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Ploidy manipulation and induction of alternate cleavage patterns through inhibition of centrosome duplication in the early zebrafish embryo

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Abstract

Background—Whole genome duplication is a useful genetic tool because it allows immediate and complete genetic homozygosity in gynogenetic offspring. A whole genome duplication method in zebrafish, Heat Shock, involves a heat pulse in the period 13 – 15 minutes post-fertilization (mpf) to inhibit cytokinesis of the first mitotic cycle. However, Heat Shock produces a relatively low yield of gynogenotes.

Results—A heat pulse at a later time point during the first cell cycle (22 mpf, HS2) results in a high (>80%) frequency of embryos exhibiting a precise one-cell division stall during the second cell cycle, inducing whole genome duplication. Coupled with haploid production, HS2 generates viable gynogenetic diploids with yields up to 4 times higher than those achieved through standard Heat Shock. The cell cycle delay also causes blastomere cleavage pattern variations, supporting a role for cytokinesis in spindle orientation during the following cell cycle.

Conclusions—Our studies provide a new tool for whole genome duplication, induced gynogenesis and cleavage pattern alteration in zebrafish, based on a time period prior to the initiation of cell division that is sensitive to temperature-mediated interference with centrosome duplication. Targeting of this period may also facilitate genetic and developmental manipulations in other organisms.

Introduction

Ploidy manipulation methods were initially adapted to facilitate genetic analysis in vertebrates by George Streisinger and colleagues using the zebrafish model system (Streisinger et al., 1981; Streisinger et al., 1986). Such methods, originally applied to generate clonal lines and determine genetic distances, became the foundation of many subsequent studies aimed at gene discovery through forward genetics approaches. Genetic screens using zebrafish haploid embryos have been used extensively to identify embryonic lethal recessive mutations (Walker, 1999). Ploidy reduction to generate zebrafish haploids, carrying only maternally-derived functional DNA, can be readily induced by fertilizing eggs

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in vitro with sperm that has been treated with ultraviolet light to damage the paternal DNA. (Although egg activation per se in this organism is solely dependent on water exposure (Kane and Kimmel, 1993), fertilization by the (UV-treated) sperm remains essential for development (Walker, 1999), likely because the sperm provides the only source of centrioles to the zygote (Kessel et al., 1983; Yabe et al., 2007).

Haploid zebrafish embryos, however, die at about 2–3 days post-fertilization (dpf) (Walker, 1999), thus restricting the use of such screens to early developmental processes. Diploidization of haploid embryos through whole genome duplication results in gynogenesis, and allows development to proceed to later stages including adulthood (Streisinger et al., 1981; Streisinger et al., 1986). Because gynogenesis results in the immediate homozygosis of mutations present in a single heterozygous individual, it allows bypassing one generation, compared to genetic schemes using solely natural crosses, when attempting to observe the effect of homozygosis for recessive mutations. Thus, gynogenesis-based genetic schemes have the potential to result in a significant reduction in the time and space required for a genetic scheme, a benefit that is particularly relevant when target traits occur in juvenile or adults (Beattie et al., 1999) or are intergenerational (Pelegri and Schulte-Merker, 1999; Pelegri et al., 2004).

Gynogenesis in zebrafish has been primarily achieved through two main ploidy manipulation approaches, both of which rely on the inhibition of cell division without affecting ongoing DNA replication (Streisinger et al., 1981; Pelegri and Schulte-Merker, 1999). In one approach, inhibition of cytokinesis during the second meiotic division results in a diploid oocyte, which upon fertilization undergoes embryonic development. Fertilization with UV-treated sperm generates diploid gynogenotes. A second approach relies on the inhibition of cytokinesis for the first embryonic cell cycle. In this case, fertilization with UV-treated sperm results in haploid zygotes that after whole genome duplication become diploid gynogenotes.

Ploidy manipulation approaches have traditionally relied on physical treatments such as heat shock, hydrostatic pressure or cold shock to interfere with cytokinesis. In the zebrafish, the most effective treatment targeting meiosis II is hydrostatic pressure (during the time period 1.4 – 6 minutes post-fertilization (mpf)) using the method known as Early Pressure. On the other hand, the most widely used method for targeting the first embryonic mitosis in zebrafish is a heat pulse during the period 13 – 15 mpf, using the method known as Heat Shock.

Currently, Early Pressure is the most commonly used method of ploidy manipulation in zebrafish because it results in higher yields of gynogenotes than Heat Shock (Streisinger et al., 1981; Pelegri and Schulte-Merker, 1999). However, it can be argued that Heat Shock has a greater intrinsic potential as a genetic tool than Early Pressure because Heat Shock-derived gynogenotes are homozygous at all genomic loci, as diploidization occurs during a mitotic division. In contrast, Early Pressure-derived gynogenotes exhibit only partial homozygosity, specifically heterozygosity in chromosomal regions distal to sites of recombination (Streisinger et al., 1986; Pelegri and Schulte-Merker, 1999). Thus, when generating gynogenetic families from heterozygous carrier females, Heat Shock-derived gynogenotes

exhibit allelic homozygosity at a high and fixed frequency (50%), regardless of chromosomal position, whereas Early Pressure-derived gynogenotes exhibit frequencies of allelic homozygosity that are variable, ranging from near 0 to 50% depending on the centromere to locus distance. This characteristic in Heat Shock-derived progeny both increases the yield of progeny homozygous for a given mutation present in the mother and provides a fixed expected ratio that facilitates discrimination between genetic mutations and non-genetic syndromes. Because of the predicted advantages conferred by methods that lead to genome-wide homozygosity through the inhibition of embryonic mitosis (e.g. Heat Shock), we have pursued the development of such methods to induce whole genome duplication and gynogenesis.

