ROLE OF OSTEONECTIN IN MORPHOLOGICAL DEFORMITIES INDUCED BY UV RADIATION IN FISH EMBRYOS

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ABSTRACT

UVR exposure is known to cause developmental defects in a variety of organisms including aquatic species but little is known about the underlying molecular mechanisms. In this work we used zebrafish (Danio rerio) embryos as a model system to characterize the UVR effects on fish species. Larval viability was measured for embryos exposed to several UVR spectral treatments by using a solar simulator lamp and an array of UV cut-off filters under controlled conditions in the laboratory. Survival rate and occurrence of development abnormalities, mainly caudal (posterior) notochord bending/torsion, were seriously affected in UV exposed larvae reaching values of 53% and 72% respectively, compared to non-UV exposed larvae after 6 days post fertilization (dpf). In order to elucidate the molecular mechanisms involved, a matricellular glycoprotein named osteonectin and the expression of a DNA-repair related gene, p53, were studied in relation to UVR exposure. The results indicate that osteonectin and p53 expression were increased under UVR exposure due to wavelengths shorter than 335 nm (i.e. mainly UVB) and 350 nm (i.e. short UVA and UVB), respectively. Furthermore, parallel experiments with microinjections of osteonectin capped-RNA showed that malformations induced by osteonectin overexpression were similar to those observed after a UVR exposure. Consequently this study shows a potential role of osteonectin in morphological deformities induced by solar UV radiation in zebrafish embryos.
INTRODUCTION

The ultraviolet (UV) region of the spectrum is generally classified into UVC (200–280 nm), UVB (280–315 nm) and UVA (315–400 nm) but only the UVB and UVA components reach the Earth’s surface while the UVC radiation is completely absorbed by the stratospheric ozone layer. The UVB reaching the Earth’s surface has increased during the last decades as a result of the stratospheric ozone depletion (1,2). After the Montreal Protocol there are some early signs of stratospheric ozone recovery (3) however ozone is also affected by factors such as changes in the temperature and dynamics of the stratosphere which are, in turn, affected by climate change. This is delaying, perhaps indefinitely, a full recovery of ozone and consequent reduction in UVB. Moreover, global change can also affect UVA and UVB in the aquatic environment through variations in cloud cover and the amount of the coloured dissolved organic matter, among other factors (4,5). While this increased UVB and the potential for long term variation in UVR has motivated a variety of studies on their effects in both terrestrial and aquatic ecosystems, there are still major gaps in our understanding of the mechanisms involved. Several experiments have demonstrated significant alterations generated by UVR in organisms from different environments such as the induction of cutaneous malignant melanomas in mammals (6,7), skeletal malformations and low hatching success in amphibians (8-10) and decreased survival and oxidative stress in different fish species (11-13). However, very little has been done to establish the molecular basis of the mentioned alterations produced by the exposure to UVR.

It is well known that DNA damage caused by UV radiation provokes adaptive cellular responses, which include DNA repair events, activation of several signalling cascades, and changes in transcription (14). The repair of UV-induced DNA lesions is launched during and immediately after a UV exposure. At the same time, a cellular response,
either a replication arrest or apoptosis takes place (14, 15). Recent studies have shown that apoptosis of cultured cells is led by \textit{p53} gene after a DNA-damaging event. Cellular \textit{p53} is normally maintained at a low expression level, but rapidly increases upon exposure to harmful agents such as UVR (16). For example, in zebrafish an enhanced rate of apoptosis associated with a high \textit{p53} expression was observed after UV exposure (17). Furthermore, a mutation in \textit{p53} may inhibit the apoptotic process and triggers carcinogenesis (18, 19).

In skin, the cellular events are coupled with paracrine events and the following photoprotective responses, such as changes in the extracellular matrix. However, the role of matrix proteins in protective mechanisms after a UV exposure is still unclear. Three matricellular senescence-associated proteins, i.e. fibronectin, osteonectin and SM22, were increased in human skin diploid fibroblasts (hdfs) 72 h after several exposures to UVB (20). Furthermore osteonectin is also associated with an aggressive tumor phenotype in certain types of cancer such as melanomas (21). Multiple biological functions have been associated with this protein since it was first described as the major non-collagenous constituent of vertebrate bones. In zebrafish, \textit{osteonectin} expression appears early in development and it is required for skeletal development (22).

