proteins. As shown in Fig. 23, Gr was greatly reduced in embryos injected with *gr*MO-ATG-1, as compared to the *gr*-5mmMO and WT embryos. In rescue morphant, the intensity of Gr signal seems equivalent to those of *gr*-5mmMO and WT samples, but it is somehow overestimated as more proteins were loaded (see the intensity of the α-tubulin band in Fig. 23). These results show that *gr*MO-ATG-1 is able to bind to its *gr*-mRNA target sequence and to curtail downstream translation.

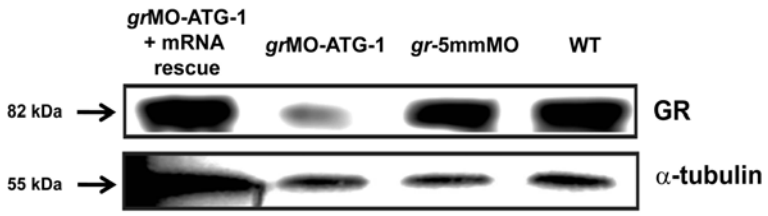


Fig. 23. Western blot analysis showed that the Gr protein from 48 hpf embryos is markedly reduced after injection with 8.2 ng of *gr*MO-ATG-1in comparison to mRNA rescue, *gr*-5mmMO and WT. A band of ~82 kDa was produced with the Gr antibody used at 1:800, while the α-tubulin antibody at 1:1000 gave the expected band of ~55 kDa.

35.1.2. *In vitro* transcription and translation inhibition by Morpholinos

In order to confirm the specifity and to measure the efficiency of translation inhibition by MO, an *in vitro* transcription and translation system (TNT Quick coupled reticulocyte lysate system and Transcend™ biotinylated lysine-tRNA) was adopted. To this end, the full length of zebrafish *gr* mRNA and trout *gr2* mRNA were synthesized, after checking by sequencing that no base changes in the transformed plasmid could interfere with the translation process. The transcription/translation reaction mixtures were incubated with *gr* mRNA from zebrafish alone and together with 8.2 ng *gr*MO-ATG-1. *Gr2* mRNA from trout was also used to verify the efficiency of the *in vitro* transcription and translation system, whereas a control reaction was performed without added RNA. This assay shows that *gr*MO-ATG-1 efficiently blocks translation of *gr* mRNA *in vitro*. (Fig. 24).

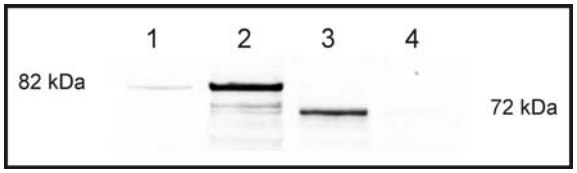


Fig. 24. *In vitro* inhibition of zebrafish *gr* mRNA translation by *gr*MO-ATG-1. 1 = inhibition of zebrafish *gr* mRNA using the *gr*MO-ATG-1; 2 = *z-gr* mRNA translation product; 3 = *t-gr* mRNA translation product; 4 = negative control. The molecular weight of zebrafish Gr is about 82 kDa, whereas the that of trout Gr2 is about 72 kDa.

35.2. RNA rescue of MO phenotype

As proof-of-specificity, I compared the phenotypes induced by injection of two different non-overlapping 5'-UTR oligos targeted to block translation of the same mRNA. As both sequences induced the same phenotype, this supports the assumption that it is due to the knockdown of the targeted messenger. To further confirm this conclusion, the *gr*MO-ATG-1 was co-injected with *in vitro*-transcribed mRNA encoding the full length of the rainbow trout glucocorticoid receptor 2 (*t-gr2* mRNA), as this procedure was intended to rescue the MO phenotype.

Actually, embryos injected with the *gr*MO-ATG-1 and *t-gr2* mRNA at different concentrations (2, 4, 6 and 8 ng/µl) showed varying degrees of rescue of the *gr*MO-ATG phenotype. As shown in Table 13, the injection of *t-gr2* mRNA at the concentration of 6 ng/µl resulted in 85% of the injected embryos displaying a normal phenotype during development (Fig. 20). At 5 dpf, the swim bladders were inflated and no persistent yolk sac was found. Only 15% were abnormal, while intervening mortality rate was 14%. With lower doses of *t-gr2* mRNA (2 and 4 ng/µl), the embryos presenting the *gr*MO-ATG phenotype were 67 and 47%, respectively. Mortality was also higher. However, the further increase of the *t-gr2* mRNA concentration up to 8 ng/µl did not improve rescuing, because mortality was greatly increased. These results confirms that the MO phenotype was definitely caused by Gr knockdown.

Table 13. Results of *gr*MO-ATG-1 rescue by *t-gr2* mRNA.

***gr*MO-ATG-1 (8.2 ng) + various concentrations of *t-gr2* mRNA**	**n.**	**Dead (%) (Means ± SD)**	**n. surviving**	**Normal (%) (Mean ± SD**	**Abnormal (%) (Mean ± SD)**
+ 2 ng/µl mRNA	343	37 ±10	216	32 ± 5	68 ± 5
+ 4 ng/µl mRNA	312	34 ± 8	215	53 ± 2	47 ± 3
+ 6 ng/µl mRNA	330	14 ± 6	284	85 ± 4	15 ± 4
+ 8 ng/µl mRNA	387	44 ± 12	216	84 ± 4	16 ± 6

Data were pooled from 3 experiments. *gr*MO-ATG-1 dose was 8.2 ng per embryo. The percentages of normal and abnormal embryos were calculated from surviving embryos at 5 dpf.

35.3. Inhibition of zebrafish *gr* pre-mRNA splicing

To verify the effectiveness of splice-site-targeted MO, the exon-skipping *grMO*-SP was injected into 1-cell embryos at the concentration of 62.5 ng. Then, RT-PCR was performed on total RNA with *gr*-specific primers (GLU-F1 and GLU-R). The *gr* primary transcript was detected in both the WT controls and *gr*MO-SP-injected embryos, but in the latter case at lower expression levels, indicating a knockdown effect on *gr* transcripts. The predicted mis-spliced transcript was also detected as a lower band, and the size of this product is consistent with the loss of the targeted exon (Figure 25).

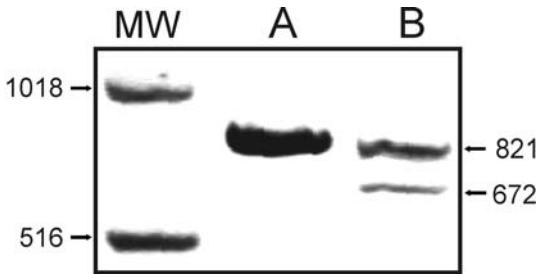


Fig. 25. Splice-site-targeted MO can alter splicing of *gr* mRNA in zebrafish. **A**: non-injected embryos; **B:** *gr*MO-SP-injected embryos at 1 dpf; MW = DNA ladder.

36. Microarray analysis of MO effects on gene expression

Microarray technology was employed to identify differential expression of genes between *gr*MO-ATG-1 and *gr*-5mmMO-injected embryos and WT controls. Both MOs were injected at 8.2 ng, and samples were collected at 5 and 10 hpf, corresponding to gastrula and early segmentation stages, respectively. Total RNA samples were quality-checked with the Agilent 2100 bioanalyzer that use the RNA integrity number (RIN) software tool. Accordingly, integrity is 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 is facilitated, comparison of samples is enabled and repeatability of experiments is ensured. A RIN number of 10 indicates high RNA quality, whereas a RIN number of 1 indicates low RNA quality. RNA with a RIN number > 6 is of sufficient quality for gene expression profiling experiments. All RNA samples used in this experiment obtained RIN values between 9.7 and 10. The microarray analysis was carried out by Miltenyi Biotec GmbH (Germany), according to the experimental design illustrated in Table 14, whereas Table 15 reports cRNA yields and dye-incorporation rates.

Table 14. The experimental design of the microarray analysis performed by the Miltenyi Biotec GmbH; (Cy3 = control; Cy5 =sample).

Experiments	Cy3	Cy5
1	*gr*-5mmMO 5 hpf	*gr*MO-ATG-1 5 hpf
2	WT 5 hpf	*gr*MO-ATG-1 5 hpf
3	*gr*-5mmMO 10 hpf	*gr*MO-ATG-1 10 hpf
4	WT 10 hpf	*gr*MO-ATG-1 10 hpf

Table 15. cRNA yields and dye-incorporation rates.

Sample	Labeling	cRNA (ng/µL)	Volume (µL)	Cy3 (pmol/µL)	Cy5 (pmol/µL)	Incorporation rate (fmol/ng)
5MIS-5h	Cy3	122.8	55	1.1	–	9
WT-5h	Cy3	130.7	55	1.2	–	9
5MIS-10h	Cy3	228.3	55	2.5	–	11
WT-10h	Cy3	197.7	55	1.7	–	9
GR-5h	Cy5	168.7	55	–	3.5	20
GR-10h	Cy5	178.4	55	–	2.8	16

36.1. Scanning results

Fluorescence signals of the hybridized Agilent Oligo Microarrays were detected using the Agilent's DNA microarray scanner. A red spot indicates that the fluorescencet intensity of the Cy5 signal is higher than that of Cy3, which means that the corresponding gene is over-expressed in the *gr*MO-ATG-1 sample. Green spots indicate that the fluorescence intensity is higher in the control sample, while yellow spots are indicative of equal signal intensities. The representative false-color image of the microarray experiments with *gr*MO-ATG-1 and *gr*-5mmMO at 5 hpf is shown in Fig. 26.

