Induction of macrocyst wall formation by ZYG1 in *Dictyostelium discoideum*

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Abstract

Background: Macrocyst is known as the sexual developmental form in cellular slime molds. ZYG1 is known to be involved in zygote formation by cell fusion during macrocyst formation in *Dictyostelium mucoroides*, one of the species of cellular slime molds.

Aim and methods: To know the effects of ZYG1 on the asexual and sexual development in *Dictyostelium discoideum*, another species of cellular slime molds, we observed the developmental process and forms of a transformant (GFP-ZYG1^{OE}) derived from *Dictyostelium discoideum*, in which GFP-ZYG1 fusion protein is overproduced.

Results: GFP-ZYG1^{OE} cells were developed without or with an opposite mating type, V12M2 cells. When GFP-ZYG1^{OE} cells were developed without V12M2 cells, they formed macrocyst-like structures consisting of a central cell mass and a thick transparent macrocyst wall surrounding it. It was suggested that transparent zones in the macrocyst wall were formed by cell fusion. When GFP-ZYG1^{OE} cells were developed with V12M2 cells, globular bodies which are likely the macrocysts surrounded by thin cellulosic walls were found besides macrocysts. They were formed directly from zygotic giant cells without the formation of cell aggregates. The formation of the globular body is not caused by ZYG1 overproduction, because GFP^{CONT} cells, a transformant overproducing GFP protein as controls, also formed globular bodies.

Conclusion: By the use of GFP-ZYG1^{OE} cells overproducing GFP-ZYG1 fusion protein, it is revealed that ZYG1 is closely involved in macrocyst wall formation. This is the new finding concerning the function of ZYG1. In addition, the formation of a globular body, which is likely the macrocyst surrounded by a thin cellulosic wall, is demonstrated as a novel process of sexual development.

Keywords: *Dictyostelium*, cellulose, cell fusion, macrocyst wall, ZYG1

Introduction

Macrocysts are formed as a sexual developmental process in cellular slime molds. Removal of any growing medium (starvation) causes cells to stop growing and form cell aggregates. Macrocyst formation in Dm7, a wild-type strain of *Dictyostelium mucoroides* is characterized by the formation of large aggregates after starvation which are then subdivided into smaller masses (precysts), each of which is surrounded by a fibrillar sheath. A giant cytophagic cell arises at the center of each precyst, which engulfs all the

other cells in the precyst. The engulfed cells (endocytes) become oval and are eventually broken down into granular remnants. The enlarged giant cell is eventually surrounded by a thick macrocyst wall to form a mature macrocyst. The macrocyst wall has been shown electron-microscopically to consist of three layers: a thin inner layer of the least electron-density, a thick middle layer of medium electron-density, and a thin electron-dense outer layer [1]. The macrocyst wall is considered to be a product synthesized by a giant cell between the cell membrane

and the outer-most fibrillar sheath [1]. After a resting period, the macrocyst germinates to release several amoeboid cells and initiates a new life cycle [2].

There are mainly two kinds of mating systems involved in macrocyst formation: homothallic and heterothallic [3-6]. Dm7 is a homothallic strain, which forms macrocysts without mating types. Ax2, an axenic strain of *Dictyostelium discoideum* (*D. discoideum*) is a heterothallic strain, which mates with an opposite mating type strain, V12M2. In the developmental process of macrocyst formation in Ax2 cells co-cultured with V12M2 cells, zygotic giant cells attract local amoebae, resulting in the formation of precysts [7,8]. Thus giant cells appear within aggregates during macrocyst formation in Dm7 cells [1], while the giant cells are formed before distinct cell aggregation in the case of Ax2 cells co-cultured with V12M2 cells. The process of macrocyst formation, however, is believed to be basically the same between the two mating systems [9,10].

The cytophagic giant cells formed during macrocyst formation are zygotes produced by cell fusion and subsequent nuclear fusion [11]. As regulators of zygote formation, ethylene (a potent plant hormone) and 3', 5'-cyclic adenosine monophosphate (cAMP) have been identified. Ethylene induces zygote formation [11], while cAMP inhibits zygote formation [12,13].

Concerning the signal transductions involved in zygote formation, the signaling pathway via Ca^{2+} and protein kinase C (PKC) is suggested in both homothallic Dm7 and heterothallic Ax2 cells [13,14-18].