The purpose of this study was to characterize the potential molecular responses caused by UV radiation in the freshwater species zebrafish, \textit{Danio rerio}. Zebrafish is a species widely used as a model organism in laboratories because of several properties that make this species easy to work with. Some of these advantages are their small size, fast development and hundreds of embryos per spawning. Moreover external development and their transparent embryos are important characteristics for an easy phenotype observation allowing an appropriate morphological monitoring.
In the present study, we investigated potential underlying molecular mechanisms of solar UV radiation induced musculo-skeletal deformities in fish embryos. First, different exposures of full spectrum irradiance including photosynthetic active radiation (PAR, i.e. visible radiation, 400-700 nm), UVA and UVB, as well as different spectral treatments using an array of several UV cutoff filters were used to determine the embryonic sensitivity to UVR. Expression of the DNA-repair-related gene p53 and the extracellular matrix protein osteonectin were measured under all the conditions. Second, survival and malformation percentages were assessed in non UVR exposed (i.e. control) and UVR exposed embryos. Finally, osteonectin overexpression experiments were carried out in zebrafish embryos to determine the potential role of osteonectin on developmental abnormalities produced by UVR exposure and how these affect performance, health and well-being of fish species.

MATERIAL AND METHODS

Fish husbandry
Zebrafish embryos of the standard wild type Tue (Tuebingen) strain were raised at 10 hours light and 14 hours dark photoperiod at approximately 28°C. The procedures for zebrafish culture and embryo collection have been described previously (23). The designation of zebrafish developmental stages follows that of Kimmel et al. (24).

Experimental set up and UVR exposure
Zebrafish embryos were exposed in a special polychromatic incubator, the “photoinhibitron”. The incubator uses a 2500 W xenon lamp (Solar simulator lamp, Schoeffel Instrument Corp., Westwood, NJ, USA), which, after appropriate filtration,
provides PAR, UVA and UVB in similar proportions as solar irradiance (Fig. 1). The beam passes through an array of eight long-pass filters constructed using Schott (Duryea, PA, USA) WG filters (nominal 50% transmittance (T) at 280, 295, 305, 320, and 335 nm), a Schott GG filter (50% T at 395 nm) and Newport (Franklin, MA, USA) LG filters (50% T at 350 and 370 nm). For convenience, we subsequently refer to each of these long-pass filters by their wavelength of 50% T or “cutoff” wavelength. In order to obtain treatments with varying irradiance, long-pass filters were combined with neutral density screens to produce up to 10 different irradiances for each filter for a total of 80 treatments of varying spectral composition and irradiance. For embryo exposures we selected several positions for each long-pass filter in which PAR irradiance was about 600±50 µmol photons m\(^{-2}\) s\(^{-1}\) as measured with a QSL-2101 spherical sensor (Biospherical Instruments). This PAR value is approximately 30% of the maximum irradiance of a sunny day. Unweighted UVR irradiance ranged from 45.8 W m\(^{-2}\) in the most damaging treatment (280 nm cutoff) to 1.5 W m\(^{-2}\) in the least damaging treatment (395 nm cutoff), with UVA irradiance being 40.9 W m\(^{-2}\) and 1.5 W m\(^{-2}\), and UVB irradiance 4.9 W m\(^{-2}\) and 0.0 W m\(^{-2}\), respectively. Weighted irradiance in the two most damaging treatments (280 and 295 nm cutoff) calculated using the action spectra for DNA damage of Setlow normalized to 300 nm (25) was 4.74 and 1.68 W m\(^{-2}\) (Fig. 1). Weighted irradiance in the next most damaging treatment, 320 nm cutoff, was 0.014 W m\(^{-2}\). For comparison, solar exposures can reach up to 0.161 W m\(^{-2}\) at the equator (26). The light treatments are directed to 1.8 cm diameter, flat-bottom quartz cuvettes that are mounted within a temperature-regulated block and that were filled with water and fish embryos for exposure. Temperature was maintained at 28ºC and spectral irradiance was measured with a scanning monochromator (SPG 300 Acton Research, Acton, MA, USA) with a fiber optic and photomultiplier tube as previously described (27).
To determine the effect of exposure duration, 4 hours post fertilization (hpf) embryos were exposed to full spectrum irradiance (280 nm cutoff) in the photoinhibitron for 60, 120, 180 and 240 minutes and then sampled at 24 hpf for RNA extraction.