Fig. 26. Cy5/Cy3 false-color image after scanning of samples with *gr*MO-ATG-1 and *gr*-5mmMO at 5 hpf.

36.2. Image and data analysis

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. For determination of differential gene expression, FES derived output data files were further analyzed using the Rosetta Resolverâ gene expression data analysis system (Rosetta Biosoftware). This software offers, among other features, the possibility to visualize the results of the data analysis in a double-log scatter plot. A representative double-log scatter plot for the experiments with *gr*MO-ATG-1 and *gr*-5mmMO at 5 hpf is shown in Fig. 27.

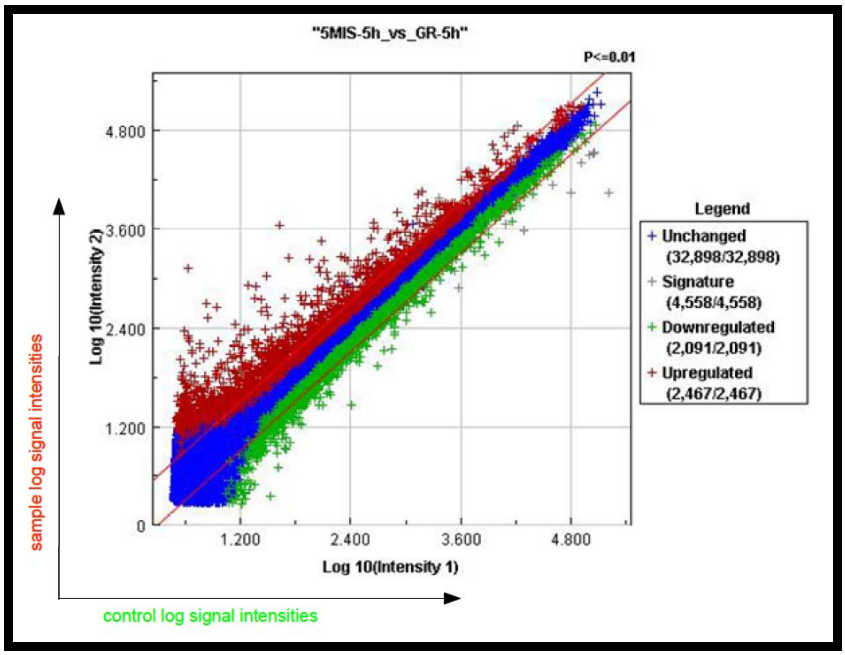


Fig. 27. Scatter plot of signal intensities of all spots. As an example the data of one array experiment is shown. The signal intensities of each feature represented by a dot is shown in double logarithmic scale. X-axis: Cy3-log signal intensity; y-axis: Cy5-log signal intensity. Red diagonal lines define the areas of 2-fold differential signal intensities. Blue cross: unchanged genes. Red cross: significantly up-regulated genes (P <0.01). Green cross: significantly down-regulated genes (P <0.01). Grey cross in legend: summary of significantly up- and down-regulated signatures.

36.3. Interpretation of microarray results

The gene lists were normalized using the Resolver® Software (Agilent technologies). Putative candidate genes with a fold change > 2 and p-value < 0.01 were listed as the pre-selected candidate genes. These were analyzed using the Functional Classification Tool (DAVID Bioinformatics Resources 2008; http://david.abcc.ncifcrf.gov/home.jsp). This tool provides a rapid way to reduce large lists of genes into functionally related groups of genes to help unravel the biological content captured by high throughput technologies. The annotation of genes was performed using the KEGG genes database (http://www.genome.jp/kegg/genes.html). The final list of dys-regulated genes from experiments 1 and 2 were clustered as 5-hpf gene expression, while experiments 3 and 4 were clustered as 10-hpf gene expression. The results show that 30 transcripts were dys-regulated at 5 hpf; of which 20 were up-regulated and 10 were down-regulated. At 10 hpf, 32 genes were dys-regulated, with 11 up-regulated and 21 down-regulated. Finally, the common dys-regulated genes from 5 and 10 hpf were 16 genes (Table 16). The lists of dys-regulated gene names are shown in Tables 17, 18 and 19.

Table 16. The number of up-regulated and down-regulated genes.

Experiment	Up-regulated/total	Down-regulated/total
5 hpf	20/30	10/30
10 hpf	11/32	21/32
5 hpf e 10 hpf	14/16	2/16

Table 17. Expression of genes up- or down-regulated at 5 hpf (gastrula period). Oligonucleotide microarray results were taken from a comparison of *gr*MO-ATG-1versus *gr*-5mmMO (experiment 1) and *gr*MO-ATG-1 versus WT (experiment 2)

Up-regulated		Down-regulated	
Gene	**Accession number**	**Gene**	**Accession number**
Forkhead box i1	NM_181735	wu:fb48e04 (Sulfatase FP2b)	NM_001003833
Glycerol-3-phosphate dehydrogenase 1 (soluble)	NM_214753	Inversin	NM_152970
Tryptophan hydroxylase 1, like	NM_001001843	Lamin β1	NM_152972
Cytochrome p450, subfamily xia, polypeptide 1	AF527755	Vesicle docking protein p115	NM_200155
zgc:77882 (Zinc finger protein 36)	NM_199649	Thioredoxin domain containing 4 (endoplasmic reticulum	NM_200892
Transgelin 2	NM_201576	Proto-oncogene tyrosine-protein kinase Fyn	NM_001007286
Actin related protein 2/3 complex, subunit 5b	NM_201509	Betaine-homocysteine S-methyltransferase	NM_001012480
Ankyrin repeat and SOCS box-containing 5	NM_001017753	Nerve growth factor receptor (TNFR superfamily, member 16	ENSDART00000022685
Claudin a	NM_131762	Gle 1 rna export mediator-like	NM_001003885
Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	BC076285	Inter'alpha (globulin) inhibitor h2	NM_213007
Transferrin-a	NM_001015057		
Rho-associated, coiled-coil containing protein kinase	NM_174863		
DNA-damage-inducible transcript 4	NM_200107		
Rkhead box protein A2, hepatocyte nuclear factor 3-beta	NM_130949		
Heme oxygenase (decyclizing); heme oxidase; haem oxidase	NM_199678		
Apelin receptor	NM_001030197		
Cathepsin H	NM_212688		
Homeobox (expressed in ES cells)	NM_131349		
Ras-homolog gene family member e	NM_199522		
Ras-like family member b	NM_200140		

Table 18. Expression of genes at 10 hpf (segmentation period). Oligonucleotide microarray results were taken from a comparison of *gr*MO-ATG-1versus *gr*-5mmMO (experiment 3) and *gr*MO-ATG-1 versus WT (experiment 4)

Up-regulated		Down-regulated	
Gene	**Accession number**	**Gene**	**Accession number**
Bcl2-associated x protein	NM_131562	zgc:76963	NM_212876
zgc:112397	NM_001020697	Sirtuin 2 (silent mating type information regulation 2, homolog	NM_199596
Gtp binding protein 1, like	NM_001024811	zgc:101886	NM_001007374
zgc:86648	NM_001004608	yy1 transcription factor, like	NM_213120
Protein tyrosine phosphatase, non-receptor type 6	NM_213181	Methyl cpg binding protein 2	NM_212736
Beta-ureidopropionase	NM_199616	zgc:92420	NM_001003463
Proto-oncogene protein	NM_200172	Hypothetical protein ankyrin repeat domain 46	NM_205596
RalA-binding protein 1	NM_207080	Dkfzp434b168 protein homolog (human)	NM_173267
Hypoxia-inducible factor 2 alpha	NM_001039806	Family with sequence similarity 50, member a	NM_001017636
Cyclin G1	NM_199481	Peptidylprolyl isomerase a, like	NM_199957
Jumonji domain containing 6	NM_170761	zgc:56702	NM_201103
		zgc:55479	NM_200158
		Chromodoamin helicase DNA binding protein 1-like	NM_200313
		Succinate dehydrogenase (ubiquinone) flavoprotein subunit	NM_200910
		Isovaleryl-CoA dehydrogenase	NM_201491
		Mitogen-activated protein kinase-activated protein kinase 5	NM_001002336
		Serine/threonine-protein kinase Chk2	NM_200045
		Tubulin beta	NM_198818
		Cohesin complex subunit SCC3	XM_681720
		Hyaluronan-mediated motility receptor	NM_199580
		Synaptosomal-associated protein, 23kDa	NM_200190

Table 19. Common dys-regulated genes between 5 and 10 hpf

Up-regulated		Down-regulated	
Gene	**Accession number**	**Gene**	**Accession number**
Insulin-like growth factor 2 precursor	NM_131433	Integral membrane protein 1	NM_201458
v-jun sarcoma virus 17 oncogene homolog (avian)	NM_199987	Minichromosome maintenance protein 6	NM_001082849
zgc:92360	NM_001002715		
Myosin regulatory light chain interacting protein	NM_199983		
zgc:91984	NM_001002228		
S-adenosylmethionine synthetase	NM_001040379		
Phosphoinositide-3-kinase, regulatory subunit	NM_213038		
PARP poly (ADP)- ribose Polimerase	NM_200501		
Matrix metalloproteinase-2 (gelatinase A)	NM_198067		
Transformed 3T3 cell double minute 2	NM_131364		
Growth arrest and DNA-damage-inducible protein	NM_200576		
Caspase 8, apoptosis-related cysteine protease	NM_131510		
Sestrin	NM_001002660		
Tumor necrosis factor receptor superfamily, mem 10	NM_194391		

37. Validation of differentially expressed genes by semiquantitative RT-PCR

In order to confirm the differential expression of genes that were identified by microarray technology, three genes were selected for validation by semiquantitative RT-PCR: *caspase 8*, *insulin-like growth factor-2 precursor* (*igf2*α) and *centaurin-*α. Three replicate samples from *gr*MO-ATG-1-injected and non-injected embryos were collected at 4, 8, 12, 24, 48 hpf. Total RNA was extracted and semiquantitative RT-PCR was performed at different number of amplification cycles.