As a gene involved in zygote formation, *zyg1* has been isolated from a cDNA library of Dm7 by time dependent differential screening (DDBJ/EMBL/GenBank, accession number AB006956) and from a genomic DNA of Dm7 by PCR (DDBJ/EMBL/GenBank, accession number AB479506). *Zyg1* encodes a protein (ZYG1; deduced Mr 29.4×10^3) consisting of 268 amino acids. According to BLAST and FASTA searches, ZYG1 is predicted to have PKC-mediated phosphorylation sites, but neither transmembrane domains nor specific signal sequences [19,20]. In a transformant over-expressing *zyg1*, the formation of zygotic giant cells is greatly enhanced, and macrocysts are formed even under unfavorable conditions [21]. Since the *zyg1* expression is enhanced by ethylene, ethylene induces zygote formation through an enhanced expression of *zyg1* [22].

From the prediction that ZYG1 (a *zyg1* product) has PKC-mediated phosphorylation sites, ZYG1 is expected to be a candidate of a substrate for PKC. Using Ax2, instead of Dm7, this has been made clear. A transformant, GFP-ZYG1^{OE} (which is named as GFP/ZYG1^{OE} in [23]) derived from Ax2, which overproduces GFP-ZYG1 fusion protein, is constructed. For this, a *zyg1* isolated from Dm7 is used, because the *zyg1* has not been found in *D. discoideum* by the Japanese *Dictyostelium* cDNA project and genome project of *Dictyostelium* [24]. Using GFP-ZYG1^{OE} cells, it has been shown that ZYG1 is actually phosphorylated by PKC [23]. GFP-ZYG1 fusion protein uniformly distributes in the cytoplasm of GFP-ZYG1^{OE} cells. In addition,

ZYG1 is translocated at regions of cell-to-cell contact where cell fusion occurs [23]. These results indicate that ZYG1 moves from the cytoplasm to the cell membrane, is phosphorylated by PKC and causes cell fusion through PKC mediated signal transduction pathways. Thus ZYG1 plays an important role in zygotic cell fusion.

The developmental process and forms of GFP-ZYG1^{OE} cells co-cultured without or with an opposite mating type, V12M2 cells, were also examined during the course of the study. We report here a new function of ZYG1 and the unique process for macrocyst formation obtained consequently by use of GFP-ZYG1^{OE} derived from Ax2 cells, which overproduces GFP-ZYG1 fusion protein.

Materials and methods

Cell cultures and developmental conditions

GFP^{CONT} and GFP-ZYG1^{OE} transformants derived from Ax2 (an axenic strain of *D. discoideum*) (Amagai et al., 2012) and V12M2 (an opposite mating type strain of Ax2) were used in this work. Vegetative GFP^{CONT} and GFP-ZYG1^{OE} cells were grown axenically by shake cultures in PS-medium (1% Special Peptone [Oxoid: Lot No. 333 56412], 0.7% Yeast extract (Oxoid), 1.5% D-glucose, 0.11% KH_2PO_4 , 0.05% $Na_2HPO_4 \cdot 12H_2O$, 40 ng/ml vitamin B₁₂, and 80 ng/ml folic acid) containing 200 µg/ml streptomycin, 10 µg/ml tetracycline and 50 µg/ml Geneticin (G418) (GIBCO BRL). To allow the cells to differentiate, they were harvested during the exponential growth phase, washed once in BSS (Bonner's salt solution: 10 mM NaCl, 10 mM KCl, 2.7 mM $CaCl_2$) [25] as a starvation medium. V12M2 cells were grown by simultaneous culture with *Escherichia coli* B/r. Vegetative cells at the exponential growth phase were harvested and washed three times with BSS for starvation. Cell culture and development were carried out in dark conditions at 22°C. For observation and time-lapse imaging under an inverted microscope (Nikon), the cells were settled on glass based dishes (Iwaki, 3910-035, 35 mm diameter) and developed under submerged conditions in BSS at $1 \sim 6 \times 10^6$ cells/cm².

Calcofluor staining

Immediately after one drop of Calcofluor (10 µg/ml; Sigma F-3397) was added into the 2 ml cell suspension, one drop of cell suspension was placed on cleaned coverslips for observation.