Subsequently, in order to establish the effect of different wavelengths of UV a second exposure experiment with 4 hpf embryos was carried out using different cutoff filters (280, 295, 320, 335, 350, 370 and 395 nm cutoff) for 150 min. Dark control group was also included as non-exposed reference sample. Samples were collected at 24 hpf for analysing *osteonectin* and *p53* expression by quantitative real time PCR (qRT-PCR). Additionally, fish exposed to UVR filtered through the 295 nm cutoff or the 395 nm cutoff filter (i.e. UVR excluded, control) were collected for *in situ* hybridization. Control and UV exposed 24 hpf embryos were fixed overnight at 4°C in 4% paraformaldehyde in 1XPBS, washed in PBS, and stored at -20°C in 100% methanol for *in situ* hybridization. Some embryos exposed to the 295 nm and 395 nm cutoff treatments were raised until 6 days post fertilization (dpf), sampling at 1, 2, 3 and 6 dpf, to test the larval viability and development abnormalities. Ethical approval for all animal studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority. Results show the mean ± SEM of 2 independent experiments, with samples analyzed each time in triplicate.

**RNA isolation and quantitative real time PCR (qRT-PCR)**

Control non-exposed and UV exposed 24 hpf embryos were collected and total RNA was extracted using Trizol reagent according to manufacturer’s protocol (Invitrogen). cDNA was synthesized from total RNA using superscript III (Invitrogen) according to
manufacturer’s recommendations. The following primer sequences were used for Q-RT-PCR: for osteonectin \( (5’\text{primer} / 3’\text{primer}) \) CCCTCTGCGTGCTCCTCTTA / GCATCGCACTGCTCAAAGAA, for \( p53 \) \( (5’\text{primer} / 3’\text{primer}) \) GGATCCTTCTTGCAAAAGCAATGGCGCA / CCGGTGAATAAGTGCAAGTTA and for \( 18S \) \( (5’\text{primer} / 3’\text{primer}) \) ACCACCCACAGAATCGAGAAA / GCCTGCGGCTTAATTGTGACT. All expression levels were normalized to \( 18S \) using the \( 2^{\Delta\Delta T} \) method (28). Quantitative real time PCR (qRT-PCR) reactions were performed using an AB 7300 Real-Time PCR System and SYBR green incorporation (Applied Biosystems). The PCR cycles for all primer sets were: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were done in triplicate and each condition was repeated 2 times. Dark control group was used as reference sample.

**Larval viability and developmental deformities percent**

Survival and developmental deformities percentages were calculated in the control (395 nm cutoff) and UV (295 nm cutoff) exposed embryos groups. Larvae were exposed for 150 min in the photoinhibitron as explained previously and were subsequently transferred to 1 L tanks under optimal growth conditions.

Survival percent was calculated as the number of embryos survived within 1, 2, 3 and 6 dpf divided by the total number of embryos and multiplied by 100. Alterations in spinal curvature were used as marker for the calculation of developmental deformities percentage. Developmental abnormalities percent was calculated as the number of abnormal embryos survived within 1, 2, 3 and 6 dpf divided by the total number of surviving embryos and multiplied by 100.
mRNA synthesis, microinjection and In situ hybridization

For mRNA synthesis, the pCS2\textsuperscript{+}-osteonectin was linearized with Not I. Capped mRNA was transcribed \textit{in vitro} using the SP6 Message Machine Kit (Ambion). Osteonectin capped–mRNA was injected into one- or two-cell stage embryos. The amount of mRNA injected was titrated for the maximal doses that could be injected (22). Approximately 1 nL of two different osteonectin capped-mRNA concentrations (200 µg/mL or 800 µg/mL) was injected per embryo. Approximately 150 embryos were used. Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense osteonectin probe as previously described (22). Control embryos were injected with 1 nL of eGFP (Green Fluorescent Protein) capped-mRNA (500 µg/mL).

