Silk Sericin Hydrolysate is a Potential Candidate as a Serum-Substitute in the Culture of Chinese Hamster Ovary and Henrietta Lacks Cells

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Received 16 August, 2018; Editorial decision 15 December, 2018

Abstract

The silk sericin hydrolysate (SSH) from the waste of silk processing as a substitute of fetal bovine serum (FBS) was used for the culture of Chinese hamster ovary (CHO) cells and Henrietta Lacks (Hela) strain of human cervical cancer cells. The survival ratio of these cells cultured in SSH media were similar to or higher than those in FBS media. Especially after the serum was replaced by low concentration of SSH at 15.0 μg/ml for 5 d, the proliferation of both cells was also similar to or higher than that of FBS group; the percentages of CHO and Hela cells in S-phase were 28.9 and 28.0%, respectively. The former is nearly two times that of FBS group, the latter is also higher than the control group. Reverse transcription-polymerase chain reaction (RT-PCR) revealed that among the differentially expressed genes, the relative expression of CXCL12 gene of CHO cells in SSH group increased, was three times that of serum group, and the relative expression of LCN2 gene of Hela cells increased 2.8 times, indicating that these related genes were activated to promote cell growth and proliferation. These results fully illustrated the hydrolysated sericin has a potential use as serum substitutes in cell culture.

Key words: sericin, serum, substitute, tumor, cell cycle

Cell culture is one of the most common methods in cell biology research, with the continuous development and innovation in the field of modern biology and medicine, the use of this technology is becoming more and more universal. The medium of cell culture often needs to add various nutrients, generally add the animal serum, such as fetal bovine serum (FBS), because it contains plentiful nutrients and physiochemical compounds which are required in cell growth (De Castro et al. 2006, Pedraza et al. 2008). But it still has some shortcomings, such as the instability between batches makes it difficult to control the quality of each batch. Virus contamination and mycoplasma contamination which always exist and hard to avoid, resulting in an obstacle to the purification of the subsequent product, meanwhile, more and more scholars have begun to worry about the safety and defects of FBS (Yao et al. 2004, Sato et al. 2009), they constantly propose the need to actively discover and develop the substitute of FBS (Van der Valk et al. 2010). By now many researchers have made great efforts to develop diversified serum-free media products (Lange et al. 2007, Chen et al. 2014).

The cocoon shell of the silkworm is mainly composed of silk fibroin and sericin which is wrapped in its outer layer for adhesion and protection (Kundu et al. 2008). In the process of silk processing, the sericin is usually treated as waste and is discharged into the river with the degummed waste liquid, it not only leads to serious environmental pollution, but also results in a great waste of biological resources. Our research team has developed a pollution-free and environmentally-friendly silk degumming solvent—the strongly alkaline electrolysis water method (Cao et al. 2013), silk protein surfactant (Wang et al. 2015) and alkyl polysaccharide (Wang and Zhang 2017), which could greatly improve the problems of the environment and subsequent sericin recycling, our team also reported a method of recovering sericin from silk processing waste and performing degradation (Wu and Zhang 2014). The recovered macromolecule sericin crude products were hydrolyzed with protease (Wu et al. 2014), and prepared sericin of various molecular weight ranges. Sericin of different molecular weight may have different effects on cells.

Early in 2005, there was a report on silk sercin used for insect cell culture, sercin polypeptides could protect SF9 insect cells from death which due to acute deficiency of serum (Takahashi et al. 2005). As a mitotic factor, sercin could better promote the proliferation and attachment of mammalian cells (Terada et al. 2005, Martinez-Mora et al. 2012, Nayak et al. 2013). It could also significantly promote the proliferation of hybridoma cells (Terada et al. 2002). Sercin had a corresponding effect on human marrow stromal cells (hMSC)
The cell density of CHO cell was 8.3 $\times 10^3$ cells/well, the cell density of Hela cell was 2.2 $\times 10^4$ cells/well, used 100 μl of culture medium containing 10% FBS and 1% double antibody (mixed solution of specific operation was as follows: CHO cells at a density of 2.5 $\times 10^5$ cells/well and Hela cells at a density of 6.7 $\times 10^5$ cells/well were inoculated respectively into 6-well plates and cultured overnight at 37°C. Afterward, the cells were washed with cold PBS to remove ethanol, slowly and fully resuspended with 1 mg/ml RNase A solution at 37°C water bath for 30 min in the dark to digest RNA in the cells, and finally stained PI using a standard method. After dyeing, the red fluorescence was detected by flow cytometry at the excitation wavelength of 488 nm, corresponding to the BD Flow Cytometer FL2 detection channel, and the light scattering was detected. Then FLOWJO software was used to analyze the cell cycle.