Here, we identify a time window corresponding to a period towards the end of the first cell cycle, (22 – 24 mpf), different from that in the standard Heat Shock protocol (13 – 15 mpf), where a heat pulse results in a one-cycle cell division delay approaching near-complete penetrance. In this alternate method, even though the heat pulse occurs during the first cell cycle, the first cell division occurs normally and it is the second cell cycle that undergoes a one-cycle cell division stall. Subcellular analysis suggests that this one-cycle stall is caused by centrosomal duplication defects induced by the heat shock one cycle earlier, during the time of treatment. This one-cycle stall results in whole genome duplication and, when coupled to haploid production, leads to diploidized gynogenotes that can develop into viable and fertile adults. The procedure generates a fixed ratio of gynogenotes with 50% being homozygous for alleles present in heterozygous carrier mothers, consistent with the inhibition of embryonic mitotic division. A significant fraction of blastomeres that undergo a cell division stall also exhibit aberrant cleavage patterns, supporting a role for cytokinesis in the determination of cleavage orientation. Our data identify a heat shock-sensitive developmental period, prior to the initiation of blastomeric cell division, which can be exploited for genetic and developmental manipulations.

Results

A heat pulse during the first cycle promotes a stall in cell division during the second cycle

The standard Heat Shock method characteristically results in a relatively low yield of viable gynogenotes (9% in unselected lines (Pelegri and Schulte-Merker, 1999)). We hypothesized that heat pulses at later times in early development might also lead to cell division inhibition and whole genome duplication, possibly at improved efficiencies compared to this method. In order to identify additional time periods when the embryos are potentially sensitive to cell division inhibition by heat shock, we scanned early development with a 2-minute heat pulse of 41.4°C (normal temperatures range from 24 – 29°C) every 3 minutes during the 13 to 43 mpf developmental time period, and observed the consequences for cell division. We found that heat pulses throughout the period 13 – 28 mpf resulted in high fractions of embryos exhibiting cell division delay (Fig. 1). To our surprise, embryos treated during the later stages of this time period exhibited a cell division stall at the 2-cell stage (Fig. 1H) as opposed to the 1-cell stage stall expected for standard Heat Shock (Fig. 1D). Quantification of the effects (Fig. 1J) shows that a heat pulse in accordance with the standard Heat Shock protocol (13 – 15 mpf) results primarily (58.5%) in a delay in the cell division at the one-cell

stage (inhibition of first cell cycle cytokinesis). On the other hand, heat pulses at later times (16 – 25 mpf) resulted in embryos exhibiting a cell division delay primarily at the two-cell stage (inhibition of second cell cycle cytokinesis), with a peak in penetrance (82.3%) at 22 mpf. After stalling for exactly one cell cycle, embryos delayed during either the first or second cell cycle resume cleavage (Fig. 1E, I), remaining one cell cycle behind age-matched control embryos in terms of their total number of blastomeres.

In contrast to heat pulses within the period 13 – 28 mpf, which resulted in a temporary one-cycle stall followed in most cases by apparently normal development, heat pulses after 28 mpf resulted in a variety of defects including complete cell division arrest, asynchronous division, incomplete cytokinesis and unequal blastomere sizes (Fig. 1J and data not shown). This 28 mpf time point, roughly flanked by pronuclear fusion (20 mpf) and the first cytokinesis (35 mpf) corresponds to the initiation of blastomere division. This suggests that heat pulses applied as or after blastomeres begin to actively cleave have deleterious effects on multiple cellular processes essential for the cell cycle and cell division. Therefore, we did not further consider treatment at these later time points as a means to induce whole genome duplication. In all subsequent experiments, we initiated heat pulses at the time point of peak penetrance, 22 mpf. We have termed this alternate heat shock-based protocol, involving a 2-minute heat pulse during the first cell cycle (22 mpf) that interferes with the second cell division, Heat Shock 2 (HS2).

Heat shock at 22 mpf inhibits centrosome duplication

To determine how HS2 results in a stall in the second cell cycle, we fixed embryos at various time points after the heat pulse and labeled the fixed embryos to detect spindles and centrosomes, using antibodies against α - and γ -tubulin, respectively (Fig. 2). During the first cell cycle, at 37 mpf (15 minutes after the heat pulse; Fig. 2A,D) treated embryos looked largely indistinguishable from control embryos in terms of spindle formation, centrosomal and nuclear material distribution, and furrow formation and cell division. One cell cycle later, at 52 mpf, cells in control embryos contain a pair of centrosomes at each pole of a spindle apparatus (Fig. 2B). In contrast, cells in heat pulsed embryos contain a single mass of centrosomal material at the center of a monoaster, with condensed chromosomes remaining near the center of the monoaster (Fig. 2E and insert). At 67 mpf, at a time that corresponds to the third cell cycle in control embryos (Fig. 2C), HS2-treated embryos are able to form normal bipolar spindles with well-focused pairs of centrosomal material (Fig. 2F and insert), showing that they have recovered from the one cell cycle lag. Afterward, they resume centrosome cycling and bipolar spindle formation in subsequent cell cycles (data not shown). The formation of monoasters instead of bipolar spindles during the second cell cycle likely cause the stall in cytokinesis, since the midzone in bipolar spindles, but not monoasters, contain active signals to induce cell division furrowing (Rappaport and Rappaport, 1974; Rappaport, 1996). The observed defect in chromosomal and microtubule arrangement during the second cell cycle, caused by a heat shock during the first cell cycle, is consistent with the HS2 treatment interfering with the ability of centrioles to replicate during the first cell cycle.

