LETTERS

Pregnenolone stabilizes microtubules and promotes zebrafish embryonic cell movement

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Embryonic cell movement is essential for morphogenesis and the establishment of body shapes^{1,2}, but little is known about its mechanism. Here we report that pregnenolone, which is produced from cholesterol by the steroidogenic enzyme Cyp11a1 (cholesterol side-chain cleavage enzyme, P450_{scc})³, functions in promoting cell migration during epiboly. Epiboly is a process in which embryonic cells spread from the animal pole to cover the underlying yolk. During epiboly, *cyp11a1* is expressed in an extra-embryonic yolk syncytial layer⁴. Reducing *cyp11a1* expression in zebrafish using antisense morpholino oligonucleotides did not perturb cell fates, but caused epiboly delay. This epiboly defect was partially rescued by the injection of *cyp11a1* RNA or the supplementation of pregnenolone. We show that the epiboly delay is accompanied by a decrease in the level of polymerized microtubules, and that pregnenolone can rescue this microtubule defect. Our results indicate that pregnenolone preserves microtubule abundance and promotes cell movement during epiboly.

Cyp11a1 (also known as P450_{scc}) catalyses the first step of steroid synthesis, the conversion of cholesterol to pregnenolone (P₅). P₅ is then further converted to 17 α -hydroxypregnenolone by Cyp17 or to progesterone (P₄) by 3 β -hydroxysteroid dehydrogenase (Hsd3b), for the production of other steroids (Fig. 1a). Steroids regulate salt and sugar homeostasis and sexual characteristics in the body, but the functions of steroids during early embryogenesis are unclear. To study their functions, we characterized the genomic structures of zebrafish *cyp11a1* and *hsd3b* (Fig. 1b), and examined their expression during early embryogenesis by *in situ* hybridization. After fertilization, zebrafish blastomeres located above the yolk undergo rapid cleavage. At this time, *cyp11a1* and *hsd3b* were expressed in the cytoplasm of these blastomeres (Fig. 1c). The blastomeres then move downward to cover the yolk in a process called epiboly. During epiboly, *cyp11a1* and *hsd3b* were downregulated from blastomeres, and a new expression domain appeared at the junction between blastomeres and yolk. This expression domain, called the yolk syncytial layer (YSL), is an extra-embryonic layer composed of yolk syncytial nuclei that move ahead of blastomeres during epiboly.

To test the function of *cyp11a1* during early embryogenesis, we injected antisense morpholino (mo) oligonucleotides into zebrafish embryos at the one-cell stage to block its expression (Supplementary Fig. S1). At 100% epiboly, when wild-type blastomeres completely covered the yolk, embryos injected with *cyp11a1*-mo1 could not cover the yolk, leaving an unclosed yolk plug (Fig. 2). Control embryos injected with morpholino oligonucleotides against a random sequence, an unrelated sequence from *dlx3*, *hsd3b* or *cyp17*, or a 5-base mismatched *cyp11a1*-mo1 were all normal (Supplementary Table S1). The epiboly delay started before gastrulation, when wild-type blastomeres cover 50% of the yolk (50% epiboly). At this time, blastomeres of the *cyp11a1*-mo1 morphants covered only about

Figure 1 | Two steroidogenic genes, *cyp11a1* and *hsd3b*, are expressed in blastomeres and the yolk syncytial layer during early zebrafish embryogenesis. **a**, Steroidogenic pathways. Cyp11a1 catalyses the conversion of cholesterol to pregnenolone (P₅), which is then converted to 17 α -hydroxypregnenolone (17OH-P₅) and progesterone (P₄) by Cyp17 and Hsd3b, respectively. DHEA, dehydroepiandrosterone. **b**, Genomic structures of zebrafish *cyp11a1* and *hsd3b*. Shaded and open boxes indicate coding and non-coding regions of exons, respectively. Arrows indicate transcriptional start sites; lines below the boxes indicate the locations of morpholino oligonucleotides (mo). **c**, Detection of *cyp11a1* and *hsd3b* expression by whole-mount *in situ* hybridization at the cleavage and 50% epiboly stages. Arrowheads indicate yolk syncytial layer.

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44% of the yolk (data not shown). Thus, *cyp11a1*-mo1 morphants show an early epibolic defect.