Quantitative RT-PCR
Differentially expressed gene (DEG) was verified by quantitative RT-PCR (qRT-PCR). We performed 10 qRT-PCR involved ‘inhibit DNA binding’ (ID1, ID2), ‘cell cycle progression’ (FN1, MBD4, MMP1) and ‘cell proliferation and migration’ (INHBA, LCN2, CBY1, CXCL12, SCNN1A). The β-actin gene was selected as the reference gene. Primers were designed by Oligo software (version 7.60). Experiments were repeated for three times.

Statistical Methods
The experimental data were processed by Origin 8.5 statistical software, and the results were expressed as mean ± SD. All data comparison used one-way analysis of variance; it had statistical significance when $P < 0.05$.

Results
Cell Morphology and Overall Survival Ratio
The morphology of the cells was analyzed by cell photographs which were continuously shot for a week with a microscope, and representative photomicrographs of cells on day 1 and day 5 were selected.
As a result, it was found that CHO cells could analogously grow well in SSH medium and FBS control medium, and also showed normal cell morphology (Fig. 1A–E). CHO cells cultured in SSH medium showed diffuse fibroblast-like cell morphology with extensive cell–cell contacts. This was the same as the cells cultured in FBS medium (Fig. 1A). In the first to fifth day, the cell proliferated rapidly, but the morphology of the cells was still similar to that of the FBS control group, especially when treated with 15 μg/ml SSH media (Fig. 1B). The typical cell morphology of the HeLa cells (Fig. 2A–E), particularly a subconfluent monolayer of cell status with an unoccupied surface, cell boundaries and condensed nuclear chromatin, was shown in FBS and SSH media. Unaltered cell morphology indicated that SSH could support cell growth of HeLa cells. Furthermore, no significant differences in cell morphology were observed between cells cultured in SSH media with the concentration at 15 μg/ml and FBS media based on cell size, shape and profile (Fig. 2B).

Cell proliferation is an important vital characteristic of the organism, single cell organisms produce new individuals in the form of cell division, multicellular organisms produce new cells by cell division for replenishing aging and dead cells in the body. MTT is often used to detect the capacity of cell proliferation, its detection principle is that succinate dehydrogenase in mitochondria of living cell

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**Fig. 1.** Microscope photos (x200) of CHO cells cultured in FBS or SSH on 1 d (a–e) and 5 d (A–E). a&A: FBS, b&B: SSH (15 μg/ml), c&C: SSH (30 μg/ml), d&D: SSH (60 μg/ml), e&E: SSH (120 μg/ml)
can make the exogenous MTT reduce to water-insoluble blue-violet crystal formazan, and the crystal is deposited in cells, while dead cells do not have this function. Dimethyl sulfoxide (DMSO) can dissolve formazan in cells, the absorbance value (OD) is measured at 490 nm by a microplate reader, within the range of a certain number of cells, the amount of MTT crystals is proportional to the number of cells. The number of viable cells is determined by the measured OD value, the bigger the OD value, the stronger the cell activity. After morphological observation, we measured the overall cell survival rate by MTT assay. Cells were cultured by SSH with different concentrations; it was found that 15 μg/ml SSH was the most suitable for two cell lines (Fig. 3). Specifically, in the first 2 d, the OD values of CHO cells in the medium of the FBS and different concentrations of sericin alkaline hydrolysate were similar. On the third to seventh day, the absorbance values of the low-dose SSH (15 μg/ml) were comparable to those of the FBS group, while the OD values of the other several concentrations of SSH were slightly lower than those of the FBS group (Fig. 3a). HeLa cells showed a higher overall survival rate in the first 5 d of the 15 μg/ml SSH medium (Fig. 3b). On the sixth day and the seventh day, the absorbance values were slightly lower than the FBS group. While the other concentrations, especially the high concentration of 120 μg/ml, the OD values were far lower than the FBS group. In conclusion, it was found that 15 μg/ml SSH medium was the best choice for serum-free growth of both cells.