HS2 promotes changes in ploidy number

HS2-treated embryos that undergo a one-cell cycle stall continue to develop to form 24 hours post-fertilization (hpf) embryos that lack any overt defects (data not shown, see also Fig. 4E). However, at 5 dpf HS2-treated embryos that undergo a cell division delay exhibit a distended heart cavity and do not develop an inflated swim bladder, invariably resulting in lethality (Fig. 3B). We surmised that these phenotypes constitute a lethal syndrome associated with tetraploidy, as a one-cycle stall in early zebrafish embryos is known to promote whole genome duplication (Streisinger et al., 1981; Walker, 1999; Yabe et al., 2007) and the day 5 phenotypes observed in HS2-treated embryos are similar to those found in tetraploid embryos induced by mutations in centriolar components (Yabe et al., 2007). We directly confirmed that the stall at the 2-cell stage caused by HS2 results in whole genome duplication: chromosome counts in HS2-treated embryos that had undergone a delay at the 2-cell stage were consistent with tetraploidy (Fig. 3D) whereas mock-treated control embryos remained diploid (Fig. 3C).

Coupling HS2 with haploid production results in viable gynogenotes

Zebrafish eggs can be fertilized in vitro with UV-treated sperm, whose DNA is rendered unable to contribute to the embryo by the treatment, to result in haploid embryos (Streisinger et al., 1981; Walker, 1999). Such haploid embryos exhibit a characteristic morphology at 24–48 hpf, including a significantly reduced degree of axis extension (Fig. 4B,D). Coupling this procedure with methods that induce whole genome duplication, such as inhibition of second polar body extrusion by Early Pressure, inhibition of the first cell division through standard Heat Shock (13 – 15 mpf), or paternal mutations in centriolar components, can induce diploidization of haploid zygotes yielding gynogenetic diploids (Streisinger et al., 1981; Walker, 1999; Yabe et al., 2007). We tested whether HS2 can similarly induce gynogenetic development. We performed HS2 on zygotes derived from females homozygous for recessive mutant alleles of pigmentation genes, such as *golden*, *albino*, and *casper* (a double mutant line for the pigmentation genes *nacre* and *roy*) after fertilization with UV-treated sperm from normally pigmented (wild-type) males (diagrammed in Fig. 4A). In this scheme, the pigment mutant marker (Fig. 4C) allows confirmation that embryos of normal morphology at 24 hpf are indeed gynogenetic diploids, as these exhibit the phenotype corresponding to the maternal recessive pigment marker, while potential “escaper” embryos, derived from incompletely inactivated sperm, show pigmented cells (such escapers, if present, are excluded from the analysis). Indeed, treatment of haploid zygotes with HS2 results in diploid gynogenetic embryos, as assessed by their maternal pigment phenotype and normal morphology in 24–48 hpf embryos (Fig. 4E and data not shown), as well as viability at 5 dpf (data not shown, see below). Diploidization of chromosomal content in HS2-treated haploids was also confirmed by chromosome spreads: HS2-treated haploids that underwent a cell cycle delay exhibit a diploid chromosomal count, whereas untreated control embryos remained haploid (Fig. 4F, 4G).

HS2 results in improved efficiency of gynogenesis compared to the standard Heat Shock method

We directly compared the effectiveness of the standard Heat Shock and HS2 methods to generate gynogenotes using a line carrying the *golden* mutation. In three independent experiments, pools of eggs from 6 *golden* females were fertilized in vitro with UV-treated sperm derived from males of wild-type pigmentation, and the zygotes were divided into three fractions that underwent different treatments: control untreated, heat-shocked at 13 – 15 mpf (standard Heat Shock), and heat-shocked at 22 – 24 mpf (HS2). The resulting embryos were allowed to develop without selection for cell division stall, to determine overall yield relative to the initial number of fertilized eggs, and diploidization was assessed by a normal morphology at 24 hpf. As expected, 100% of control untreated zygotes developed into 24 hpf embryos with haploid morphology (data not shown). In all experiments HS2 produced statistically significant increases (chi-square test, p-value = 0.016) in the yields of gynogenetic diploids (average: 24%, range: 11%–52%) compared to standard Heat Shock (average: 16% range 6%–20%; Fig. 5A). Although HS2-derived clutches exhibited a relatively wide range of gynogenote yield, certain clutches could show notably high yields. In particular, the HS2 yield for the pooled clutch in Trial 1 (52%) was substantially higher than yields in the other two HS2-treated clutches (11%–20%) and all clutches treated with standard Heat Shock (6% – 20%). In addition, in Trial 1 the yield of HS2-treated embryos is about 4-fold higher than that of embryos from the same pool of zygotes treated with standard Heat Shock. These observations suggest that there are particular genetic backgrounds in which HS2 treatment produces high yields of gynogenotes.

HS2-derived gynogenotes can survive past 5 dpf and live to be viable and fertile adults, and we have generated such gynogenetic adults from *golden* (Fig. 5B) and *casper* females (Fig. 5C; induction of adult gynogenotes from *albino* females was not attempted as our initial (non-gynogenetic) *albino*-carrying lines exhibited less robust growth). Because inhibition of embryonic cell division is expected to result in homozygosity throughout the genome, such adults are expected to be free of any lethal or, if they are able to produce viable offspring, sterile mutations. We are currently using such HS2-derived adults as founders for lethal- and sterile-free homozygous lines, and one such line has already been established. Selection of stocks through multiple generations of gynogenetic development has been shown to increase gynogenetic yield (Streisinger et al., 1981), possibly through the selection of appropriate genetic backgrounds, and it is possible that propagation of lines through HS2 will similarly result in increased efficiency of HS2-mediated gynogenesis.