37.1. *Caspase 8*

Caspases are proteases that cleave target substrates that have a specific peptide sequence. During apoptosis, the activation of caspases, which can be induced by nuclear, metabolic, or externally activated stimuli, takes place in a cascade manner and leads to nuclear engulfment and cell death. Multiple forms of caspase have been found in vertebrates including zebrafish. Class I caspases, such as caspase-2, -8, -9 and -10, promote the upstream part of the cascade reaction via N-terminal prodomains bound to specific death adaptor molecules. Class II caspases with short N-terminal prodomains, *e.g.* caspase-3, -6 and -7, act as downstream effectors by targeting cellular proteins for proteolytic cleavage. Activated caspase-8 is known to promote the apoptotic signal by directly cleaving and activating downstream caspases.

The semi-quantitative RT-PCR analysis revealed that the transcripts of *caspase 8* were more expressed in *gr*MO-ATG-1-injected embryos at all developmental stages (Fig. 28). This is in agreement with the results obtained with the microarray analysis where, both at 5 and 10 hpf, the transcript of this gene resulted up-regulated in MO-treated embryos.

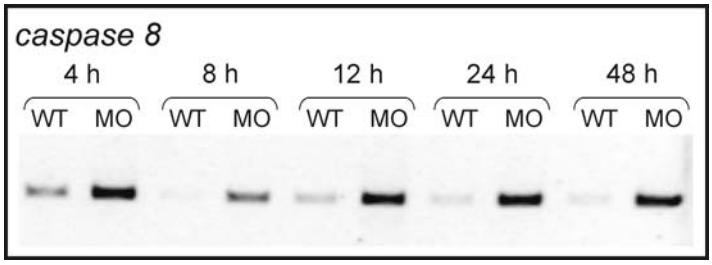


Fig 28. Representative **s**emiquantitative RT-PCR analysis of *caspase 8* transcripts. Gene expression was analyzed in embryos at 4, 8, 12, 24 and 48 hpf. The products were amplified for 32 cycles. WT = non-injected embryos, MO = *gr*MO-ATG-1-injected embryos.

37.2. I*nsulin-like growth factor-2α precursor* (*igf2α*)

The insulin-like growth factor 2α (IGF2) signaling system plays an important role in embryonic growth. It is a critical regulator of somatic growth during fetal and postnatal development, primarily through its stimulatory effects on cell proliferation and survival. At a cellular level, the IGFs function as autocrine and paracrine growth factors to control mitosis, apoptosis, differentiation and chemotaxis and may also have endocrine effects.

The semi-quantitative RT-PCR analysis revealed that the transcripts of zebrafish *igf2α* were, after 8 hpf, more expressed in *gr*MO-ATG-1-injected embryos compared with the expression in WT (Fig. 29). On the other hand, at 4 hpf, the transcription level seems slightly lower in MO-treated embryos. The microarray results showed that, both at 5 and 10 hpf, this transcript was up-regulated by the possible absence of the Gr protein. The influence of *gr*MO-ATG-1 seems to start only after the 4 hpf, but remains evident at least until 48 hpf, although at a less extent at the last two time points.

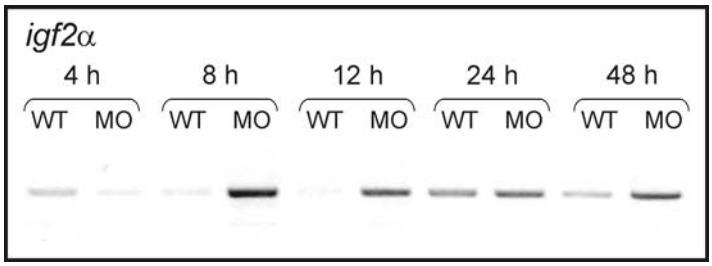


Fig. 29. Representative **s**emiquantitative RT-PCR analysis of *igf2α* precursor transcripts. Gene expression was analyzed in embryos at 4, 8, 12, 24 and 48 hpf. The products were amplified for 26 cycles. WT = non- injected embryos, MO = *gr*MO-ATG-1-injected embryos.

37.3. *Centaurin-α1*

Centaurin-α1, also known as p42IP4BP, belongs to a recently identified family of ARFGAP proteins. These proteins are involved in regulating various biological processes, including membrane ruffling, membrane trafficking, actin cytoskeleton regulation and presynaptic vesicular structures (Vashisht *et al*., 2009). Centaurin-α1 is expressed mainly in the neurons of hippocampus, cortex, cerebellum and hypothalamus (REF) (CIT). It is up-regulated in neurons of patients with Alzheimer's disease. Moreover, it interacts with the protein kinase CKIα, which hyperphosphorylates β-amyloid precursor protein in the brains of Alzheimer patients (Kanamarlapudi 2005). Centaurin-α1 expression during zebrafish embryogenesis was never, up to now, analysed.

The semi-quantitative RT-PCR analysis revealed that the transcripts of centaurin were up-regulated in *gr*MO-ATG-1-injected embryos from 4 hpf until 12 hpf (Fig. 30). At 24 hpf and 48 hpf, transcripts were not different between WT and *gr*MO-ATG-1-injected embryos using

34 PCR cycles. However, by lowering the PCR cycle number, a higher expression of this transcript is evident also at 24 and 48 hpf.

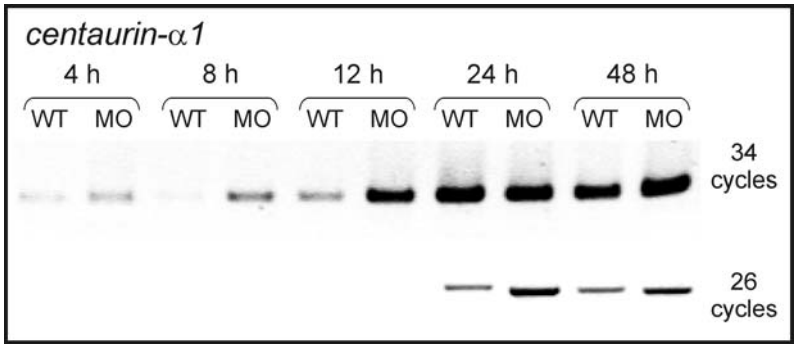


Fig. 30. Representative **s**emiquantitative RT-PCR analysis of *centaurin-α1* transcripts. Gene expression was analyzed in embryos at 4, 8, 12, 24 and 48 hpf. The products were amplified for 34 and 26 cycles (only 24h and 48h samples). WT = non-injected embryos, MO = *gr*MO-ATG-1-injected embryos.

In conclusion, the RT-PCR results confirmed the MO-induced changes and extended the temporal expression patterns of these genes, as previously evidenced by microarray analysis. For a more thorough assessment of the transcriptional influence of GR on candidate target genes during early embryogenesis in zebrafish, qRT-PCR should be applied in order to monitor changes in transcript number between *gr*MO-ATG-1-injected and non-injected embryos.

38. Analysis of apoptosis in injected embryos

To determine cell death levels during the development, *gr*-5mmMO- and *gr*MO-ATG-injected embryos were incubated in an acridine orange solution at 28.5°C. Acridine orange is a hydrophilic DNA-intercalating agent which interacts with DNA of non-condensed, fragmented chromatin, as in cells undergoing apoptosis. Healthy cells remain unstained by this dye. Fluorescent staining with acridine orange indicated cell death in the brain, otic and trunk regions in *gr*MO-ATG-injected embryos, (Fig. 31C and 31D), while no significant apoptosis was not detected in *gr*-5mmMO-injected embryos (Fig.31 A and 31B). These data indicate that increased cell death is likely one of the factors responsible for the small head and short body observed in *gr*MO-ATG-injected embryos.

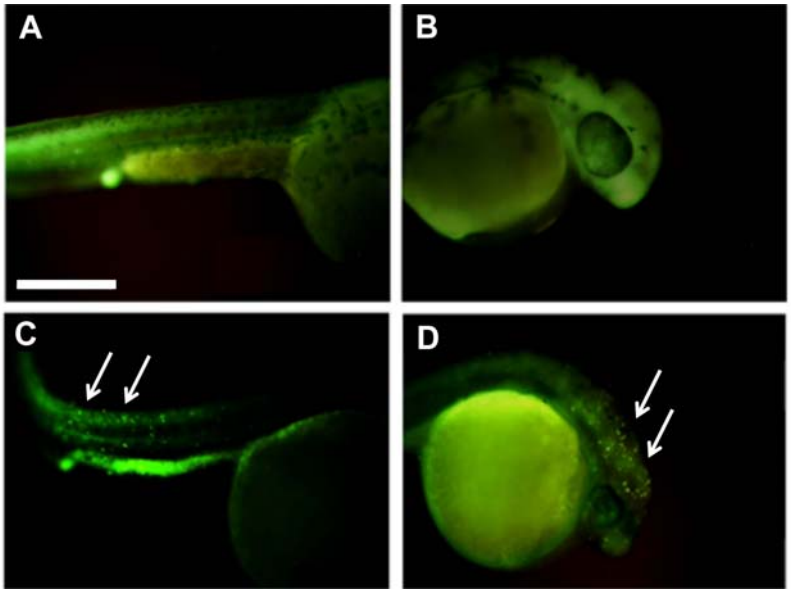


Fig. 31. Acridine orange staining in the trunk and head regions of *gr*-5mmMO-injected embryos (A and B) and *gr*MO-ATG-injected embryos (C and D) at 24 hpf. Apoptic areas are indicated by the arrows. Scale bars = 250 μm

39. *In situ* hybridization

Next, I analyzed *gr*MO-injected embryos for potential patterning defects using several developmental markers.