Imaging of living cells

Glass dishes with adhered living cells were placed on an inverted microscope for observation (Nikon, Japan). Time-lapsed images were acquired at one frame per 20 seconds using a CCD camera (Leica Dc250, Germany).

Observations and photographs

Developing cells and forms were observed and photographed under an inverted microscope, a phase-contrast microscope (Nikon, Tokyo) and a fluorescence microscope using UV excitation

(Olympus, Tokyo).

Results

GFP-ZYG1^{OE} cells construct macrocyst-like structures without cells of the opposite mating type

The GFP-ZYG1^{OE} cells and GFP^{CONT} cells, transformants derived from Ax2 cells, are confirmed by western blot to overproduce GFP-ZYG1 fusion protein and GFP protein, respectively, at least during early development [23].

When GFP-ZYG1^{OE} cells were developed without V12M2 cells, GFP-ZYG1^{OE} cells formed loose aggregates without sheath materials at 1 day of development after starvation (**Figure 1a**). After 3 days of development, loose aggregates changed to tight aggregates, each of which was surrounded by a sheath. Cells located at the center seemed to be optically denser than peripheral cells (**Figure 1b**, arrows). The optically denser part of an aggregate became a tight aggregate (a central cell mass) at 7 days of development (**Figure 1c**, an arrow). The peripheral cells surrounding the central masses were eventually lost, thus resulting in the formation of a macrocyst-like structure consisting of a central cell mass and a thick transparent wall (a macrocyst wall) surrounding it. Since the peripheral cells did not become transparent synchronously, three types of developmental forms eventually coexisted (**Figures 1d-1f**). As described above, the structure consisting of a central cell mass and peripheral cells surrounding it (**Figure 1d**), and the macrocyst-like structure consisting of a central cell mass and a thick transparent wall surrounding it (**Figure 1e**) were observed as the first and second forms. In addition, empty walls without central cell masses were sometimes observed as the third form (**Figure 1f**).

Although macrocyst-like structures are morphologically similar to normally formed macrocysts, it is presently unknown whether or not the central cell mass is a zygotic giant cell containing endocytes.

When the macrocyst-like structure was stained with Calcofluor before the completion of a transparent wall, the whole of the central cell mass was stained. As the outer surface of the peripheral cells surrounding the central cell mass was also stained, each peripheral cell was easily recognized. Comparing the size (diameter) of the cells, the cell shown by an arrow was about 4 times larger than that shown by an arrowhead (**Figure 2a**). Large-sized cells seem to fuse with each other to increase their size.

Some macrocyst-like structures were in process of forming transparent walls by losing peripheral cells. Since some lines consequently became visible in the macrocyst-like structure, it was realized that the central cell mass was surrounded by several parts of transparent zones (**Figure 2b**).

Apparently, there was no significant difference in the macrocyst walls in either macrocyst-like structures or macrocysts. To confirm this similarity, the localization of GFP-ZYG1 fusion protein and cellulose in the macrocyst-like structure and macrocyst was examined by staining with Calcofluor. In the

Figure 1. Development of GFP-ZYG1^{OE} cells. Starved GFP-ZYG1^{OE} cells were developed without their mating cells at a density of 6×10^6 cells/dish. (a) A phase-contrast photomicrograph at 1 day of development. Most of the cells finished aggregation and form loose aggregates. (b) A phase-contrast photomicrograph at 3 days of development. Loose aggregates change to tight aggregates, each of which is surrounded by a sheath (arrows). The cells located at the center of an aggregate seem to be optically denser than the peripheral cells. (c) A phase-contrast photomicrograph at 7 days of development. The optically denser part of an aggregate becomes a tight aggregate (a central cell mass, arrow). This structure looks like a macrocyst (a macrocyst-like structure). (d) A bright-field photomicrograph at 18 days of development. This shows a macrocyst-like structure consisting of a central cell mass and peripheral cells surrounding it. (e) A bright-field photomicrograph of a macrocyst-like structure at 17 days of development. This shows a complete macrocyst-like structure consisting of a central cell mass and a transparent macrocyst wall surrounding it. (f) A bright-field photomicrograph at 19 days of development. This shows a cell mass consisting of only transparent zones. Bars: 100 μ m.