When we injected a fluorescein-conjugated control morpholino oligonucleotide into embryos at the one-cell stage, the fluorescent signal was distributed throughout the entire embryo at 30% epiboly. When we injected the same morpholino into the yolk at the 1,000 (1k)-cell stage, the fluorescent signal was restricted to only the YSL (Supplementary Fig. S2a). Thus, injecting oligonucleotides into the yolk at the 1k-cell stage affects only YSL function. At 100% epiboly, embryos injected with *cyp11a1*-mo1 at the 1k-cell stage failed to close their yolk plug, although the extent of epibolic delay was less severe than that for embryos injected at the one-cell stage (Supplementary Fig. S2b). This result indicates that *cyp11a1* expressed in the YSL participates in the epibolic process.

The YSL is known to be involved in mesoderm induction⁵ and nutrient production^{6,7}. To test whether *cyp11a1* in the YSL is involved in germ layer induction, we examined cell fates in *cyp11a1*-mo1 morphants using mesodermal (*tbx6* and *shh*), mesendodermal (*gsc*) and endodermal (*sox17*) markers. The cell fates of mesoderm and endoderm did not change upon reduction of *cyp11a1* expression by *cyp11a1*-mo1 (Supplementary Fig. S3). Although cell fates of the *cyp11a1*-mo1 morphants appeared normal, the length of their anterior–posterior axis was reduced (Supplementary Fig. S3d) and their somites were wider (data not shown). This indicates a problem with the convergent extension movement that builds up body axis by narrowing and lengthening dorsal tissues. Not all types of cell movements were affected, as involution movement seemed normal (Supplementary Fig. S4).

To confirm the function of *cyp11a1*, we used a different morpholino oligonucleotide, *cyp11a1*-mo2, which targets the junction between exon 4 and intron 4 of *cyp11a1*, and affects zygotic *cyp11a1* RNA splicing and epibolic movement (Fig. 1b and Supplementary Fig. S5). This morpholino also caused epibolic delay. The epibolic delay caused by each of *cyp11a1*-mo1 and *cyp11a1*-mo2 was partially rescued when embryos were co-injected with *cyp11a1* mRNA (Supplementary Figs S2c and S5d). This result shows that *cyp11a1* contributes to epibolic movement.

Cyp11a1 encodes a steroidogenic enzyme (Fig. 1a), and we predicted that reducing *cyp11a1* expression would result in a decrease in steroid products. Indeed, the level of the Cyp11a1 product pregnenolone from *cyp11a1*-mo1 morphants 10 h after fertilization was about half of that in wild-type embryos (Supplementary Fig. S1b), and incubation of *cyp11a1*-mo1 morphants with pregnenolone from the early cleavage stage partially rescued the epibolic defect (Fig. 3a).

Figure 2 | Reduced *cyp11a1* expression results in epibolic delay.

a, Phenotypic examination of embryos under a dissecting microscope. Wild-type (WT) embryos and embryos injected with control, *cyp11a1*-mo1, *cyp11a1* 5-mis (5-base mismatched *cyp11a1*-mo1), *hsd3b* or *cyp17* morpholino oligonucleotides (mo) at the one-cell stage reach 100% epiboly. Embryos injected with *cyp11a1*-mo1 cannot close the yolk plug.

This indicates that pregnenolone and/or its metabolite is involved in zebrafish epiboly.

To determine whether pregnenolone is metabolized further, we incubated embryos with radioactive pregnenolone and checked the formation of metabolites by thin-layer chromatography. We found that some pregnenolone could be metabolized into progesterone, but that very little 17 α -hydroxypregnenolone was produced, even when Hsd3b activity was knocked down (Supplementary Fig. S6). When tested, progesterone could not rescue the epibolic defect—instead, it worsened the severity of the growth delay (data not shown). As a concentration of only 0.1 μ M pregnenolone was enough to rescue the epibolic defect (Fig. 3a), and this concentration is lower than the K_m for either Cyp17 (5 μ M; ref. 8) or Hsd3b (0.2–1.6 μ M; ref. 9), it is likely that pregnenolone, rather than its metabolite, rescues the epibolic delay.