Statistical analysis

Results are given as mean ± SEM. First one-way analysis of variance (ANOVA) was applied followed by the Student-Newmans-Keuels (SNK) test to check differences between particular groups. Data were log-transformed when necessary to achieve normality and homogeneity of variance (INSTAT\textsuperscript{tm}, GraphPad Software V2.04a). The level for accepted statistical significance was \(p<0.05\). Significant differences in the figures are indicated by asterisks.

Exposure response curves were fitted using a non-linear data analysis program describing a sigmoid curve (Sigma Plot, Scientific Graphic Software, Version 9.0).

RESULTS
Time series of full spectrum exposure on the matricellular protein osteonectin and the DNA repair-related gene p53 expression

The expression levels of the matricellular protein osteonectin and the DNA repair-related gene p53 after different UVR exposure times (60, 120, 180 and 240 min) under full spectrum irradiance (280 nm cutoff) on 4 hpf embryos were determined by the Quantitative real time PCR (qRT-PCR) in 24 hpf embryos. Results showed a time-dependent increase of the expression of both genes in response to a full spectrum exposure (Fig. 2). The maximum increase in expression was obtained after a 180 min exposure when osteonectin and p53 expression levels reached a 2.2±0.16 and 1.77±0.15 fold increase, respectively. Furthermore, an exposure-response curve was fitted to the measured levels in each expression time series (Fig. 2), from which the exposure time to reach 50% of the maximum was estimated to be 150 minutes for osteonectin and 140 minutes for p53.

UV action spectra for the matricellular protein osteonectin and the DNA repair-related gene p53 expression

Once the estimated exposure time to reach fifty percent of the maximum increase in gene expression in response to the full spectrum exposure was determined, we examined the effect of excluding various portions of the UV spectrum on the expression of the osteonectin and p53. For that purpose, a 150 min time exposure experiment with 4 hpf embryos using different cutoff filters (280, 295, 320, 335, 350, 370 and 395 nm) was carried out.

The increase in osteonectin and p53 gene expression was higher when embryos were exposed to UVB than when UVB was excluded from the spectra, reaching values around a 2.0 fold increase compared to those non UV exposed embryos (395 nm cutoff).
Significant differences were also observed for the spectral response between *osteonectin* and *p53* gene expression. *Osteonectin* levels increased significantly only when wavelengths shorter than 335 nm were included in the exposure spectra while *p53* expression was induced by longer wavelengths, when wavelengths shorter than 350 nm were included in the spectra. Moreover, spectral treatments including only longer wavelengths of the UVA region and PAR did not produce significant increases in *osteonectin* and *p53* gene expression compared to non UV exposed embryos.

To verify the *osteonectin* expression increase in UV exposed embryos, *in situ* hybridization was carried out in UVR excluded (control, 395 nm cutoff) and UV exposed embryos (295 nm cutoff). By 24 hpf, *osteonectin* transcripts were significantly increased in the caudal fin fold, notochord, somites and the otic vesicle of UV treated embryos compared to embryos where UVR was excluded (Fig. 4 arrow heads). Alterations in spinal curvature were also identified in the UV-exposed group (see Fig. 4, arrow). The results obtained by *in situ* hybridization agree with those observed by qRT-PCR, indicating a significant increase of *osteonectin* expression after UV exposure.

**Larval viability and morphological phenotypes**

We examined the percent survival and incidence of development abnormalities for UVR excluded control (395 nm cutoff) and UV (295 nm cutoff) exposed embryos within 1, 2, 3 and 6 dpf. As indicated in Table 1, in the UVR excluded control groups, 94 ± 1.3% of the embryos survived up to 6 d. In contrast, only 53 ± 8.6% of embryos exposed to UV survived up to 6 d, which is significantly lower from the control treatment group. Significant reduction of survival rate in UV (295 nm cutoff) exposed embryos was also found at 1, 2 and 3 days after treatment.
The incidence of fish with developmental abnormalities was also significantly higher in UV exposed embryos in all developmental stages analyzed (Table 1). The number of abnormalities increased with time after exposure in the UV exposed larvae from 5 ± 0.6 % and 48 ± 10 % in the control and UV exposed embryos, respectively at day 1 to 7 ± 1 % and 72 ± 4.6 %, respectively at day 6.