macrocyst-like structure, GFP-ZYG1 fusion protein was localized at the whole central cell mass and the outer layer of the macrocyst wall, co-localizing with Calcofluor staining (**Figure 3a**). In the macrocyst formed in GFP^{CONT} cells co-cultured with V12M2 cells, GFP protein was localized at the whole giant cell and the outer layer of the macrocyst wall, co-localizing with Calcofluor staining (**Figure 3b**). As a result, GFP-ZYG1 fusion protein and GFP protein were co-localized with cellulose in both macrocyst-like structures and macrocysts. This indicates the similarity of the macrocyst wall in both macrocyst-like structures and macrocysts. However, as GFP^{CONT} protein also showed the same localization as GFP-ZYG1 fusion protein, co-localization of GFP-ZYG1 fusion protein with cellulose does not depend on ZYG1 overproduction.

When GFP^{CONT} cells were developed without V12M2 cells, they formed loose aggregates, but never macrocyst-like structures. Therefore, the formation of macrocyst-like structures in GFP-ZYG1^{OE} cells was concluded to be due to ZYG1 overproduction.

Macrocyst formation by GFP-ZYG1^{OE} and GFP^{CONT} cells co-cultured with V12M2 cells

To elucidate the function of ZYG1 protein during macrocyst formation, GFP-ZYG1^{OE} cells were developed with V12M2 cells. As a result, mainly two types of developmental forms were found: naturally-occurring mature macrocysts surrounded with a thick macrocyst wall (**Figure 4Aa**) and globular bodies (**Figure 4Ab**). These forms were also found when GFP^{CONT} cells were developed with V12M2 cells as controls (**Figure 4Ac**). In consequence, there seemed to be no essential differences in the developmental forms between GFP-ZYG1^{OE} and GFP^{CONT} cells. In macrocysts, two types of macrocyst wall were observed. One was lined with peripheral cells (**Figure 4Aa**, arrowed), and another with few peripheral cells (**Figure 4Aa**, arrowhead).

Macrocysts formed by GFP^{CONT} cells co-cultured with V12M2 cells also showed two types of macrocyst wall. Although most of the macrocyst walls were clear zones in mature macrocysts developed for 21 days (**Figure 4Ad**), macrocyst walls in which peripheral cells were still retained were sometimes observed even after 21 days of development (**Figure 4Ae**).

With regard to globular bodies, there has been no precise description yet. The mature globular bodies were filled with endocytes (**Figure 4B**, upper panel, arrowhead). Apparently, a thick macrocyst wall was not formed in the globular body. However, as the periphery of the globular bodies was stained with Calcofluor (**Figure 4B**, lower panel, arrowed), it is evident that they have a thin wall consisting of cellulose.

To precisely examine how globular bodies are formed, the developmental process in a mixed culture of GFP-ZYG1^{OE} and V12M2 was observed under phase-contrast microscopy and also recorded using time-lapsed imaging. Cell aggregates (arrowhead) and giant cells (arrows) were formed at 1 day of development after starvation, although many cells remained as non-aggregated single cells (**Figure 5a**). After 4 days of development, giant cells full of granular endocytes appeared (**Figure 5b**, arrows). This was followed by formation of globular bodies after 6 days of development (**Figure 5c**, arrows). As the developmental process did not progress synchronously, giant cells and globular bodies coexisted for several days of development.

When GFP^{CONT} cells were developed with V12M2 cells as controls, they showed a similar developmental process as

Figure 4. Formation of globular bodies. (A) Final structures formed by co-cultures of GFP-ZYG1^{OE} or GFP^{CONT} cells with V12M2 cells. (a) and (b) Starved GFP-ZYG1^{OE} cells were mixed with V12M2 cells at a ratio of 1:1 at a density of 6×10^6 cells/dish and developed. (a) A bright-field photomicrograph of macrocysts with thick macrocyst walls at 5 days of development. Two kinds of macrocyst wall are observed: one is lined with peripheral cells (arrow), and another with few peripheral cells (arrowhead). (b) A phase-contrast photomicrograph of globular bodies at 6 days of development. (c-e) Starved GFP^{CONT} cells were mixed with V12M2 cells at a ratio of 1:1 at a density of 6×10^6 cells/dish and developed. (c) A phase-contrast photomicrograph of a macrocyst surrounded with a macrocyst wall (arrow) and globular bodies (arrowheads) at 7 days of development. (d) A bright-field photomicrograph of a macrocyst with a clear macrocyst wall at 21 days of development. e A bright-field photomicrograph of a macrocyst with a macrocyst wall filled with cells at 21 days of development. Bars: 100 μ m. (B) Formation of cellulosic walls in globular bodies. Starved GFP-ZYG1^{OE} and V12M2 cells were developed as described in A. At 12 days of development, globular bodies were photographed after staining with Calcofluor. Upper panel: A phase-contrast photomicrograph of globular bodies. They contain endocytes (arrowhead). Lower panel: a fluorescence photomicrograph corresponding to the upper panel. As the periphery of globular bodies is stained with Calcofluor (arrows), it is evident that the globular body is surrounded by a thin cellulosic wall. Bars: 50 μ m.