HS2 results in the direct homozygosis of mutations present in heterozygous carrier mothers

Due to whole genome duplication during the embryonic mitotic cell cycles, HS2, like standard Heat Shock (Streisinger et al., 1981; Pelegri and Schulte-Merker, 1999), is expected to result in homozygosity throughout the entire genome. If the mother is heterozygous at any given locus, segregation of the alleles through meiosis followed by whole genome duplication during a stalled mitosis is expected to result in progeny that are homozygous for each allele and which are present in equal (50:50) proportions. We tested

whether HS2 produces this genetic segregation behavior by producing gynogenetic embryos from females heterozygous for one of various visible pigment mutations, such as *albino*, *golden* and *nacre* (within the *casper* background, the pigment phenotype for *nacre* but not *roy* can be scored within the tested developmental time, up to 5dpf). In all cases, recessively pigmented embryos were present in the diploidized gynogenetic clutches at a frequency not significantly different from 50% (Fig. 6; chi-square tests, *golden*: p-value = 0.75; *albino*: p-value = 0.45; *nacre*: p-value = 0.83), consistent with gynogenetic embryos being homozygous for either the mutant or wild-type allele in the expected (50:50) distribution. Thus, HS2 allows the homozygosis of mutations present in one copy in a carrier female in a single generation and with a high and predictable frequency.

Manipulation of the cleavage pattern through HS2

Previous studies have proposed a model in which the simultaneous formation of the bipolar spindle for a given cycle and furrowing for the immediately preceding cell cycle contributes to the creation of the alternating pattern of cell cleavage orientation observed in the early zebrafish embryo (Wühr et al., 2010). In this model, a zone of microtubule depolymerization at the furrows, termed aster-aster interaction zone, sets the stage for the generation of asymmetric forces on the developing mitotic spindle (Fig. 7A). Such an asymmetry results in the alignment of the spindle parallel to the furrow, in turn generating a cleavage division plane that is rotated 90° with respect to the furrow for the previous cycle. In the case of HS2-treated embryos that exhibit a cell division lag an uncoupling of spindle positioning from furrow formation occurs (Fig. 7B). According to this model, such uncoupling is predicted to result in deviations from the expected cleavage pattern after resumption of cell cleavage. The HS2-induced cell division stall in the second cell cycle allows testing this idea, because the first cell cleavage plane formed prior to the furrow stall can serve as a spatial landmark to assess cleavage orientation in subsequent cell cycles. Indeed, a high fraction of HS2-treated embryos, when resuming cleavage after a one-cycle delay during the second cell cycle, exhibit abnormal cleavage patterns (Fig. 7C). While the normally alternating pattern of spindle orientation results in an invariant square (2 × 2) cellular arrangement at the 4-cell stage, HS2-derived embryos with a second division stall exhibit a range of cellular arrangements, such as rhombus (diamond-shape, Fig. 7D), T-shape (Fig. 7E) and linear (4×1, not shown). Also as predicted by the model, the unexpected patterns of cleavage plane positioning only occur during the cell division immediately following the division stall. Irrespective of their spatial arrangement when blastomeres resume cleavage, in subsequent cycles (3 examined cycles) blastomeres continue to divide in the expected 90° alternating orientation pattern (Fig. 7F and data not shown). Together, these observations provide support for a model in which embryos with large cells use cues from the aster-aster interaction zone at the ongoing cytokinetic furrow to direct spindle orientation (and thus cleavage plane positioning) for the immediately following cell cycle (Wühr et al., 2010).

Discussion

Ploidy manipulation methods have the potential to facilitate genetic analysis of vertebrate development. Methods that increase ploidy in particular allow the generation of tetraploids and gynogenetic diploids. Generating gynogenetic diploids can simplify genetic schemes

because it allows the direct homozygosis of mutations from a single heterozygous individual. Unlike haploid embryos, such gynogenotes survive past embryonic development to become juvenile and ultimately fertile adults, allowing the analysis of gene variants that affect such later developmental stages. In various vertebrate model systems, such as the zebrafish (Pelegri and Schulte-Merker, 1999; Beattie et al., 1999; Pelegri et al., 2004), and *Xenopus tropicalis* (Noramly et al., 2005), gynogenetic methods have been exploited in genetic screens designed to identify mutations affecting adult and fertility (parental-effect) traits. Our studies document an additional tool for whole genome duplication and gynogenesis, which likely relies on the interference with centriole duplication during the first cell cycle.

HS2 provides an alternative gynogenetic method

Traditional gynogenetic methods have strengths and weaknesses. Inhibition of cytokinesis of meiosis II by the Early Pressure method results in higher yields than the standard Heat Shock method, possibly due to an intrinsic greater ease to inhibit cytokinesis in the case of a much smaller cell (the polar body) and better post-diploidization survival due to partial heterozygosity of Early Pressure-derived embryos. However, Early Pressure can interfere with genetic analysis and identification of new mutations due to the partial and, for newly induced mutations, unpredictable fraction of homozygosity in Early Pressure-derived gynogenotes (which varies with chromosomal location). On the other hand, inhibition of cytokinesis of the first embryonic mitosis by standard Heat Shock results in a high, fixed and predictable fraction of homozygosity regardless of chromosomal location. These characteristics would, in principle, make Heat Shock the gynogenetic method of choice, but this method suffers from lower yields.

Here, we report that a heat pulse at a time corresponding to the stage immediately following pronuclear fusion and the initiation of the first embryonic cell cycle results in inhibition of cytokinesis of the second embryonic mitosis, leading to whole genome duplication. We refer to this heat pulse treatment as HS2 and show that it results in gynogenetic development at improved frequencies compared to the standard Heat Shock protocol.