The type of developmental abnormalities were similar in all developmental stages analyzed, with caudal (posterior) notochord bending/torsion the most frequent developmental abnormalities recorded in the UV (295 nm cutoff) exposed embryos (Fig. 4 and Fig. 5).

**Osteonectin injection mimics the morphological phenotypes observed in embryos exposed to damaging UV radiation**

To link the increase of osteonectin expression with the high incidence of developmental abnormalities found in UVR exposed embryos, approximately 0.2 ng or 0.8 ng of osteonectin capped-mRNA were injected into one- or two-cell stage embryos. After injection the embryos were raised to 24 hpf and their phenotypes were scored. The expression of osteonectin was dose dependent, as it was higher when a larger amount of capped-mRNA was injected (Fig. 6).

Phenotypic malformations observed, mainly caudal (posterior) notochord bending/torsion, were similar to those observed after a damaging UV exposure (Fig. 5, Fig. 6).

**DISCUSSION**

Ultraviolet radiation is widely mentioned as a damaging environmental factor for organisms in both terrestrial and aquatic systems (29, 30). The effects derived from a
deleterious UV exposure are known to cause irreparable effects at different levels from organism survival and reproduction (8-11,) to cellular metabolism and viability (13, 14). However, the molecular responses triggered in an animal organism after a UV exposure are not yet understood. Previous studies have already established that the zebrafish system can be an important tool to investigate the biological effects of UV light in vertebrate development (11,17). Moreover, it has been demonstrated that zebrafish have a competent antioxidant response and photorepair system to repair UV induced DNA damage (17). This photorepair system includes up-regulation of \textit{p53} gene and cell cycle arrest (31).

In this study, UVR exposure was performed using a special polychromatic incubator, the “photoinhibitron”, under controlled conditions in the laboratory. Under natural conditions the direct effects of UV radiation on specific molecular targets are difficult to assess due to the interaction with other environmental factors and changes in irradiance caused by the variability in cloud cover, atmospheric composition and/or the amount of the coloured dissolved organic matter, among others. The incubator uses a solar simulator lamp which, after appropriate filtration, emits PAR, UVA and UVB in similar proportions as those observed under natural conditions (27). This produces a reliable UVR-dependent response in zebrafish embryos where the damage induced by short UV wavelengths is counteracted by the repair mechanisms activated by longer wavelengths. Our results show that using this experimental setup, exposure to UV can cause a DNA damage response in zebrafish embryos (Fig. 2). We demonstrate that UV exposure can induce an exposure-dependent increase in the DNA repair-related gene \textit{p53} expression. Therefore, like mammalian cells, zebrafish embryos do show an increase in \textit{p53} gene expression in response to UVR.
In parallel with p53 induction, we also demonstrated a UV exposure-dependent increase in the expression of the matricellular protein, osteonectin. Osteonectin is a multifunctional protein that modulates cell-matrix interaction and cell function, but does not seem to have a direct structural role in the matrix (32). Osteonectin is an evolutionary conserved matricellular protein (22, 33). Within all vertebrates, osteonectin is expressed in a temporally and spatially specific manner with strong expression during embryogenesis in developing tissue such as the notochord, somites and embryonic skeleton (22, 34, 35). A marked reduction in osteonectin expression occurs once adulthood is reached, although it has been shown that re-emerges in response to tissue injury, remodelling and inflammation (36). However, the precise function of osteonectin, in particular during early embryogenesis is largely unknown. Its dynamic expression patterns during embryogenesis and its sequence homology with other vertebrates, suggests a conserved function of osteonectin in vertebrates (22). Consequently, osteonectin potentially influences important physiological and pathobiological processes as a regulator of cell-matrix interactions.

To date, there is little information on the direct effect of UV on osteonectin gene expression regulation. Aycock et al., (37) showed that osteonectin was present in relatively high quantities in UV-induced squamous cell carcinoma, however was undetectable in skin from the non-irradiated control group. Additionally, osteonectin-null mice were tumour resistant, developing no squamous cell carcinoma in response to UV radiation. Therefore, they suggested that osteonectin had a critical role in mediating skin tumor formation in response to UV irradiation.