shown in a mixed culture of GFP-ZYG1^{OE} and V12M2 cells. They were forming aggregates and giant cells (arrowed) at 1 day of development (Figure 5d). After 5 days of development, giant cells full of granular endocytes appeared (Figure 5e, arrows). This was followed by the formation of globular bodies after 6 days of development (Figure 5f, arrows).

Time-lapse recording revealed that free-living giant cells ruffled their lamellipodia. As the free-living giant cells contained small particles, amoebae engulfed by them possibly changed to small particles (endocytes) [1]. It was sometimes observed that a giant cell itself was engulfed by another giant cell (Movie 1). The free-living giant cells containing endocytes suddenly stopped moving, retracted their lamellipodia and formed globular bodies (Figure 6 and Movie 2). Occasionally,

Figure 5. The developmental process of globular body formation by GFP-ZYG1^{OE} or GFP^{CONT} cells co-cultured with V12M2 cells. a- c Starved GFP-ZYG1^{OE} and V12M2 cells were developed as described in the legend of Figure 4. (a) A phase-contrast photomicrograph at 1 day of development. Large aggregates (arrowhead) and free-living giant cells (arrows) are formed, though many cells remain as single cells. (b) A phase-contrast photomicrograph at 4 days of development. Giant cells filled with granular endocytes are formed (arrows). (c) A phase-contrast photomicrograph of globular bodies (arrows) at 6 days of development. d- f Starved GFP^{CONT} and V12M2 cells were developed as described in the legend of Figure 4. (d) A phase-contrast photomicrograph at 1 day of development. Cells are forming an aggregate. A free-living giant cell (arrow) is observed. (e) A phase-contrast photomicrograph at 5 days of development. Giant cells filled with granular endocytes are formed (arrows). (f) A phase-contrast photomicrograph of globular bodies (arrows) at 6 days of development. Bar: 100 μ m.

globular bodies returned to the giant cells spreading their lamellipodia. In this case, however, they finally became globular bodies.

Discussion

In the present study, the developmental process and forms of GFP-ZYG1^{OE} cells overproducing GFP-ZYG1 fusion protein were examined. As a result, the interesting findings concerning the function of ZYG1 and the developmental process of macrocyst formation were obtained.

When GFP-ZYG1^{OE} or GFP^{CONT} cells were co-cultured with V12M2 cells, they formed macrocysts and many free-living giant cells. The free-living giant cells contained endocytes, retracted their lamellipodia and developed directly into globular bodies. Globular bodies have the characteristics of a macrocyst including the formation of giant cells containing endocytes and a wall stained with Calcofluor, though they do not form a thick macrocyst wall. Consequently, the globular body is likely the macrocyst formed directly by a giant cell without the process of cell aggregation.

Since globular bodies were also observed in a mixed culture of GFP^{CONT} and V12M2 cells, the formation of the globular body

Figure 6. The time-lapse imaging of a globular body formation. Starved GFP-ZYG1^{OE} and V12M2 cells were developed as described in the legend of **Figure 4**. Time-lapse imaging was performed at 20-second intervals. A free giant cell containing endocytes suddenly stops movement, retracts its lamellipodia and then forms a globular body. Bar: 100 μ m.

would not be caused by ZYG1 overproduction.