39.1. *Chordin*

Chordin is an extracellular antagonist to bone morphogenetic proteins (BMPs) and is secreted by the dorsal "organizer". Zebrafish chordin is expressed in both the organizer region and transiently in axial mesoderm. Under normal circumstances, the interaction between chordin and BMPs prevents BMPs from activating their receptors and creates a functional gradient of BMPs, which is responsible for the dorso-ventral patterning in zebrafish embryos (Miller-Bertoglio *et al*., 1997). Injection of zebrafish *chordin* mRNA into the ventral side of *Xenopus* embryos induced secondary axes. Ectopic overexpression in zebrafish resulted in an expansion of paraxial mesoderm and neurectoderm at the expense of more lateral and ventral

derivatives, producing a range of defects similar to those of dorsalized zebrafish mutants (Leung *et al*., 2005).

The *in situ* hybridization results (Fig. 32), obtained with the probe produced using the plasmid kindly offered by Dr. Marnie Halpern (Milller-Bertoglio *et al*., 1997), show that in early gastrulation (50% epiboly), the *chordin* mRNA expression was confined to deep cells in the embryonic shield both in *gr*-5mmMO and morphant embryos. A weaker expression seems to be present in morphant samples.

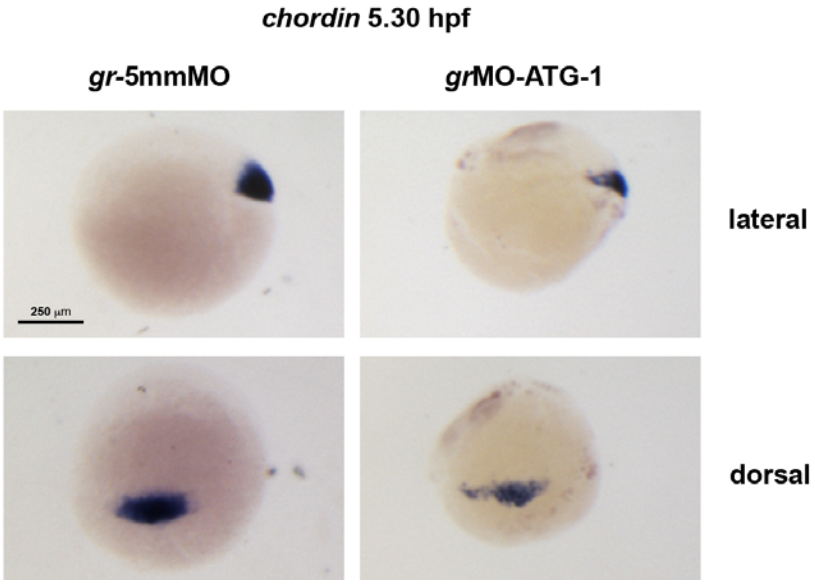


Fig. 32. Expression of *chordin* at 5.3 hpf. A weaker expression seems to be present in morphant samples.

39.2. *Egr2b*

Egr2b (early growth regulator 2b), also known as krox20, is the third and fifth rhombomere marker in the hindbrain (Oxtoby and Jowett, 1993). In the developing mammalian CNS, homologous sets of genes are expressed, representing a genetic network regulating the development of the hindbrain segments, termed rhombomeres (de Vellis and Carpenter, 1999). Rhombomeres appear in the floor of the fourth ventricle during early development and may be analogous to segments of the developing body plan of invertebrates. Rhombomeres are cell lineage-restriction units and express a number of genes in accord with their boundaries. A class of these genes are the *hox* genes, whose expression may be activated by other transcriptional regulators, including *krox-20*. Disruption of *krox-20* activity also disrupts

the pattern of *hox* gene expression and rhombomere formation, suggesting that this gene acts upstream of the *hox* genes.

The *in situ* hybridization analysis of *egr2b* was performed with the probe kindly offered by Dr. Trevor Jowett (Oxtoby and Jowett, 1993). As shown in the Fig. 33, at 15 and 24 hpf, *egr2b* expression is restricted to two regions of the neuroepithelium of the prospective hindbrain, rhombomeres 3 and 5. Staining for *egr2b/krox-20* transcripts in the hindbrain of morpholino-treated embryos indicates that its pattern of expression is similar to that of *gr*-5-mmMO embryos, demonstrating that, at these stages of development, this area was not greatly affected by *gr*MO. The only visible effect in morphants is constituted by a slight increase of *egr2b* expression in both rhombomeres, increase that is more evident from the dorsal view.

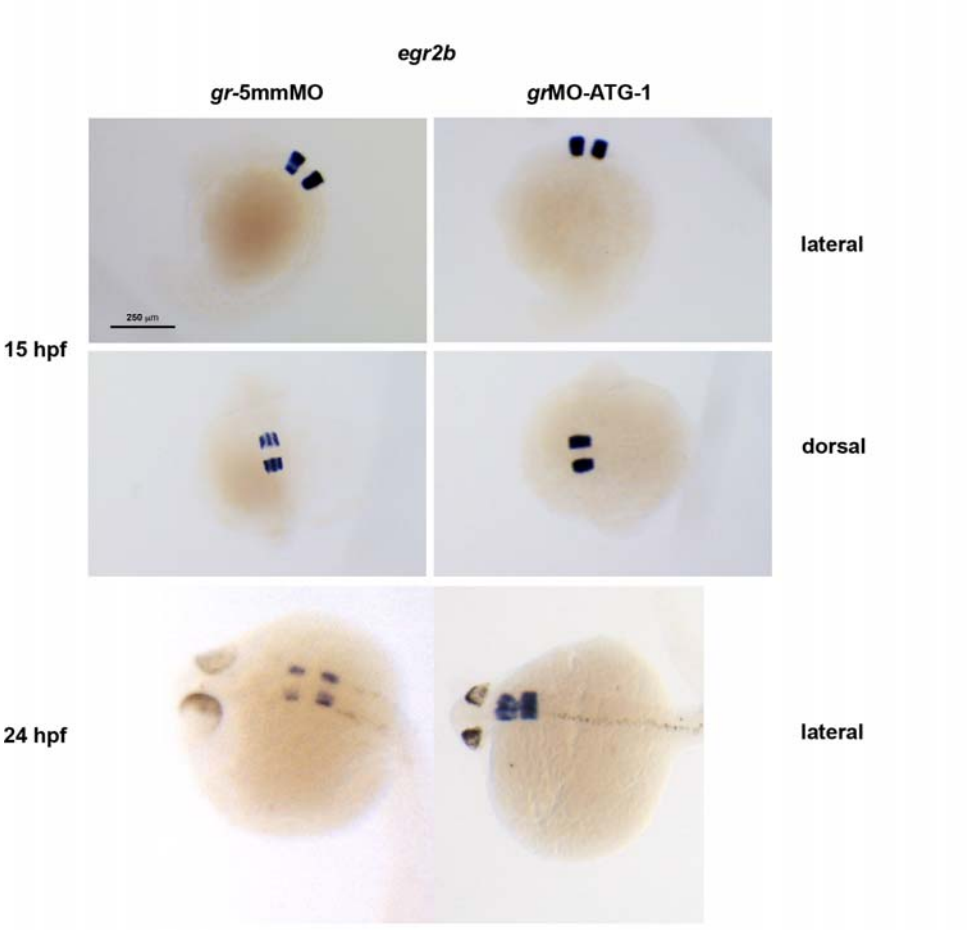


Fig. 33. Expression of *egr2b* at 15 and 24 hpf. The morphants show a slight increase in total abundance of *egr2b*, but its pattern of expression is the same as that of *gr*-5mmMO embryos.

39.3. *Pax2a*

Pax2a (paired box gene 2a) is a member of the family of transcription factors that includes Pax2, Pax5 and Pax8 (the Pax2/5/8 family) in mammals. The proteins in this family share a paired-type domain and a partial homeobox as DNA-binding motifs, an octapeptide for protein-protein interaction, and a transactivating/inhibiting domain at the carboxy terminus (Pfeffer *et al*., 1998). The paired-box genes play an important role in the regionalisation of the cephalic neural plate. Pax2 plays a key role establishment of the midbrain and isthmus organiser in mice (Favor *et al*., 1996), as does the homologous *pax2a* gene in zebrafish (Lun and Brand, 1998).

The phenotypes of homozygous mutant mice range from strong defects in the development of midbrain, eye, ear and kidney (Favor *et al*., 1996) to nearly normal development of the midbrain-hindbrain (MHB) territory (Torres *et al*., 1995). In *pax2a*-deficient zebrafish, a loss MHB boundary was reported (Lun and Brand, 1998).