Fig. 2. Microscope photos (x200) of Hela cells cultured in serum or alkaline hydrolyzed sericin on 1 d (a–e) and 5 d (A–E). a&A: FBS, b&B: SSH (15 μg/ml), c&C: SSH (30 μg/ml), d&D: SSH (60 μg/ml), e&E: SSH (120 μg/ml).
Cell Cycle Distribution

The cell cycle distribution of CHO and Hela cells were analyzed by Flow Cytometry. Fig. 4 were representative images analysis of 80–90% fusion of both cells. As could be seen from the figure, in the 15 μg/ml SSH group, the proportion of cells in S phase of both cells increased compared with the FBS group, among them the proportion
of S phase of CHO cells was 1.89 times that of FBS group (Table 1), correspondingly the proportion of cells in G2/M phase decreased. Figure 5 and Table 2 were the ratio of the cycle distribution of CHO cells from 0 d to 7 d, compared with cells in FBS media, the percentage of S phase cells in SSH media did not increase significantly in the first 5 d, G0/G1 and G2/M phases were also not accompanied by a reduction in proportion (Fig. 5a–d). While on the fourth day to the seventh day, the percentage of S phase cells in SSH media increased, G0/G1 and G2/M phases were accompanied by a reduction in proportion (Fig. 5e–h). These results indicated that there were similar (or greater) amounts of active cells in DNA synthesis in media of 15 μg/ml SSH.

Figure 6 and Table 3 showed the proportion of Hela cells cycle distribution in the period of 0 to 7 d, compared with cells in FBS media, the percentage of S phase cells in SSH media did not increase significantly in first 5 d, G0/G1 and G2/M phases were also not accompanied by a reduction in proportion (Fig. 6a–f). These results indicated that there were similar (or greater) amounts of active cells in DNA synthesis in SSH media. While on the sixth day and the seventh day, the percentage of S phase cells in SSH media increased, G0/G1 and G2/M phases were accompanied by a reduction in proportion (Fig. 6g and h).

The above results indicated that the two cells in the 15 μg/ml SSH group, could show similar or better cell cycle distribution compared to the control medium of FBS. Demonstrating that no deleterious distortion occurred in the cytogenetic process in the SSH medium.

Comparison of Related Gene Expression

In order to compare and study the cell cycle and other expression of related genes, in this experiment, the expression of 10 genes involved ‘inhibition of DNA binding (ID1, ID2)’, ‘cell cycle progression (FN1, MFS4, MMP1)’, and cell proliferation and migration (INHBA, LCN2, CBY1, CXCL12, SCNN1A) were detected by qRT-PCR. According to the results obtained in the experiment, it was found that the cell morphology, the overall survival ratio and the cell cycle distribution of the two cells in the low concentration of SSH were significantly better than those in the high concentration of SSH, moreover, the culture effect of 15 μg/ml SSH was similar to that of FBS. Therefore, this study selected this concentration of SSH to detect the 10 related genes of CHO and Hela cells, and FBS as a control to do a comparative study. The results showed that after CHO cells were cultured in SSH media, the relative expression of the related gene MFS4 involved in cell cycle progression were flat with the FBS group, the relative expression of the other two genes FN1 and MMP1 were increased by 2.2 and 2.9 times, respectively, compared with the FBS group (Fig. 7a). CBY1 and LCN2, which were involved in cell proliferation and migration, were slightly higher or lower than those in FBS group, the expression of SCNN1A gene was significantly reduced by about 40%, while the relative expression of INHBA and CXCL12 genes increased significantly, compared with FBS, they increased by 160 and 210%, respectively. The relative expression of ID1 and ID2 genes involved in DNA binding were respectively decreased by 20 and 40%. These results indicated that in the genes involved in cell cycle progression, two genes were increased, one gene was flat; the relative expression of INHBA and CXCL12 genes involved in cell proliferation and migration was significantly increased. After Hela cells were cultured in SSH media (Fig. 7b), MFS4 gene involved in cell cycle progression was flat with FBS, while the relative expression of MMP1 and FN1 increased significantly by 30 and 100%; the gene involved in cell proliferation and migration in addition to the expression of SCNN1A gene decreased by 60%, the relative expression of the other four genes CXCL12, CBY1, INHBA, and LCN2 respectively increased 30%, 120, 130, and 180%. Similarly, the relative expression of ID1 and ID2 genes involved in inhibition of DNA binding decreased by 70 and 60%, respectively. From the above results, it was found that 15 μg/ml SSH substituted FBS to culture both CHO and Hela cells, in the gene of cell cycle in addition to MFS4 which was flat, the expression of the other two genes FN1 and MMP1 increased significantly, especially CHO cells; and inhibited the expression of the DNA-binding gene more or less; while in the genes involved in cell proliferation and migration, the expression of SCNN1A gene was inhibited, the expression of the other two genes INHBA and CXCL12 were significantly promoted.