When GFP-ZYG1^{OE} cells were developed without V12M2 cells, they formed macrocyst-like structures. The macrocyst-like structure consisted of a central cell mass with a macrocyst wall surrounding it (**Figure 1e**). When GFP^{CONT} cells were developed without V12M2 cells, they formed loose aggregates, but never macrocyst-like structures. Therefore, the formation of macrocyst-like structures is apparently due to ZYG1 overproduction. It is presently unknown whether the central cell mass is a zygotic giant cell containing endocytes. Considering that ZYG1 is known to induce cell fusion [21,23], it is supposed that globular bodies formed only by cell fusion may be formed by ZYG1 overproduction. However, the fact that the GFP-ZYG1^{OE} cells failed to form globular bodies without the help of mating type cells seemed to indicate that the functional zygotic giant cells might not be formed only by ZYG1 overproduction. On the other hand, it is most likely that the wall formation in a macrocyst-like structure may be induced by ZYG1 overproduction.

In the macrocyst-like structure, GFP-ZYG1 fusion protein, as well as cellulose, was localized at the whole central cell mass and the outer layer of the macrocyst wall (**Figure 3a**), as macrocysts formed by co-cultures of GFP^{CONT} with V12M2 cells (**Figure 3b**). This indicates the similarity in macrocyst walls of macrocyst-like structures and macrocysts. Consequently, according to the descriptions by Filosa and Dengler (1972), the thick transparent zone of the macrocyst-like structure corresponds to the thin inner layer and thick middle layer of the macrocyst wall in the macrocyst. The outer layer of the macrocyst-like structure, stained with Calcofluor, corresponds to the electron-dense, thin outer layer of the macrocyst wall in the macrocyst.

The formation process of transparent zones in macrocyst-like structures was also suggested in the present study: that is, peripheral cells in the macrocyst-like structure increase their size by cell fusion and eventually become transparent zones (**Figure 2**). Coupling with the enlarged cells, GFP-ZYG1 fusion protein may be pushed toward the cell membrane and co-localized finally with cellulose at the outer layer of the macrocyst wall (**Figure 3**). Since ZYG1 is known to induce cell fusion [21], it is quite possible that ZYG1 may induce cell fusion in the early stages of macrocyst wall formation.

In macrocysts formed by GFP-ZYG1^{OE} or GFP^{CONT} cells co-cultured with V12M2 cells, two types of macrocyst wall were observed; a clear zone (**Figure 4Aa**, arrowhead and **4Ad**) and a zone filled with cells (**Figure 4Aa**, arrowed and **4Ae**). From this, it is supposed that the cell fusion process could be involved also in the wall formation of the macrocyst. The macrocyst wall is considered to be a product synthesized by a giant cell between the cell membrane and the outermost fibrillar sheath [1]. Both giant and periphery cells are possibly involved in the wall formation. However, at present, the accumulation of evidence is insufficient to clarify the precise mechanism of wall formation.

In the absence of a *zyg1* in GFP^{CONT} cells, how is the macrocyst wall formed in the macrocysts? Since the existence of a *zyg1* homologue in V12M2 genome has been suggested by the genomic Southern analysis [21], ZYG1 could be supplied by V12M2 cells.

The idea of macrocyst wall formation by cell fusion of peripheral cells suggests that peripheral cells surrounding a giant cell might be necessary for the formation of transparent zones in a macrocyst wall. This may explain the reason why a globular body formed from a free-living giant cell has no thick transparent zones in a macrocyst wall. From the observations that the globular body is not surrounded by peripheral cells, cell fusion and subsequent vacuolization may not occur outside of a giant cell, thus resulting in lack of transparent zones of a macrocyst wall.

Recently, by BLAST search [26,27], uncharacterized ZYG1-like protein has been reported from prokaryotes such as *Enterobacter cloacae* (DDBJ/EMBL/GenBank, accession number AYIG01000002, E value: 3e-93) and *Escherichia coli* (DDBJ/EMBL/GenBank, accession number AWCH01000020, E value: 3e-91). As cellular slime molds except axenic strains such as Ax2, feed on bacteria phagocytotically as a food supply, their cells contain a lot of bacteria. It might be that a long time ago a bacterial *zyg1* gene incorporated into the genome of cellular slime mold. Considering the functions of ZYG1, the information about cyst formation also might originate from bacteria. We hope that these interesting ideas will be further examined in the future.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	AA	FT	TU	TA	YM
Research concept and design	✓	--	--	--	--
Collection and/or assembly of data	✓	✓	✓	✓	--
Data analysis and interpretation	✓	--	--	--	✓
Writing the article	✓	--	--	--	--
Final approval of article	✓	--	--	--	--

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