The *in situ* hybridization analysis performed with the probe for *pax2a*, kindly offered by Dr. Hee-Chan Seo and Dr. Anders Fjose (Krauss *et al*., 1991), showed no differences in the pattern of between *gr*-5mmMO and *gr*MO embryos at 15 hpf (Fig. 34).

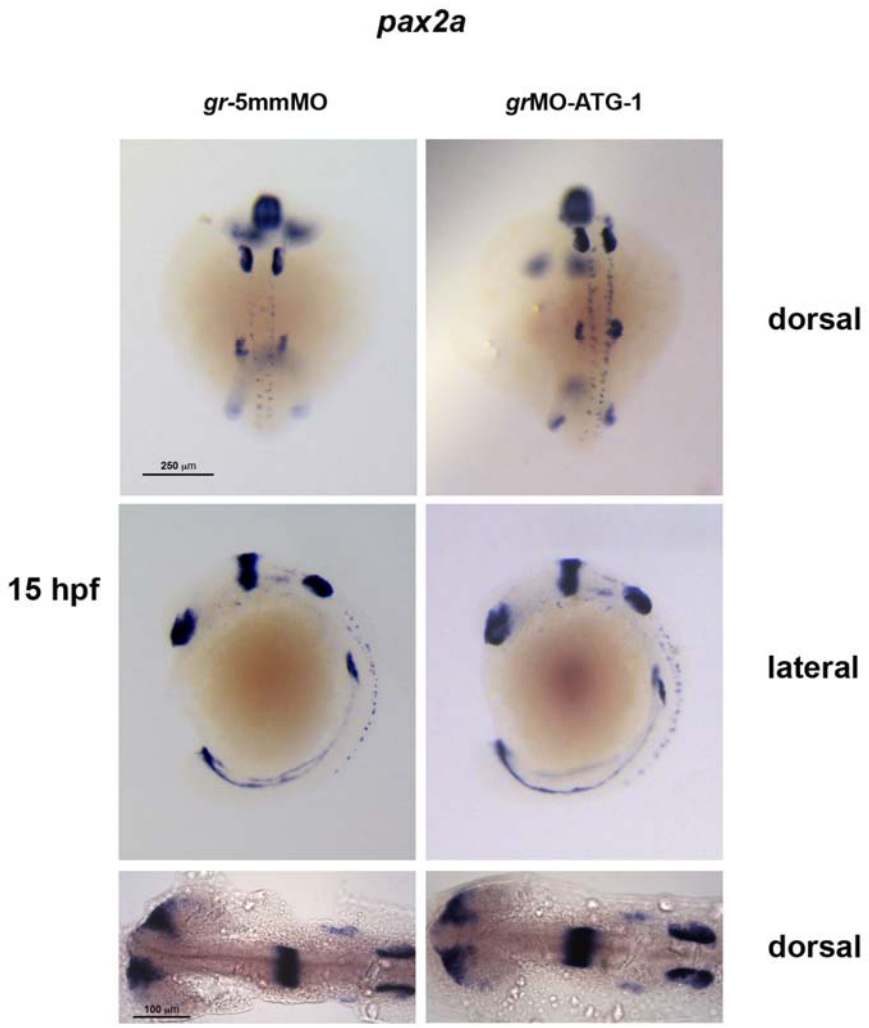


Fig. 34. Expression of *pax2a* at 15 hpf. No differences is evident between *gr*-5mmMO and *gr*MO embryos.

39.4. *Emx1*

In mammalian embryos, *emx1* and *emx2*, two vertebrate homeobox genes related to the *Drosophila empty spiracles* (*ems*) gene, play an essential role in rostral brain development. In zebrafish, the expression of *emx1* gene is detected around the10-somite stage in the pineal gland primodium, the developing anterior brain and the pronephric primodium within the intermediate mesoderm. Later, this gene is expressed in the telencephalon, as in mammals, and can be detected in the olfactory placode and in a small group of cells in the forebrain at 25

hpf. In the mesoderm, *emx1* expression is gradually concentrated in the posterior pronephric duct during somitogenesis, and becomes expressed predominantly in the urogenital opening at 25 hpf (Kawahara *et al*., 2002).

Also with this marker, analysed with the probe kindly offered by Dr. Atsuo Kawahara (Kawahara *et al*., 2002), a much higher expression was detected in morphant samples with respect to *gr*-5mmMO zebrafish (Fig. 35).

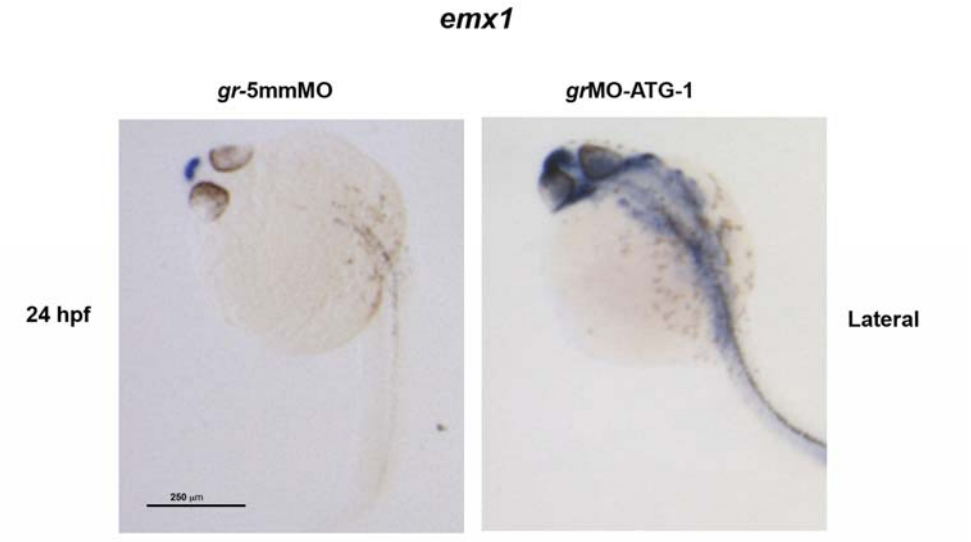


Fig. 36. Stronger expression of *emx1* in morphants as compared to controls at 24 hpf.

39.5. *Six3a*

The transcription factor Six3a contains a homeodomain and a Six domain and is specifically expressed in the anterior neuroectoderm including the eye field and specific parts of the abutting surface ectoderm in all vertebrates analyzed so far (Carl *et al*., 2002).
In addition, at subsequent stages of development, *six3a* is expressed in the developing retina. Overexpression of *six3a* results in an enlarged forebrain in zebrafish. In medaka, inactivation of Six3a leads to the complete absence of forebrain and eyes, indicating the key role of Six3a in the patterning of the anterior neural plate and establishment of retinal identity (Carl *et al*., 2002).

The *in situ* hybridization experiments were performed with the probe for *six3a* kindly offered by Dr. Anders Fjose (Wargelius *et al*., 2002). At 15 hpf, morphants exhibited a

higher expression of this marker, as shown in the Fig. 36. At 48 hpf, however, the expression pattern seems the same in *gr*-5mmMO and *gr*MO-ATG-1 treated samples, but the head is smaller in morphant fish.

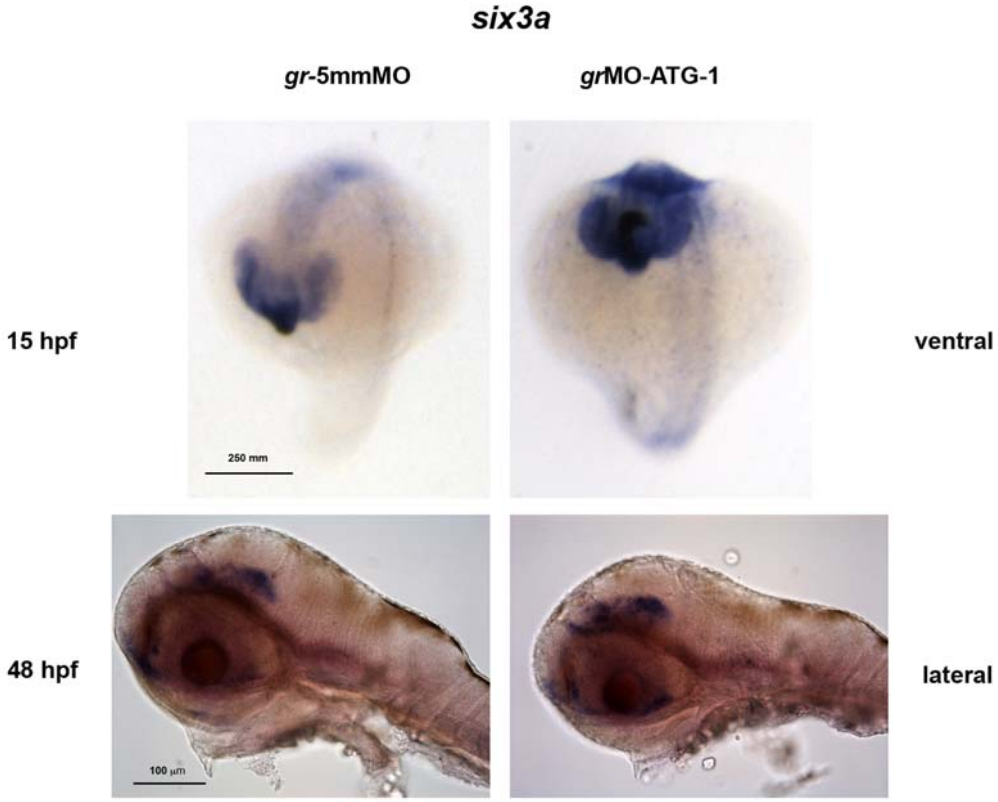


Fig. 36. Expression of *six3a* at 15 and 48 hpf. The expression is higher in morphants at 15 hpf but similar to controls at 48 hpf. Note the smaller head in morphants at 48 hpf.