HS2 likely targets centriolar biogenesis prior to initiation of cell division

Though the heat pulse occurs prior to the first cell division, HS2 results in a stall during the second cell division. This situation is in contrast to prior work that suggested that standard Heat Shock acts by inhibiting the spindle apparatus during the *ongoing* cell cycle (Streisinger et al., 1981). However, other studies indicate an alternative mechanism of cell division stalling based on the inhibition of centriole duplication. As first shown in *C. elegans* (O'Connell et al., 2001) and later zebrafish (Yabe et al., 2007), defects in centriolar biogenesis result in precise cell cycle stalls, but such stalls occur one cell cycle after the effect on centriolar duplication. This behavior is due to the fact that single unpaired centrioles at each spindle pole are sufficient for spindle function and dynamics (O'Connell et al., 2001). Thus, a reduction from a normal centriolar pair at the centrosome to a single centriole, caused by a centriolar duplication defect, does not interfere with the cell division in which the defect occurs. In zebrafish, males homozygous for a mutation in the centriolar component *cellular atoll/sas-6* yield embryos that stall in the first cell cycle, resulting in

whole genome duplication (Yabe et al., 2007). This stall is likely caused by the presence of a single centriole instead of the two centrioles normally present in zebrafish sperm (Kessel et al., 1983; Delattre and Gönczy, 2004), presumably due to defects in centriole duplication during spermatogenesis (O'Connell et al., 2001). Although the *cellular atoll/sas-6* paternal effect occurs at low penetrance (in 5% or less of embryos (Yabe et al., 2007)), precluding its practical application, this effect highlighted the potential for centriolar biogenesis manipulation to promote changes in ploidy.

The effect of HS2 is consistent with such an induced defect in centriolar biogenesis (Fig. 8). In this case, a defect in daughter centriole formation during the first cell cycle caused by the heat pulse does not affect the formation of the spindle during that same cycle, likely due to the presence of unpaired centrioles at each spindle pole. However, this defect, coupled to completion of the first cell cycle, generates in the second cell cycle blastomeres containing a single unpaired centriole. These blastomeres are therefore unable to generate a bipolar spindle. The lack of bipolar spindles in the second cycle results in the observed cytokinesis failure, as furrow induction depends on signals from opposing astral microtubules (Rappaport and Rappaport, 1974; Rappaport, 1996). Because DNA synthesis occurs independently of the centriolar cycle (Dekens et al., 2003; Yabe et al., 2007), such cytokinesis failure results in whole genome duplication. Recovery from the effects of the heat pulse allows centriole duplication in the following cell cycle, resulting in the restoration of centriolar pairs and the resumption of normal cell division.

Several studies in teleosts have reported a cell division stall similar to the one we observed after HS2, where a physical treatment occurring during the first cell cycle affects cell division corresponding to the second cell cycle in embryos of the rainbow trout (Zhang and Onozato, 2004) and the olive flounder (Zhu et al., 2007). These previous studies report monoaster instead of bipolar spindle formation during the stalled cell cycle, as well as duplication of chromosome number in the resulting cells. Our studies confirm the basis of this phenomenon, providing for the first time direct visualization of centrosomal components and their defective replication in treated embryos. More importantly, our studies demonstrate this phenomenon can be used to generate viable and fertile diploid gynogenotes, which undergo direct homozygosis of mutations present in heterozygous carrier mothers. Our studies also show that HS2 allows experimental manipulation of orientation of cleavage planes (see below).

Possible centriolar basis for the first cycle cell division stall caused by standard Heat Shock

Our data suggests an alternative explanation for the basis of the effect of standard Heat Shock, which as mentioned above has been generally interpreted as inhibiting assembly of the spindle apparatus corresponding to the first mitotic division. We find that HS2, with its heat pulse at 22 – 24 mpf, occurring just prior to formation of the mitotic spindle at 25 mpf, does not affect the first mitotic division. This indicates that the mitotic spindle is not sensitive to heat treatment immediately prior to its formation, and further raises the question of how standard Heat Shock, which relies on a heat pulse at an even earlier time point (13 – 15 mpf), may affect the spindle. On the other hand, our studies involving HS2 suggest that

centriole duplication is heat sensitive. These observations suggest that the 13 – 15 mpf heat pulse for standard Heat Shock may not directly lead to spindle apparatus disassembly but may interfere with some aspect of centriole reconstitution and/or biogenesis. For example, studies in *Drosophila* that show that a fully formed centriole in the sperm is accompanied by an incompletely formed daughter centriole that finishes its formation after fertilization (Blachon et al., 2009) and standard Heat Shock could potentially interfere with a possible need to mature a second sperm-derived centriole. An effect on the maturation of a second sperm-derived centriole could cause the stall in cell division for the first cell cycle observed after standard Heat Shock, due to the lack of a properly formed bipolar spindle during that cycle, and an additional cell cycle would be required to restore a functional centriolar pair to resume normal cell division. Further studies will be required to test this possibility.

It is also worth noting that the Late Pressure gynogenetic protocol, developed concurrently to standard Heat Shock (Streisinger et al., 1981) and generally assumed to have a similar mechanistic basis, involves hydrostatic pressure during a time period (22.5 – 28 mpf) that is similar to that of HS2. Thus, it is possible that, like HS2, Late Pressure may promote whole genome duplication by inhibiting the second cell division rather than the first one.

HS2 provides improved yields of fully homozygous embryos through whole genome duplication

A basic initial question in our studies was whether we could find conditions that increase gynogenesis yield through promotion of whole genome duplication, over that of standard Heat Shock. We specifically tested whether the overall yield of gynogenote production could be improved by applying heat pulses at later time points in development, thus bypassing early developmental periods which may be sensitive to this physical treatment. Treatments at stages immediately before and during the initiation of blastomere division cycling (31 – 52 mpf) did not produce satisfactory yields, as they caused a variety of defects during cell division and cytokinesis resulting in low embryonic survival. On the other hand, heat pulses at time points earlier in the first cycle induce a cell division stall without causing noticeable additional defects. Our studies highlight a period during development, between fertilization and the initiation of rapid embryonic cell cycling, which is conducive to the manipulation of centrosome replication and ploidy through a heat pulse. This developmental window could prove to be a useful target to induce diploidization in other organisms.