Zebrafish epiboly is driven by yolk microtubule arrays, which extend from yolk syncytial nuclei along the animal–vegetal axis of the yolk^{10–12}. To investigate whether the epibolic function of *cyp11a1* is related to microtubules, we visualized microtubules by staining them with an antibody against α -tubulin. We observed no significant difference between wild-type and *cyp11a1*-mo1 morphants during epiboly (data not shown). We therefore tested the stability of microtubules by treating embryos at 50% epiboly with the microtubule-disrupting agent nocodazole. While almost completely disrupted by 10 μ g ml⁻¹ nocodazole¹¹ (data not shown), yolk microtubules remained intact with treatment of 1 μ g ml⁻¹ nocodazole, but the yolk microtubules of *cyp11a1*-mo1 morphants were thinner than those of wild-type embryos (Fig. 3b). This result shows that yolk microtubules of *cyp11a1* morphants are more sensitive to nocodazole treatment, indicating that they are less stable^{13,14}. When *cyp11a1*-mo1 morphants were incubated with pregnenolone, yolk microtubule structure was restored (Fig. 3b). Therefore, pregnenolone can stabilize yolk microtubules *in vivo*.

In addition to the imaging study described above, we examined microtubule content using a biochemical method. Protein extracts from wild-type embryos and *cyp11a1*-mo1 morphants at 50% epiboly were centrifuged at 100,000 g to separate polymerized tubulin (pellet) from free tubulin (supernatant). When embryos were treated with the tubulin-polymerizing drug taxol, the amount of polymerized tubulin in the pellet was increased (Fig. 3c). In *cyp11a1*-mo1 morphants, the amount of polymerized tubulin in the pellet was much less than for wild type, indicating a lower abundance of polymerized microtubules. When the extract from *cyp11a1*-mo1 morphants was incubated with pregnenolone, the amount of polymerized tubulin in the pellet increased in a dose-dependent manner (Fig. 3d). This indicates that pregnenolone can increase the amount of polymerized tubulin *in vitro*.

To visualize the interaction between pregnenolone and microtubules, we conjugated the fluorescent dye fluorescein to pregnenolone (Supplementary Fig. S7). The resulting F-P₅ also increased the abundance of polymerized tubulin in *cyp11a1*-mo1 morphant extracts (Supplementary Fig. S8), indicating the fluorescein moiety does not interfere with pregnenolone activity. When incubated with *cyp11a1*-mo1 morphant extract for 30 min, F-P₅ co-localized with polymerized α -tubulin (Fig. 4). When fluorescein alone was incubated with morphant extract, it did not co-localize with α -tubulin and microtubules were not detected. This indicates that pregnenolone binds to microtubules, or to something that associates with microtubules in cell extracts. This binding results in increased microtubule content *in vitro*.

Microtubule dynamics provide an important force for cell movement and neurite outgrowth^{15,16}. However, the regulators of microtubules are largely unknown, except for a few identified proteins such as CLASPs, MAP2c, MACF7 and Op18, which stabilize microtubules in various cell types^{13,17,18}. Here we show that pregnenolone is a regulator of yolk microtubules—it maintains the abundance of yolk microtubules and promotes epibolic movement. Pregnenolone

Figure 3 | Pregnenolone rescues epiboly defect and increases microtubule content. **a**, The effect of different concentrations of pregnenolone on the percentage of epiboly. Error bars show s.d. * $P < 0.05$; ** $P < 0.01$. **b**, Microtubule structure of nocodazole-treated wild-type embryos (16 out of 18 embryos showed normal microtubule structure, $n = 16/18$), *cyp11a1-mo1*-injected embryos ($n = 19/23$) or *cyp11a1-mo1* morphants incubated with 20 μM P₅ ($n = 19/19$) at 50% epiboly. Enlarged images show

higher magnifications of the areas in the rectangles. **c**, **d**, Microtubule content assay. Embryo extracts from wild-type (WT) or *cyp11a1-mo1*-injected embryos at 50% epiboly were incubated with or without 20 μM taxol or different concentrations of P₅, centrifuged to separate free tubulin in the supernatant from polymerized microtubules in the pellet, then probed with an α-tubulin antibody. mo, morpholino oligonucleotide.

promotes microtubule assembly through binding to microtubule-associated protein 2 from rat brain¹⁹. This provides insight into the mechanism of cell movement controlled by pregnenolone, not only during zebrafish embryogenesis but probably also for neurite outgrowth and other types of cell movement.

Here we describe a new function for *cyp11a1* expressed in the zebrafish YSL. The YSL is known to have a role in morphogenesis during gastrulation²⁰, but how it participates in morphogenetic cell movement was not clear. We now show that the YSL gene *cyp11a1* is important for the production of pregnenolone, which preserves the abundance of microtubules, thus promoting cell movement.