39.6. *Myod1*

In vertebrates, the skeletal muscles of the trunk and limbs originate in embryonic structures called somites. Two genes, *myod1* and *myf-5*, are expressed in somites and are implicated as determination factors of the skeletal muscle lineage. *myod1* belongs to a group of structurally related proteins, the myogenic basic helix-loop-helix (bHLH) family of transcription factors, which has a major regulatory role in myogenesis (Weinberg *et al*., 1996). These bHLH transcription factors act sequentially in myogenic differentiation. *Myod1* is one of the earliest markers of myogenic commitment and regulates skeletal muscle differentiation. *Myod1* may also play a role in regulating muscle repair.

The whole mount *in situ* hybridization for *myod1* transcripts was performed with the probe offered by Dr. Gianfranco Bellipanni (Weinberg *et al*., 1997). As indicated in Fig. 37, the expression of this early muscle differentiation marker is normal in treated embryos at 15 hpf.

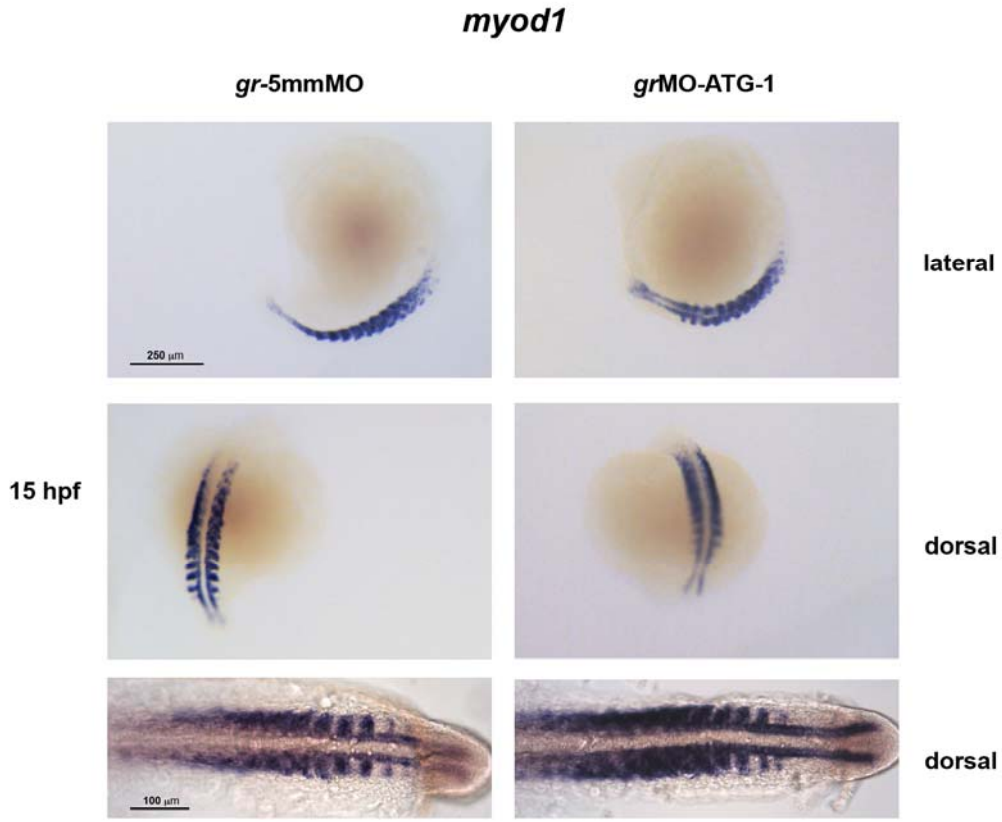


Fig. 37. Expression of *myod1* at 15 hpf. No differences is evident between *gr*-5-mmMO and *gr*MO embryos.

39.7. *Gr*

Gr expression was analysed by whole mount *in situ* hybridization with a probe containing the whole coding region of this protein. This analysis showed *gr* expression at 5 hpf (50% epipoly), while no signal was found at 10 hpf, in agreement with the RT-PCR result that shows a minimum of *gr* mRNA concentration at 8 hpf. At 15 hpf, the expression of *gr* was detected in the head and trunk regions, as observed also at 24 hpf (Fig. 38).

Because this *in situ* experiment doesn't show a very localized pattern of expression, we are preparing a new probe containing the 3'-UTR region in order to verify this widespread expression.

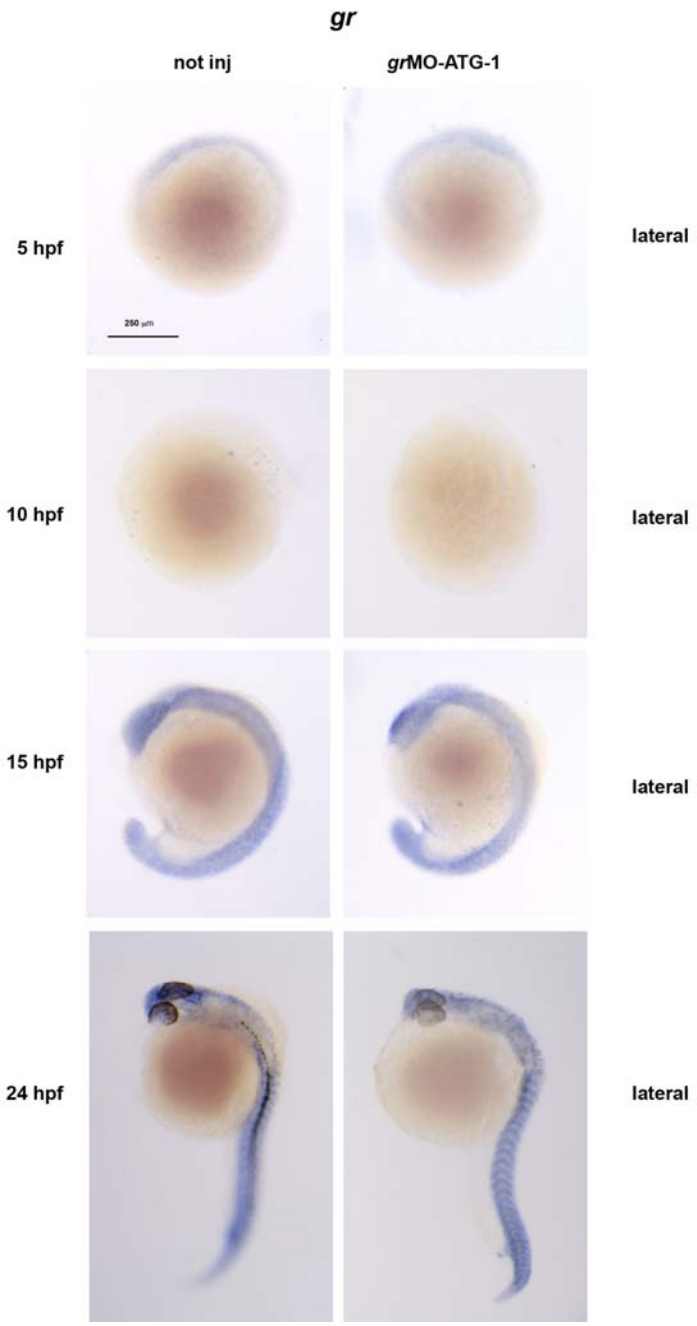


Fig. 38. Expression of *gr* at four developmental stages. Note the disappearance of the signal at 10 hpf and its stronger level in morphants at 24 hpf.

DISCUSSION

40. Expression analysis of steroid hormone receptor mRNAs during zebrafish embryogenesis

In zebrafish, mRNAs for steroid hormone receptors synthesized during oogenesis appear to be differentially available for translation during early embryogenesis, thus hinting at a possible role during subsequent development. In this work, I provide a simultaneous survey on the occurrence and dynamics of the maternal mRNAs for nine steroid receptors from ovulation to 48 hpf, in order to integrate and extend experimental evidence from previous reports.

In zebrafish, two *er* subtypes have been identified, *er*α and *er*β, which are phylogenetically related to the corresponding tetrapod subtypes. In addition, two *erβ* variants have been isolated from different fish species, including zebrafish, namely *erβ1* (also known as *esr2β*) and *erβ2* (also known as *esr2α* or *erβa*) (Lassiter *et al*., 2002; Bardet *et al*., 2002; Menuet *et al*., 2002; Tingaud-Sequeira *et al*., 2004). My results show that, during zebrafish early development, the content of *erα* mRNA is initially very low and is only slightly increased by embryonic transcription during the pharyngula stage. A similar profile was also reported by Bardet *et al*. (2002), although their data start from 12 hpf.

erβ1 and *erβ2* were found to display completely dissimilar profiles, as also observed by Bardet *et al*. (2002), Lassiter *et al*. (2002) and Tingaud-Sequeira *et al*. (2004). As determined by qRT-PCR, there was a rather high concentration of maternal *erβ2* mRNA just after fertilization, which began to decline during blastulation to reach a minimum at the gastrula stage with a small recovery later on by embryonic transcription. Conversely, maternal *erβ1* mRNA does not appear to be relevant, since the messenger for this receptor was found to be strictly dependent upon embryonic expression starting at 12 hpf.