Specifically, the up-regulated genes were associated with cellular energy metabolism and DNA replication such as cellular respiration and cell cycle progression, while the down-regulation of genes were associated with extracellular matrix transport, ribonucleoprotein complex, cell growth inhibition such as inhibition of DNA binding (ID1, ID2), transcriptional activation and fibrinolysis. In addition, it was consistent with the observation of the phenotype and metabolic status of the cells, genes associated with these functions and related biological processes were activated to promote cell survival and proliferation.

Table 1. The proportion in different phases of both cells at 80–90% cell confluence

| Group | G0/G1 | S   | G2/M |
|-------|-------|-----|------|
| CHO   | FBS   | 45.62% | 15.37% | 40.95% |
|       | SSH (15 μg/ml) | 40.84% | 29.05% | 32.14% |
|       | Multiple (SSH/FBS) | 0.895 | 1.890 | 0.785 |
| Hela  | FBS   | 54.07% | 24.75% | 20.25% |
|       | SSH (15 μg/ml) | 50.25% | 27.40% | 24.19% |
|       | Multiple (SSH/FBS) | 0.929 | 1.107 | 1.195 |

Discussion

The purpose of this experiment is to systematically evaluate the performance of sercin as a serum substitute. In this experiment, we selected CHO cells and HeLa cell lines. These two cells are very representative, CHO cells represent fibroblast-like cells, HeLa cell lines represent tumor cells.

Using sercin instead of FBS to culture different types of cells is still less, which greatly limits the sercin as a serum substitute used in the cell culture industry (Fujita et al. 2010).

CHO cells belong to fibroblast-like cells; they can either adhere to the wall for growth or grow in suspension (Kumagai et al. 2003). CHO cell is one of the best expression systems for exogenous eukaryotic genes. It is also the most commonly used mammalian cell in biopharmaceuticals (Jayapal et al. 2007). In genetic engineering drugs that have been listed and are undergoing clinical studies, about 60 to 70% are mammalian cells expressing products, while the products expressed by CHO cells account for most of them (Moreira 2007). CHO cell lines are one of the most commonly used and most effective expression systems for exogenous proteins; it can be used as a mammalian host for the treatment of recombinant proteins (Weikert et al. 1999, Berquist et al. 2010, Bosques et al. 2010). Compared with other expression systems, CHO expression system has the following advantages (Sung et al. 2004, Kildegaard et al. 2013), with an accurate post-transcriptional modification function, the expressed protein is closest to the native protein molecule in terms of molecular structure, physicochemical properties and biological function.
(Barnes et al. 2003); with the efficient amplification and expression ability of recombinant genes, the integration of foreign protein is stable; with the function of extracellular secretion of products, and rarely secrete its own endogenous protein, which facilitates the separation and purification of downstream products; it can be cultured in suspension or in serum-free medium to achieve high-density culture. In recent years, in order to reduce production costs and the potential harm caused by blood products, animal cell production have begun using serum-free medium. However, the serum-free medium often leads to poor cell viability, poor adherence, poor ability in suspension or in serum-free medium and FBS medium for CHO Cells for different days. The number of cells in the G0/G1-, S- or G2/M-phase is given as percentages of the total cell population.

Fig. 5. Cell cycle distribution in the SSH medium (15 μg/ml) and FBS medium for CHO Cells for different days.
to secrete foreign proteins and other shortcomings. In addition, there were researchers who attempted to transfer insulin-like growth factor IGF genes and transferrin genes into CHO cells to obtain "super-CHO" that could secrete essential proteins by itself, cells could grow well in serum-free medium without the need to add transferrin and insulin in the culture media (Pak et al. 1996).