Our direct comparisons of sibling embryos indicate that, in multiple trials, gynogenesis yields from HS2 are consistently higher than those from standard Heat Shock. In our most productive case, HS2 led to a 52% diploidization rate in day 1 embryos, compared to 13% for sibling embryos exposed to standard Heat Shock, a 4-fold increase in yield. Thus, our initial observations indicate that HS2 results in a higher yield of viable gynogenotes. We note that HS2 yields were particularly variable in our experiments, ranging from 11% to 52%, and we are currently testing whether specific parameters such as maternal age influence the effectiveness of the treatment. Moreover, previous work by Streisinger and coworkers has shown that propagation of lines through gynogenesis gradually improves gynogenetic yields from individuals within those lines (Streisinger et al., 1981). Such yield improvement over generations upon propagation through gynogenesis likely results from

both the removal of recessive deleterious mutations after homozygosis and selection for genetic variants that confer a favorable response (increased stalling frequency and post-stalling survival) to the gynogenesis-inducing treatment. Thus, we expect that ongoing HS2-mediated propagation of gynogenetic lines will result in lines that yield viable gynogenotes at a rate of 50% or higher. Coupled to the large number of embryos (typically 150 – 300) that can be readily obtained from individual zebrafish females, such an improvement in efficiency should allow ploidy manipulation as an effective genetic tool in this organism.

Cleavage pattern manipulation through HS2

Due to the early rapid divisions in the early zebrafish embryo, centriolar duplication and initiation of spindle formation during a given cycle occurs concurrent with cytokinesis for the previous cycle. It has been proposed that this temporal overlap allows positioning of the spindle in an orientation that is orthogonal to that of the previous cell cycle, in turn (due to the role of the spindle midzone in furrow formation) generating the alternating pattern of cleavage furrow orientation characteristic of the early zebrafish embryo (Wühr et al., 2010). Specifically, interactions between asters of the forming spindle and a microtubule-free “aster-aster interaction zone” at the furrow have been proposed to generate asymmetric forces that result in forming spindles being aligned orthogonal to the orientation of spindles from the previous cell cycle. The one-cycle time lag caused by HS2 allowed us to further test this hypothesis by dissociating the normally concurrent events of spindle formation and orientation (for a given cycle) and furrow completion (for the previous cycle). Because the one-cycle lag in HS2-treated embryos occurs in the second cell cycle, the presence of the normally formed first cleavage furrow provided positional context to an observer, which allowed detecting cleavage orientation changes. We find that, upon resuming cell division after a one-cycle lag, a high fraction of HS2-derived embryos exhibit aberrant spindle orientation and corresponding cell arrangement variants. Our results highlight a “one-cell cycle memory” mechanism for cleavage plane orientation and provide further support for the previously presented hypothesis that cytoskeletal elements in overlapping cell cycles interact to generate the pattern of alternating spindle positioning that underlies the zebrafish embryonic pattern (Wühr et al., 2010). More generally, we show that HS2 provides a means to generate cell cleavage pattern variants to test mechanisms of patterning in the early embryo.

In summary, our studies establish HS2 as an effective genetic tool to induce whole genome duplication, including gynogenetic production involving direct genetic homozygosis. Independently of its effect on ploidy, HS2 also allowed us to induce cleavage pattern variants in the early embryo, allowing us to test models of spindle positioning. Considering that heat shock sensitivity of centriolar function is likely conserved, the presented strategy could be applicable to other animal species.

Experimental Procedures

Genetic Stocks

All stocks were raised and maintained in standard conditions at 28.5°C (Brand et al., 2002). Wild-type stocks were standard AB lines. Mutations in the pigmentation genes *golden*,

albino, and *nacre* were obtained from the Zebrafish International Resource Center (ZIRC). The *nacre* mutation was obtained in a *casper* (*nacre*, *roy* double mutant) background but only the *nacre* marker could be scored in 24 hpf embryos. Homozygous individuals were crossed to wild-type AB fish to generate heterozygotes. These heterozygotes were used for the homozygosity ratio experiments. Heterozygotes were also used to generate new homozygous lines in a hybrid AB background, which were able to grow more robustly than the original homozygous line, and which were used to generate viable HS2-derived gynogenotes. Embryos were fertilized and incubated in E3 embryonic medium (Brand et al., 2002) up to 5 days of development. Work with zebrafish was approved by the appropriate committee (University of Wisconsin – Madison assurance number A3368-01)

Heat Shock Procedure

In vitro fertilization and heat shock protocols were carried out as previously described (Pelegrí and Schulte-Merker, 1999) and are briefly described below. For sperm preparation, testes of 6 AB males were removed and placed in a mixture of 990uL of Hank's Premix and 10uL of sodium bicarbonate solution. The testis were then homogenized by pipetting and were either left untreated or were exposed to two 15W UV lights at a distance of 20cm for 90 seconds. For in vitro fertilization, females were anesthetized, blotted dry, and stripped of eggs into a clean, dry petri dish. 1ml of sperm solution was added to the eggs along with 1mL of E3 embryonic medium. After one minute, the plate was flooded with additional E3 medium and the fertilized eggs were collected using a tea strainer and incubated in E3 medium in a water bath at 28.5°C until the designated Heat Shock time point. At the time to begin heat shock, embryos in the strainer were briefly blotted on a paper towel and transferred to E3 medium in a water bath at 41.4° for two minutes. At the end of the heat shock period, the embryos in the strainer were again briefly blotted on paper towel and transferred back to 28.5°C E3. At 30 mpf the embryos were transferred to a petri dish containing E3 medium and allowed to develop normally. Statistical analysis for embryos in Figs. 1, 5 and 6 was carried out in Microsoft Office Excel.

Immunofluorescence and Imaging

Embryos labeled for α - and γ -tubulin were dechorionated and fixed in microtubule fix buffer as previously described (Theusch et al., 2006). Primary antibodies used were anti- α -tubulin (Sigma, monoclonal B5-1-2, 1:2500) and anti- γ -tubulin (Sigma, antiserum IgG fraction, 1:2000), which were detected using, respectively, Cy-3 conjugated goat anti-mouse (Jackson Immuno Labs, 1:100) and Alexa-488 Conjugated goat anti-rabbit (Molecular Probes, 1:100) secondary antibodies. DNA was visualized in the embryos by labeling with DAPI (0.5ug/mL) in PBS for 30 minutes at room temperature.