Interestingly, Lassiter and Linney (2007) have demonstrated that the brain aromatase gene, *cyp19a1b*, is strongly expressed in zebrafish between 24 and 48 hpf, and my data support the assumption that the estrogen synthesized at hatching may interact with the ascending *erβ1* of embryonic origin. There is substantial evidence on the role of estrogen during zebrafish

organogenesis, as it is required for the development of the central nervous system and retinal epithelium as well as for normal cardiovascular functioning and body growth (Hamad *et al*., 2007; Nelson *et al*., 2008). At present, it seems that mainly *erβ2* can initially interact with maternal estrogen taken up into the yolk from the granulosa cells of the follicular envelope, but the regulatory significance of this interaction remains to be defined.

The gene encoding Ar is present as a single copy gene in the zebrafish genome (Gorelick *et al*., 2008; Hossain *et al*., 2008). Maternal *ar* mRNA was detectable from fertilization to the blastula stage reaching a minimum at gastrulation with limited increment thereafter. A comparable expression profile has been recently reported by Hossain *et al*. (2008). Such a dynamics points to a possible binding interaction with maternal androgens of thecal origin permeated through the oolemma, though the actual rate of mRNA translation is presently undetermined.

It is established that *ar* mediates the masculinizing effects of androgens on different parts of the fish reproductive system (Ikeuchi *et al*., 2001; De Waal *et al*., 2008), but little is known about its role in embryos before sexual differentiation. Notably, Gorelick *et al*. (2008) have observed the presence of *ar* mRNA in the zebrafish pronephros by whole-mount *in situ* hybridization between the 14- and 18-somite stages (16–18 hpf), hence after the drop of the corresponding maternal mRNA. Recent studies have revealed a differential expression of *ar* mRNA between two groups of zebrafish embryos, thus suggesting a precocious sex-related divergence (Jörgensen *et al*., 2008; De Waal *et al*., 2008).

Until now, three distinct *mpr* subtypes, named α, β, and γ, have been described in teleost fish (Thomas *et al*., 2004). They mediate meioprogestogen induction of the oocyte resumption of the second meiotic division before ovulation in zebrafish, as in other teleosts (Hanna *et al*., 2006, Thomas, 2008). There are no previous reports on *mpr* expression during zebrafish embryogenesis, but my data indicate merely a low persistence of the maternal mRNAs of *mprβ* and, to an even lower level, of *mprα* from ovulation until the blastula stage with further fading concentrations thereafter. Whether these mRNAs are simply a carry over of their occurrence during oocyte maturation or are actually translated in the early blastomeres remains to be established.

As determined by RT-PCR, *pr* mRNA was revealed in ovulated oocytes, but disappeared completely afterwards to rise again with embryonic transcription at 24 and 48 hpf. Hence,

maternal *pr* mRNA does not seem to be involved in the first life stages of zebrafish. Probably, meioprogestogens, which in fish include 20β-dihydro and 21-hydroxy derivatives of progesterone and 17α-hydroxyprogesterone (Thomas *et al*., 2002), limit their action to the terminal differentiation of the oocytes through genomic and non-genomic mechanisms. On the other hand, Bertrand *et al*. (2007), using whole-mount *in situ* hybridization, have detected a sharp rise of embryonic *pr* mRNA since epiboly (8 hpf) and during somitogenesis with subsequent prevalent rostral expression. Their figures show a *pr* mRNA dynamic pattern very similar to that of *gr* mRNA, which is highly expressed as both maternal and embryonic species. Since their antisense mRNA probe was designed on the coding region of the *pr* gene, which bears strong homology (>60%) to the *gr* gene, a cross-reactivity might explain the discrepancy about the onset and intensity of embryonic *pr* mRNA expression with respect to my data that were obtained with specific primers.

Unlike several other teleosts, in which two *gr* genes have been found, only one *gr* gene was identified in zebrafish (Prunet *et al*., 2006). My results demonstrate that the maternal and embryonic *gr* mRNAs were the most expressed messengers among the nuclear receptor mRNAs investigated. The turning point in their respective concentrations was set at the 75% epiboly stage (8 hpf), as previously found (Belvedere *et al*., 2005). This pattern is also in agreement with the report by Schaaf *et al*. (2008), in which maternal *gr* mRNA was shown to decline until the tail bud stage (10 hpf), when messenger abundance began to be restored by embryonic transcription.

My analysis, instead, diverges from that by Aslop and Vijayan (2008), who measured a pronounced decrease of maternal *gr* mRNA abundance from 1.5 to 25 hpf with a subsequent rebound until 49 hpf. In my analysis, the rebound associated with the activation of embryonic transcription is detectable at a much earlier time. Schaaf *et al*. (2008) have also established that zebrafish, like humans, co-expresses two C-terminal splice variants of *gr*, the canonical *grα* and a minor *grβ* isoform that acts as a dominant negative inhibitor. The *grβ* mRNA shows a more marked drop at the tail bud stage (10 hpf), when the *grα*/*grβ* mRNA ratio is maximal.

As to *mr* mRNA, the results obtained agree with those by Aslop and Vijayan (2008), indicating a low maternal abundance and a rise from 12 to 48 hpf, which, in their study, continues afterwards reaching 52-fold the 1.5 hpf value by 97 hpf. Given the net prevalence

of maternal *gr* transcripts over those for *mr*, that cortisol is a high affinity ligand for both receptors, and that cortisol deposited in the yolk from maternal circulation was shown to decrease 70% by 25 hpf (Aslop and Vijayan, 2008), it is evident that *gr* rather than proposed *mr* is more likely to mediate maternal cortisol action.

While embryonic mRNAs for steroid receptors are expected to be translated into proteins acting as transcription factors on steroid gene targets, the functional significance of the corresponding maternal mRNAs is more intriguing. The facts that they are deposited in the developing zygote with distinctive abundances and are differentially degraded, hint at a possible implication in early development. There are, however, three aspects that must be clarified by future research. First, how maternal mRNAs, presumably displaced with the germinal vesicle to the animal pole are partitioned among the blastomeres arising by synchronous division until MBT. Second, how steroid hormones, particularly corticosteroids, estrogens and androgens trapped inside the lipophilic milieu of the yolk, are brought into contact with their cognate receptors, when translated from their maternal mRNAs. Third, whether maternal steroid receptors exert a transcriptional control in the maternal programming of the zygotic genome activation before and after MBT (Mathavan *et al*., 2005), given the rather fast degradation of their most abundant mRNAs and the predominance of gene replication over transcription at those stages.

41. Morpholino knockdown of glucocorticoid receptor in zebrafish during embryogenesis

In the first part of my study, I have analyzed the transcripts of steroid hormone receptors during zebrafish embryogenesis, and found that *gr* mRNA is highly expressed at both maternal and zygotic stages. GR is known to play a major role in metabolic, immune and behavioral processes in vertebrates.

In the second part of this thesis, I decided to analyze the function of Gr during zebrafish embryogenesis using antisense MO technology. Typically, analyses of gene function were performed with genetic mutagenesis approaches. Knock-out mutants of various genes are available for model organisms, such as mouse and zebrafish (Malicki *et al*., 2002; Brown and Nolan, 1998). The disadvantage of this approach is that the methodologies to establish a mutant are difficult and time-consuming. MO was introduced, in recent years, to assess gene function in developmental biology. This technology has already been successfully applied to zebrafish, *Xenopus* and others species.

In this study, I used two MOs, designed to block the translation of *gr* in zebrafish. The effect of Gr deficiency was first evident as a delay in development during gastrulation, when the three germ layers are formed and the zygotic transcripts begin to be produced. After hatching, the MO phenotype includes small head and eyes, short body, pericardial edema, disrupted melanophore patterning, failure to inflate the swim bladder, tail curling, and abnormal swimming behaviour. These effects are accompanied by an increased level of apoptotic cell in the head and trunk region. Thus, the *gr* transcripts may have important roles in the control of nervous system development.

41.1. Confirmation of specificity of Gr morphant phenotypes

Morpholino phenotypes need to be cautiously controlled to confirm the specific phenotypes of *gr*MO.

The specificity of the effects of the MOs used in this work was confirmed by several evidences:

- The two non-overlapping-oligo strategy is commonly used to confirm specificity of a MO knock-down, because it is assumed that MOs with different sequences would not cause the

same non-specific phenotype (Eisen and Smith 2008). Therefore, I used two non-overlapping sequences targeting the translation site of *gr* mRNA of zebrafish (*gr*MO-ATG-1 and -2) and found that the embryos displayed the same phenotype.

- The 5-mismatch MO oligo was used at that same concentration of *gr*MOs and no *gr*MO phenotypes were observed. This suggests that the *gr*MO is specific for its target.
- One of the best controls is to rescue the MO-induced phenotype with a co-injection of the full length mRNA from another fish species or the zebrafish mRNA mutated in the region recognized by the Morpholino. Since in both cases the 5'-UTR sequence is different, the Morpholino cannot bind to same transcript and the protein encoded by the co-injected mRNA can complement the knockdown protein. In our study, the rescue mRNA approach using the complete coding sequence of trout *gr2*, successfully recovered the Gr normal phenotype.
- Finally, the inhibition of *gr* mRNA translation into Gr protein was verified using Western blotting analysis and with a protocol based on *in vitro* transcription and translation processes. Both these approaches showed the reduction of Gr protein concentration in *gr*-morphants.