Regarding the spectral dependence of gene expression, exposure to a combination of UVB and UVA radiation produced a greater osteonectin and p53 expression increase than UVA alone (Fig. 3), thus probably indicating a higher capability of UVB to
produce cellular damage in zebrafish. However, significant differences in the expression of both genes were observed in the shorter wavelengths of the UVA, in which $p53$ was activated by less damaging spectral treatments than $osteonectin$. Longer wavelengths of UVA did not produce a significant expression increase of both genes compared to embryos exposed to non-damaging PAR. Interestingly, the highest expression of both genes occurred in the WG320 treatment, even though embryos received lower exposure to DNA damaging irradiance than the other treatments with UVB. This could have occurred because DNA damage was so high in these latter treatments that incipient apoptosis had already decreased embryonic capacity for gene expression by 24 hpf (20 h after exposure).

Previous work in zebrafish has demonstrated the capacity of UVA to activate a mechanism, the photoenzymatic repair (PER), which repairs the DNA damage caused by UVB exposure (38). The evidence of the mentioned repair system is the initial detection of photolyase enzyme in 3 hpf zebrafish embryos (39). The induction of PER partially compensates for a considerable decrease in tolerance of UVB exposure at this developmental stage (39). It is suggested that the higher UVB tolerance at the egg stage may be related to other (dark) repair mechanisms as well as possible shielding by the chorion and other maternally derived photoprotective compounds. In conclusion, it has to be considered that sensitivity to UV radiation may vary between developmental stages.

Additionally a decrease in survival percent and an increase in developmental abnormalities were observed in UV-exposed embryos (Table 1). Decreased survival in the WG295 treatment is expected given the very high exposure to DNA damaging irradiance (Fig. 1). The increase of $osteonectin$ expression detected by Q-RT-PCR and $in situ$ hybridization could be an additional cause for these mentioned effects in UV
exposed embryos. The phenotypic abnormalities revealed by previous overexpression and loss-of-function studies (40) also support this possibility. It has been shown that injection of osteonectin RNA into early blastomeres is associated with head and axis defects in Xenopus. Histological analysis revealed somite malformations that corresponded with the kinked axis (40).

In this study we also show that ectopic expression of osteonectin affects zebrafish development. Microinjection of capped and poly(a)-tailed full-length zebrafish osteonectin mRNA into 1-2 cell zebrafish embryos generated phenotypic malformations, with caudal (posterior) notochord bending/torsion as the most frequent deformity.

The fact that similar phenotypic malformations linked to an increase in osteonectin gene expression were found in UV–exposed and in ectopic osteonectin expression experiments therefore suggests osteonectin expression as one of the possible molecular mechanisms of UV-radiation induced phenotypic anomalies.

In summary, the present study has demonstrated that zebrafish osteonectin plays a critical role in mediating UV-radiation induced phenotypic developmental anomalies thus further unveiling its function in the regulation of embryonic development. However, the precise osteonectin-mediated signal transduction mechanism remains to be determined.

ACKNOWLEDGEMENTS

This work was partly funded by a PhD grant (FPI BES-2009-016797) and a postdoctoral grant (JAEDoc) to ETN and RMC, respectively, by an Smithsonian Institution postdoctoral grant to CS and by the MICIN AGL2008-00392/ACU.
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evidence for more than one osteonectin in Salmonidae. Biochimie 87 (5), 411-420.


LEGEND TO THE FIGURES

**Figure 1.** Spectral irradiance from the xenon lamp used to expose zebrafish embryos to UVR in the polychromatic incubator “photoinhibitron” and solar irradiance. The thick lines show the unweighted irradiance normalized to 1 at 400 nm to facilitate comparison among spectra from the most damaging treatment in the photoinhibitron (280 nm cutoff; dashed line), a treatment very similar to solar spectrum (320 nm cutoff; dashed-dotted line) and solar spectrum (solid line). Weighted irradiance ($E_{\text{eff}}(\lambda)$, mW m$^{-2}$ nm$^{-1}$) for the 295 nm cutoff using the Setlow action spectra for DNA damage (25) normalized to 300 nm is also shown (thin solid line).