Metaphase chromosome spreads were carried out as previously described (Yabe et al., 2007) using cells from 24-hour embryos.

Live imaging of embryos, juvenile fish, and chromosome spreads were performed using a Zeiss Axioplan2 microscope and Openlab imaging software. Immunofluorescent images of α - and γ -tubulin-labeled embryos were acquired using Zeiss LSM 510 confocal microscope and ZEN imaging software.

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Figure 1.

Standard Heat Shock causes a cell cleavage stall at the first cell cycle, and HS2 a stall at the second cell cycle. (A–C) Control untreated embryos at the indicated time points, corresponding to the 2-, 4- and 8-cell stage. (D–F) An embryo with a one cell cycle stall during the first cell division, caused by a heat pulse initiated at 13 mpf (standard Heat Shock (HS)). (G–I) An embryo with a one cell cycle stall during the second cell division, caused by a heat pulse initiated at 22 mpf (HS2). Embryos exhibiting a cell cycle stall resume cleavage after the stall and contain half the number of blastomeres as control stage-matched embryos.

(J) Comparison of the degree and type of cell cycle stall caused by standard Heat Shock and HS2. Standard Heat Shock causes primarily a stall of the first cell division, whereas HS2 primarily causes a stall of the second cell division. Values are mean percentages across four different trials.

Figure 2.

HS2 causes defects in bipolar spindle formation during the second cell cycle, likely caused by centrosomal duplication defects during the first cycle. Immunofluorescence microscopy of whole mount blastodiscs, labeled to detect α -tubulin (red), γ -tubulin (green) and DNA (blue, only readily apparent in high magnification inserts in E,F) in control (A–C) and HS2-treated (D–F) embryos. Bipolar spindle is normal during the first cell division cycle (A,D). During the second cell division cycle, each of the two previously generated blastomeres show monoasters in HS2-treated embryos (E, insert) instead of bipolar spindles in control embryos (B). One cycle later, HS2-treated embryos resume the formation of bipolar spindles and cell cleavage (F, insert), although the number of blastomeres is halved compared to control embryos (forming 4 blastomeres in HS2-treated embryos (F) and 8 in control embryos (C)). Centrosomal material can be observed near the center of astral structures in both bipolar spindles and monoasters. In all cells, this centrosomal material appears duplicated with centers at each of the spindle poles, except in HS2-treated embryos during the second cell cycle, where centrosomal material appears as a single mass at the center of a monoaster (E, insert). The absence of a pair of centrosomes during the second cell cycle in HS2-treated embryos is presumably caused by defects in centriolar duplication during the first cell cycle, the time of heat treatment. The bipolar spindle is a dynamic structure that appears first, during metaphase, as a tight array of midzone microtubules (as in C) and later,

during anaphase, as a diastral structure (A,B,D,F). Where present, white double-arrows indicate axes of bipolar spindles for the corresponding cell cycle (1st, 2nd or 3rd, with '2nd' indicating the bipolar spindle that forms after the one-cell cycle lag). Scale bar in F corresponds to 100 μ m in (A–F). Inserts in (E,F) are amplified 9x and 4.5x, respectively. Diagrams on the bottom left of each panel illustrate an idealized spindle during metaphase (microtubules in red, centrosome in green) within a blastomere at each stage. Spindles exhibit a normal bipolar structure in all panels except in the embryo undergoing a cell division stall in (E), in which a microtubule-based monoaster emanates from a single centrosome. In the micrographs, the bipolar spindle exhibits somewhat different appearances due to its rapid reorganization during the cell cycle (Theusch et al., 2006; Yabe et al., 2007; Wühr et al., 2010): compact spindle midzone with weakly labeling asters during metaphase (as in (C)), solid asters during early anaphase (as in (B), (E), (F)), wider, hollow asters during late anaphase (as in (A), (D)). Microtubule enrichments observed between parallel spindles in (B), (C) and (F), or between monoasters in (E), correspond to the furrow microtubule array for the previous cell cycle, which is derived from astral microtubules and forms during cytokinesis.

Figure 3.

HS2 promotes whole genome duplication and tetraploidy. (A,B) Control (A) and HS2-treated (B) embryos derived from in vitro fertilization with untreated sperm, at 5 dpf. In HS2-treated embryos, the embryonic cavity (asterisk) is distended compared to controls, and the swim bladder (arrowhead) is not inflated. (C,D) Chromosome counts in cells from 24 hpf embryos. (C) Normal diploid chromosomal content observed in cells from embryos exposed to normal temperatures and which exhibit no cell division stall. (D) Tetraploid chromosomal content in cells from HS2-treated embryos and which showed a cell division stall in the second cell cycle.

Figure 4.

HS2 coupled to haploid production induces gynogenetic diploids. (A) Diagram of the gynogenetic procedure. Maternal homozygosity for pigment markers is used to confirm that the resulting progeny lack genetic material from (wild-type pigmented) males. (B–E) Phenotypes of 36 hpf embryos: (B) control wild-type (WT): diploid with normal (*gol*⁺) pigmentation, (C) control *golden*: wild-type diploid, homozygous for a mutation in the pigmentation gene *golden* (*gol*), which results in reduced levels of pigment in both melanophores and the eye, (D) haploid: haploid embryo derived from the fertilization of

eggs from *golden* homozygous females with UV-treated sperm from wild-type males. The shortened body axis reflects the characteristic haploid phenotype. (E) gynogenote: gynogenetic diploid produced by treating haploid embryos (which would otherwise develop into embryos shown in (C)) with HS2 and selected for a one-cycle division lag during the second cell cycle. In both (D) and (E), *golden* pigmentation of the embryo indicates only maternal genetic material was transferred to the embryo. (F,G) Chromosome counts in cells from 24 hpf embryos. (F) Haploid chromosomal content in haploid embryos depicted in (D). (G) Diploid chromosomal content in gynogenetic diploids depicted in (E).