These data confirm the specificity of the phenotypes obtained with the use of ATG-targeted MOs.

41.2. Effects of the *gr*MO-SP morpholino

In a previous work (Mathew *et al*., 2007), antisense repression of *gr* was performed using splice variant morpholino (MO) oligonucleotides designed to block splicing of the exons encoding the Gr ligand-binding domain. As reported by the authors, transient knock-down of Gr did not elicit early developmental defects, suggesting that the Gr is not essential for early embryonic development. These results are in agreement with those obtained using our *gr*MO-SP that was designed to block splicing of the target exon 3/intron 3 boundary of the *gr* pre-mRNA. The in-frame translation process will be interrupted and the protein eventually produced will lack both DBD and LBD domains.

However the splice variant Morpholinos are only able to block splicing (and thus translation) of zygotic transcripts, whereas they couldn't work on the maternal transcripts that are already present at high levels in ovulated eggs.

In conclusion these results suggest that Gr proteins that derived from translation of maternal transcripts are essential for embryonic development. In fact the knock-down of these transcripts triggers developmental defects. Conversely, the knockdown of embryonic transcripts does not seem to have similar consequences, although this may depend on its efficiency.

41.3. Microarray analyses

In this study, the gene expression of *gr* morphants and control embryos were analyzed by microarray technology at 5 and 10 hpf, corresponding to the gastrula stage and early segmentation stage. The results obtained show that 30 transcripts were dys-regulated at 5 hpf; of which 20 were up-regulated and 10 were down-regulated. At 10 hpf, 32 genes were dys-regulated, with 11 up-regulated and 21 down-regulated. Finally, the common dys-regulated genes from 5 and 10 hpf were 16 genes.

Analyzing these results, it is possible to note that a higher number of genes were up-regulated then down-regulated (45 versus 33), thus suggesting that, in early embryonic development, Gr functions prevalently as a negative regulator of gene transcription. Moreover, the low number of genes found to be regulated by Gr is in agreement with the developmental stages analyzed: particularly at 5 hpf and also at 10 hpf the zygotic transcription has just begin and a low number of genes is presumably transcribed, apparently under Gr control.

Some of these genes, and in particular *caspase-8*, *igf2α* and *centaurin-1α,* were also evaluated by semiquantitative RT-PCR to confirm the results obtained with microarray analyses.

The insulin-like growth factor (IGF) signaling system is a critical regulator of somatic growth during fetal and postnatal development, primarily through its stimulatory effects on cell proliferation and survival. At the cellular level, the IGFs control mitosis, apoptosis, differentiation and chemotaxis. Two *igf-2* genes, *igf-2a* and *b*, are present in the zebrafish genome and play an important role in early neural and cardiovascular system developments

(Harnett *et al*., 2009), as demonstrated by knock-down experiments. *Igf2a*, that corresponds to *igf2b* in the work of Zou and coworkers (2009), was first detected by them at 50% epiboly (about 5 hpf) and was maintained at relatively high levels thereafter. Thus, it seems that *igf2a* transcripts are the products of zygotic genes activated after MBT. By *in situ* hybridization, the authors showed these transcripts were highly expressed at 48 hpf and most abundantly in the anterior part of the embryo. The authors performed also a gain of function experiment by injecting the mRNAs of all four member of the zebrafish *igf* family. All zebrafish *igf* mRNAs caused similar defects in dose-dependent manners, although there were considerable differences in their effectiveness. The mRNA-injected embryos, compared to controls, showed marked expansion of dorsal neural tissues and axial domains and a severe shortening of body length (Zou *et al*., 2009). According to my results, Gr could act as a negative regulator of *igf* expression and thus indirectly be involved in the *igf* regulation of zebrafish development.

Centaurin-α1 is an Arf GTPase-activating protein (GAP) that is highly expressed in the nervous system (Moore *et al*., 2007). This protein is involved in regulating various biological processes (membrane ruffling and trafficking, actin cytoskeleton regulation and presynaptic vesicular structures) (Vashisht *et al*., 2009). In rat, siRNA-mediated knockdown of centaurin-α1 levels leads to inhibition of dendritic branching and filopodia and spine-like protrusions in dissociated hippocampal neurons. Overexpression of wild-type centaurin-α1 in cultured hippocampal neurons in early development enhances dendritic branching, and increases dendritic filopodia and lamellipodia. In zebrafish too, this protein may be involved in important biological processes during embryogenesis and could be related to the function of Gr on neurogenesis.

Further work is required to better characterize these gene and their interplay with Gr in zebrafish development.

41.4. Gr involvement in swimming behavior

An interesting phenotypic trait observed in Gr morphants was an alteration of swimming behavior, as the larvae were unable to balance and swim normally due to whirling movements, suggesting vestibular and hearing dysfunctions and lack of muscular

coordination. Similar phenotypes were reported after knockdowns of *erβ2* (Froehlicher *et al*., 2009), histone deacetylase 3 (*hdac3*), transmembrane inner ear (*tmie*) gene (Shen *et al*., 2008), Na, K-ATPase α1a.1 and β2b (Blasiole *et al*., 2006), claudin gene (Hardison *et al*., 2005) and motility mutant embryo (Granato *et al*., 1996). This phenotype was related to the development of the inner ear, which is responsible for the transduction of equilibrium stimuli into electrical impulses. The connection between Gr deficiency and this swimming alteration is not known, but it suggests that Gr as well could be involved in the regulation of genes critical for the correct formation and function of the inner ear and its sensory tissue.

41.5. Gr may be involved in swim bladder development

The swim bladder or gas bladder is a vascularized gas-filled sac, localized in the dorso-anterior part of the body cavity, that maintains buoyancy in fish. In some species, including zebrafish, the swim bladder supports hearing function by amplifying and transmitting sound waves directly to the inner ear. During early development of the digestive system, anterior endodermal precursors give rise to epithelial/parenchymal cells in the pharyngeal and other organogenetic regions (*i.e*. liver, pancreas, and swim bladder). (Shin *et al*., 2008). Hence, similarly to mammalian lungs, the zebrafish swim bladder arises from an outgrowth of the foregut endoderm, in close temporal and spatial proximity to the liver and pancreas. Its inflation occurs at about 4-5 days of development, when larvae swallow air bubbles at the water surface and convey them by peristaltic movements through the gut and pneumatic duct into the gas bladder lumen (Sadler 2007).

Because the swim bladder and mammalian lungs share a common evolutionary origin, it is likely that their development is controlled by shared regulatory pathways. Interestingly, 11β-HSD1, the glucocorticoid-activating enzyme, is expressed in the mouse lung early in the development (Thompson *et al*., 2004)**.**

A significant number of Gr-deficient zebrafish larvae fail to inflate the swim bladder, as also observed in other morphants, such as after knockdown of prolactin (Zhu *et al* 2007) or α2 macroglobulin-like (Hong and Dawid 2008). In addition, these morphants displayed defects in the liver, pancreas and intestinal development. It is well known that the glucocorticoid affects the development of pancreas in human and mice (Breant *et al*., 2006;

Gesina *et al*., 2006). The fact that gas bladder inflation was prevented even at 10 dpf may be due to either defects in the foregut or just inability to reach the air surface to engulf air bubbles owing to whirling swimming.

The unreduced yolk sac in Gr morphants suggests an impairment of nutrient uptake which may be responsible for general cellular dysfunction (Schlegel *et al*., 2006), leading to pericardial edema and several anatomical abnormalities. Hence, these could be secondary consequences of Gr deficiency and not directly related to the mechanism of action of Gr.

41.6. Whole mount *in situ* hybridization

In order to better characterize the Gr morphant embryos, we performed a preliminary series of whole-mount *in situ* hybridization experiments with several developmental markers. Although the results are just indicative, since only a small number of markers was investigated, they show that the marker expression pattern was mostly unchanged in morphant embryos, except in the case of *emx1*, whose signal was more widespread than in controls. On the other hand, some differences were found in expression levels following the *gr* knockdown. While *chordin* was down-regulated, three markers involved in neurogenesis, *egr2b*, *emx1* and *six3.1*, were up-regulated. The fourth neurogenic marker, *pax2.1*, was pretty much unchanged, as was *myoD* involved in somitogenesis and myogenesis.

The fact that *six3.1* is up-regulated at 15 hpf is particularly intriguing, because it is supposed to promote retinal development and eye formation, while morphant embryos displayed smaller head and reduced eyes at 48 hpf. Similarly, it remains to be established the way in which the high expression of *emx1* in the olfactory placode of morphants at 24 hpf would affect the completion of the olfactory system before hatching. The evidence emerging so far from pangenomic microarray analysis and qRT-PCR, in which *igf2b*, a major embryonic growth promoter, was found to be markedly over-expressed after Gr knockdown, coupled with the above *in situ* hybridization observations, suggests that maternal and zygotic glucocorticoid receptors exert an essential developmental control, which is partly carried out by restraining the expression of genes commonly regarded as promoters of fundamental organogenic processes.

In conclusion, the knock-down of Gr in zebrafish revealed a requirement of both maternal and zygotic Gr in multiple developmental processes involved in neurogenesis and gut and accessory organs formation. There are indications that the maternal receptor is critical in early embryogenesis, but more work is needed to elucidate the functional transition from the maternal to the zygotic GR and whether they affect distinctive components of the genetic machinery.

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