Hela Cells, also known as experimental proliferating epidermal cancer cells, are a kind of cell used in biology and medical research which from the cervical cancer cell lines of an American woman, Hela. In the medical field, Hela cells are widely used in tumor research, biological experiments or cell culture, which have become a very important tool in medical research (Ristiani et al. 2009, Das et al. 2012, Cotugno et al. 2014).

In this experiment, the effects of SSH as a serum substitute on cell culture were systematically evaluated by studying morphology, the overall survival rate, cell cycle and proliferation of cells and the expression of related genes.

Morphological observation of cells is often used for cell status assessment, and there have been some studies on the effects of serum-free or serum reduction on cell cultures (Hartmann et al. 2008, Jin et al. 2014). However, previous studies were limited to cell survival. In this experiment, the morphology, cell cycle and population doubling time of cells were evaluated after the SSH substituted FBS. Our results indicated that SSH could replace FBS in cell culture, and there was no significant difference in cell morphology, it was similar to previous related experiments of sericin and morphology of cultured cells (Chang et al. 1977, Aramwit et al. 2010). In addition, in the cell cycle and population doubling time, it was found that there were related reports of promoting cell growth and shortening the population doubling time with sericin in the process of fibroblast culture (Barnes and Sato 1980).

Previous studies often used expensive cell growth-related factors rather than sericin in serum-free media, such as fibroblast growth factor, leukemia inhibitory factor and transforming growth factor (Takahashi et al. 2003, Furue et al. 2008, Chase et al. 2010, Ferruzza et al. 2013). Takahashi et al. first tried sericin, it was found that the small peptide Ser D, which was added to the medium after the decomposition of sericin, could improve the growth status of insect cells with serum-free culture (Ohnishi et al. 2012). Since then, sericin was used as a component of cell culture to promote cell proliferation in serum-free media (Goto et al. 1999, Anitua et al. 2005, Miyamoto et al. 2010). However, sericin was not considered a complete substitute for FBS, and was not fully evaluated for its effectiveness in serum-free media. For example, Miyamoto et al. using sericin and DMSO as supplement for culture and cryopreservation of human hepatocytes, it could improve cell adhesion and cell viability in serum-replaced media. Therefore, these methods were difficult to obtain or clearly explained the specific molecular mechanism of sericin promoting cell growth.

Using a series of concentration gradients to optimize the concentration of SSH, we successfully applied SSH to completely replace FBS. Our preliminary results showed that by measuring the overall cell survival rate and population doubling time, the optimum SSH concentration (15 µg/ml), compared with the addition of different extraction methods of sericin, growth factor addition and partial sericin replacement method (Terada et al. 2002), had a better ability to promote cell growth and reproduction.

We have also determined some of the previous unfinished items, detected the DNA content of the cells and analyzed the cell cycle. We found that the percentage of S phase cells did not increase significantly and there was no corresponding decrease in the proportion of G0/G1 and G2/M cell populations, especially in HeLa cells. This showed in the medium of the two cells that sericin alkaline hydrolysate instead of FBS, S phase cell aggregation suggested that SSH could increase the potential of cell proliferation (Wang et al. 2008, Krueger and Wilson 2011). It was noteworthy that the promotion of cell cycle in cyogenetic compilation did not cause harmful distortion (Pinto et al. 2012). In view of the imbalance of cell cycle would lead to the possibility of damaging cells by DNA of abnormal proliferation and cell apoptosis (Pecorino 2012), the results of this study clearly showed that hydrolysated sericin replaced FBS, could promote cell cycle progression without causing apoptosis or cell damage to the cells.