The production of steroids from extra-embryonic tissues is not unique to zebrafish. In mouse, steroids are secreted from extra-embryonic giant trophoblast cells during early pregnancy²¹, but the function of steroids in mammalian embryogenesis cannot be easily investigated^{22,23}. The *Drosophila* steroid ecdysone is activated in an extra-embryonic tissue and is involved in morphogenetic cell movements for building up the body plan²⁴, but its mechanism of action is not clear. Using zebrafish, we have shown that the steroid pregnenolone, produced from an extra-embryonic tissue, participates in early morphogenesis by acting on microtubules. Steroids usually act through a genomic mechanism, whereby steroids bind to steroid receptors and control the expression of target genes²⁵. Other non-genomic actions have been proposed for steroids²⁶. We now show that pregnenolone promotes cell movements by directly stabilizing microtubules, probably through a non-genomic mechanism.

METHODS

Microinjection and reagent treatment. Morpholino oligonucleotides (Gene Tools) were diluted to working concentrations of 0.5–3 μg μl⁻¹ in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) and injected into the yolk of embryos at the one-cell or 1k-cell stage at a concentration of 9 ng per embryo, unless noted otherwise. For RNA

Figure 4 | Fluorescein-conjugated pregnenolone co-localizes with microtubules. Detection of fluorescein and α-tubulin in the microtubule pellet after incubation of *cyp11a1-mo1*-treated embryo extracts with 20 μM taxol and fluorescein-conjugated pregnenolone (F-P₅) for 30 min followed by centrifugation. When incubated with taxol and fluorescein alone (F), no microtubule signal was detected (except for some nonspecific tubulin staining of cell debris).

rescue, 9 ng *cyp11a1*-mol and 200 pg of *cyp11a1* RNA or β -gal RNA were co-injected into embryos at the one-cell stage. Embryos were incubated with pregnenolone (0.1, 1 or 20 μ M) or progesterone (1 or 20 μ M) after dechorionation and before the 16-cell stage until the late epiboly stage. For nocodazole treatment, 1 μ g ml⁻¹ nocodazole was added to the embryo medium at 50% epiboly for 20 min at 28.5 °C.

Whole-mount *in situ* hybridization and immunostaining. Whole-mount *in situ* hybridization was performed using digoxigenin-labelled antisense RNA probes and alkaline phosphatase-conjugated secondary antibodies, as described previously²⁷. Photographs were taken using an Olympus BX50 microscope. Microtubule staining with an anti- α -tubulin antibody was performed according to a previously described procedure²⁸. Fluorescent images were captured by Zeiss LSM 510 confocal microscope. Tubulin images were processed by the 3D visualization and deconvolution LSM510 basis software (v3.0, Zeiss).

Measurement of the extent of epiboly. Embryos were hybridized with an *ntl* probe to mark the marginal blastomeres, and photos were taken using a Leica MZFLIII microscope with a CCD camera (Diagnostic Instrument Inc). The length from animal pole to vegetal pole and the height of the blastoderm for each embryo were measured by SPOT program (v.4-0-8, Diagnostic Instrument Inc) (Supplementary Fig. S9). The distance of the blastoderm margin divided by the animal-vegetal length is regarded as the percentage of epiboly.

Synthesis of fluorescein-conjugated pregnenolone (F-P₅). F-P₅ consists of fluorescein and pregnenolone bridged by a six-carbon linker via ethereal and thioetherid functionalities (see Supplementary Information). It was prepared in six steps with 14% overall yield, starting from commercially available pregnenolone.

Microtubule abundance assay. This procedure was according to the instructions provided with the microtubules/tubulin *in vivo* assay kit (Cytoskeleton). Twenty embryos injected with or without 9 ng *cyp11a1*-mol were placed in 200 μ l lysis buffer plus 1 mM GTP, 10 mM ATP and 20 μ l proteinase inhibitor cocktail, homogenized, then incubated with taxol (20 μ M), pregnenolone (0.1, 1, 20 μ M), fluorescein or fluorescein-conjugated pregnenolone at 30 °C for 30 min. A 180- μ l aliquot was transferred into a pre-warmed tube and centrifuged at 100,000 g, 30 °C for 30 min. After centrifugation, pellets were dissolved in 180 μ l cold 200 μ M CaCl₂. For western blot analysis, 2.5 μ l each of total lysate and supernatant samples, and 15 μ l of pellet samples were loaded onto the gels. For the imaging study, microtubule pellets were removed from centrifuged tubes and stained with α -tubulin antibody, followed by spotting onto a glass slide for fluorescence detection.

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