**Figure 2.** Effect of duration of exposure to full spectrum radiation (WG280) on osteonectin and p53 gene expression. Sphere stage (4 hpf) zebrafish embryos were exposed to full spectrum irradiance (280 nm cutoff) in the polychromatic incubator photoinhibitron for 60, 120, 180 and 240 min and then sampled at 24 hpf. Real-time qRT-PCR for Osteonectin and p53 was carried out on 24 hpf exposed and non-exposed embryos. Shown is the average fold change in osteonectin and p53 gene expression calculated from 2 independent experiments, with samples analyzed each time in triplicate. Samples were normalized to 18S and dark control reference group set to 1. Data are expressed as mean ± SEM. Exposure response curves (A, B insets) were fitted using a non-linear data analysis program describing a sigmoid curve.
Figure 3. UVR wavelength exposure dependent expression of the Osteonectin and p53 genes in zebrafish embryos. Sphere stage (4 hpf) zebrafish were exposed for 150 min to different UVR spectral treatments (280, 295, 320, 335, 350, 370 and 395 nm long pass filters) then sampled at 24 hpf. The figure shows the average fold change in osteonectin and p53 gene expression calculated from 2 independent experiments, with samples analyzed each time in triplicate. Samples were normalized to 18S and dark control reference group set to 1. Data are expressed as mean ± SEM. Comparisons of numerical data were evaluated by One way ANOVA followed by Student-Newmas-Keuels SNK test (INSTATtm, GraphPad Software V2.04a). Degree of freedom=95 (Treatment, 15; residuals, 80). (a) denote significant difference between both genes at that particular exposure treatment and (*) denote significant differences with dark control reference group. The significant level was p<0,05.

Figure 4. Increased osteonectin expression in UV-exposed (295 nm cutoff) zebrafish embryos. Whole-mount in situ hybridization analysis of osteonectin expression in (A) UVR excluded control (395 nm cutoff) and (B) UV (295 nm cutoff) exposed zebrafish embryos. Sphere stage (4 hpf) zebrafish were exposed for 150 min at 295 or 395 nm cutoff then sampled at 24hpf. Arrow indicates phenotypic malformation observed. (A-B) lateral views, anterior to the left. Scale bars: 100 µm.

Figures 5. Exposure of zebrafish embryos to UVR (i.e. 295 nm cutoff) yielded high frequencies of morphological malformations. Sphere stage (4 hpf) zebrafish were exposed to UVR for 150 min then sampled at 48h. (A) UVR excluded control (395 nm cutoff) and (B), UV (295 nm cutoff) exposed zebrafish embryos. Arrow indicates
phenotypic malformation observed. (A-B) lateral views, anterior to the right. Scale bars: 100 µm.

**Figure 6.** *Osteonectin* overexpression phenotype mimics the UVB exposure phenotype (295 nm cutoff) in 24 hpf embryos. (A) control sense EGFP capped-mRNA injected embryos (0.5 ng/embryo). (B) sense *osteonectin* capped-mRNA injected embryos (0.2 ng/embryo) and (C) sense *osteonectin* mRNA injected embryos (0.8 ng/embryo). Arrow indicates phenotypic malformation observed. (A-C) lateral views, anterior to the left. Scale bars: 100 µm.

**Table.1** Mean survival and deformities (± SEM) of zebrafish embryos under full spectrum radiation (295 nm cutoff) or UVR-excluded (395 nm cutoff) exposures. Data are expressed as mean ± SEM. Welch’s *t*-tests were performed to analyse UV effects (INSTATtm, GraphPad Software V2.04a). Degree of freedom=6. The significant level (*) was p<0.05.
Figure 1
35x28mm (300 x 300 DPI)
280nm cutoff filter

Fold increase of gene expression

Osteonectin  p53

- 60 min
- 120 min
- 180 min
- 240 min

150 min  140 min

0 0.5 1 1.5 2 2.5 3

100x81mm (300 x 300 DPI)
Figure 3
29x19mm (300 x 300 DPI)
Figure 4
52x17mm (300 x 300 DPI)
Figure 6
156x263mm (300 x 300 DPI)
Table 1

<table>
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Mean survival and deformities (±SEM) of zebrafish embryos under full spectrum radiation (295 nm cutoff) or UVR-excluded (395 nm cutoff) exposures. Data are expressed as mean ± SEM. Welch’s t-tests were performed to analyse UV effects. (INSTAT™, GraphPad Software V2.04a). Degree of freedom=6. The significant level was.*p<0.05.