The results of the above-mentioned cell cycle assays indicated that compared to the FBS control group, the cell division procedure of the two cells could be highly activated in the SSH medium without causing adverse changes (cell apoptosis or injury) after promoting the cell cycle. In this experiment, we first evaluated the cellular status of the relevant gene expression levels in the medium of SSH, which provides a meaningful method on promoting cell growth and proliferation at the molecular level. This was consistent with the observation of the cell phenotype, the growth function and the associated gene involved in the biological processes that were activated to promote cell growth and proliferation. Upregulation of genes was associated with cellular energy metabolism and DNA replication, such as cellular respiration. The importance of DNA packaging and cell activation in cell differentiation could also be well recognized (Mullinger et al. 1980, Kim et al. 2015, Wei et al. 2015), and they benefited from the role of integration of cells with cells and cells with matrix. Similarly, the biokinetics of cell adhesion affected the regulation of cellular and cellular and cellular-matrix interactions such as cell proliferation, differentiation, and tissue formation, etc. (Théry and Bornens 2006, Shen et al. 2012). Cell adhesion molecules were important for the interfacial interaction of signal-mediated transduction between cells and extracellular matrix (Wu and Yap 2013, 2019).

Table 2. Cell cycle distribution in the SSH medium (15 µg/ml) and FBS medium for CHO Cells for different days

| Day(s) | 0   | 1   | 2   | 3   |
|--------|-----|-----|-----|-----|
| Group  |     |     |     |     |
| G0/G1 (%) |     |     |     |     |
| S (%)   |     |     |     |     |
| G2/M (%) |     |     |     |     |

| Group  |     |     |     |     |
|--------|-----|-----|-----|-----|
| G0/G1 (%) |     |     |     |     |
| S (%)   |     |     |     |     |
| G2/M (%) |     |     |     |     |

| Day(s) | 4   | 5   | 6   | 7   |
|--------|-----|-----|-----|-----|
| Group  |     |     |     |     |
| G0/G1 (%) |     |     |     |     |
| S (%)   |     |     |     |     |
| G2/M (%) |     |     |     |     |

| Day(s) | 8   | 9   | 10  | 11  |
|--------|-----|-----|-----|-----|
| Group  |     |     |     |     |
| G0/G1 (%) |     |     |     |     |
| S (%)   |     |     |     |     |
| G2/M (%) |     |     |     |     |

Further studies indicated that the SSH medium was able to promote cell growth and reproduction, and the results were consistent with previous studies. However, it is necessary to further explore the specific molecular mechanism of sericin promoting cell growth.
Rahman et al. 2016). The down-regulated genes were predominantly extracellular matrix transporters and participated in cell growth inhibition processes, such as inhibition of DNA binding (ID1, ID2), transcriptional activation and fibrinolysis. The release of inhibition gene ID1, ID2 inhibited the DNA binding process (Sun et al. 1991, Liu et al. 2016).

In summary, compared to cells cultured in FBS control group, cells cultured in 15 μg/ml hydrolysated sericin showed similar cell morphology, similar or higher overall cell survival rate, and a higher percentage of S phase as well as similar G2/G1 ratios, indicating comparable or better cell growth and proliferation. In terms of related gene expression, the DEGs between cells in the two media were mainly enriched in the functions and biological processes related to cell growth and proliferation. The relative expression of CXCL12 gene in CHO cells increased to three times, the relative expression of INHBA gene also increased significantly, and LCN2, CBY1 genes were flat with or slightly higher than the FBS group. In Hela cells, the relative expression of LCN2 gene increased to 2.8 times, the relative expression of CBY1 and INHBA genes also increased significantly, and CXCL12 gene was slightly higher than the FBS group. The above results reflected that the gene was activated to promote cell growth and proliferation. In
addition, the relative expression of \textit{FN1}, \textit{MFSD4}, and \textit{MMP1} genes involved in cell cycle progression in both media were flat with or significantly higher than FBS group, indicating that these genes were activated to promote cell cycle progression. The results of this experiment showed that the cells cultured in the medium with 15 $\mu$g/ml sericin hydrolysate instead of FBS showed similar or even better results than those cultured in the medium containing FBS. Our results not only provided conclusive evidence for the use of sericin hydrolysate as a substitute for FBS, but also better elucidated the sustainable development of the future in cell culture and the silk industry.

Acknowledgments

We gratefully acknowledge the earmarked fund for the China Agriculture Research System (CARS-18-ZJ0502) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, P. R. China. Y-Q.Z. conceived this study, M.Z., and T-T.C. constructed the database, M.Z. and T.T.C. performed the statistical analysis and wrote the article, Y-Q.Z. and Z-G.W. revised the article. We declare no conflict of interest.

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