Figure 5.

HS2 exhibits higher yields of gynogenetic diploids than standard Heat Shock. (A) In three independent trials, pools of eggs were divided and treated with standard Heat Shock (HS) or HS2. HS2-treated embryos exhibit consistently higher yields of gynogenetic production. Bars indicate the standard error in average yield values. A chi-square test for equality of proportions performed on the combined data shows a significant difference in the average yield values between standard Heat Shock and HS2 (p-value = 0.016; $\chi^2 = 5.8$). Control untreated embryos resulted in 100% embryos with haploid morphology. (B,C) HS2-derived gynogenetic adult offspring from *golden* (B) and *casper* (C) mutant lines.

Figure 6.

Direct homozygosis of alleles present in heterozygous mothers through HS2. Pools of zygotes from 7–8 females heterozygous for recessive alleles in the pigmentation genes *golden*, *albino* and *nacre* were fertilized with UV-treated wild-type sperm and underwent HS2 to produce gynogenetic progeny. In all cases, gynogenetic diploids, as assessed by normal body morphology, were observed to exhibit pigmented or non-pigmented phenotypes in proportions not significantly different from the 50:50 expectation (*golden*: p-value = 0.75, $\chi^2 = 0.10$; *albino*: p-value = 0.45, $\chi^2 = 0.58$; *nacre*: p-value = 0.83, $\chi^2 = 0.048$), consistent with diploidization of haploid zygotes.

Figure 7.

Aberrant cleavage patterns observed in HS2-treated embryos. (A) Proposed mechanism for cleavage specification in untreated wild-type embryos (after Wühr et al., 2010). A zone of microtubule depolymerization, the aster-aster interaction zone (orange), occurring during furrow formation is responsible for asymmetric forces that orient the spindle for the following cell cycle along an axis orthogonal to the orientation of the spindle in the previous cell cycle. Because the spindle midzone determines the site of furrow formation, this results in the cleavage pattern of orthogonally alternating cleavage planes characteristic of the early

zebrafish embryo. B) HS2-treated embryos that stall cell division during the second cell cycle experience an uncoupling of furrow formation for the first cell cycle and spindle positioning after its reformation during the third cell cycle. This results in the loss of a “one-cell cycle memory” for spindle alignment and leads to aberrant spindle alignment, generating alternate cleavage patterns. Not all possible cases for spindle alignment are shown, and for clarity only the clearer examples on how they yield alternate cleavage patterns are depicted. Other possible spindle arrangements are presumed classified within the morphological categories shown according to their closest morphology. In (A) and (B) the orientation of the spindle is depicted for clarity with a red double arrow. C) HS2-treated embryos that had undergone a one-cycle cell division stall in the second cell cycle ($n=171$) were, at a time when they consisted of four blastomeres (e.g. the third cell cycle), classified for blastomere arrangement according to one of the following types: square (wild-type pattern, see Fig. 2C,F), rhombus (D), T-shape (E), and linear (not shown). Square and rhombus reflect whether the patterns can be described as being enclosed in a square quadrilateral (all corners being right angles) or an unequal quadrilateral (corner angles different from right angles). In practice, square and rhombus were distinguished by whether a line drawn through a furrow retained (square) or changed (rhombus) its angle of orientation after crossing the other furrow. Cleavage patterns other than square are rarely observed in wild-type embryos, including in in vitro fertilized but otherwise untreated embryos (square: 97%, rhombus: 3%, T-shape: 0%, linear: 0%, $n = 107$) or among the minority of HS2-treated embryos that do not undergo a cell cycle stall (square: 100%, $n = 17$). Scale bar in D corresponds to 100 μm in (D,E). (F) After resumption of cell cleavage in HS2-treated embryos, spindle alignment follows the characteristic orthogonally alternating pattern for the next cell cycles in all cells, as expected from the model. The example shown depicts cleavage of embryos with a T-shape pattern after cell division resumption. Cells and spindles derived from the top blastomere, which in the third cell cycle divided according to the normal spindle orientation, are labeled in yellow. Cells and spindles derived from the bottom blastomere, which in the third cell cycle divided with an aberrant spindle orientation are labeled in blue. Blastomere cleavage pattern shown is representative of 6 analyzed embryos (3 T-shape, 3 linear), all of which showed, after cleavage resumption, orthogonally alternating cleavage planes in all cells.

Figure 8.

Proposed mechanism of HS2 to induce gynogenetic development in zebrafish. After meiosis I (top row), mature eggs are arrested in metaphase of meiosis II. Contact with water results in egg activation and completion of meiosis II. Fertilization with untreated sperm results in embryos with a diploid component (left column). Fertilization with sperm treated with UV, to interfere with sperm DNA transmission, results in haploid embryos (middle column). Treatment of haploid zygotes with a heat pulse during the first cell cycle (22 – 24 mpf, HS2) results in the inhibition of cell division during the second cell cycle (right column). This is

caused by defective centriole replication, and coupled to ongoing DNA synthesis generates gynogenetic diploids. Mature and new centrioles are depicted as dark or light green cylinders, respectively, and new centrioles mature through the completion of the cell cycle. The timing of various gynogenetic treatments, Early Pressure (EP), standard Heat Shock (HS) and HS2, is depicted in a partial developmental time line (right). Homologous chromosomes are depicted in different colors (yellow and burned red), to highlight recombination during meiosis. In contrast to the commonly used Early Pressure method, HS2 is expected to result in full homozygosity regardless of the presence of recombinant events. See text for more details.