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Exploring the Beneficial Effects of Se-Methylselenocysteine on GC-1/GC-2 Cells: From Cellular Uptake to Metabolic Pathway Regulation in Male Reproduction

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Abstract

Male infertility, a global health issue marked by spermatogenic failure, hinges on selenium (Se) as a key element for normal spermatogenesis. Among different Se species, Se-methylselenocysteine (MeSeCys) has been developed as a natural organic Se supplement with potent antioxidant and anti-inflammatory properties, but its direct effects on male reproduction need to be further explored. This study investigated the effect of MeSeCys on GC-1 spg (GC-1) and GC-2 spd (ts) (GC-2) cell lines, which mimic early stages. Treatment with 75 μmol/L MeSeCys for 24 h markedly enhanced the viability of both cell lines, with a more pronounced effect observed in GC-1 than in GC-2 cells. Moreover, this study demonstrated that MeSeCys enters cells through SLC7A11 or LRP8 channels and elevates intracellular Se levels in both GC-1 and GC-2 cells, with higher levels observed in GC-1 cells. RNA sequencing (RNA-seq) and bioinformatics analysis revealed that MeSeCys may regulate selenocompound metabolism and the glutathione metabolism pathway in both cell lines, increasing their intracellular glutathione (GSH) levels. Importantly, in GC-1 cells, MeSeCys specifically modulates the mTOR pathway, which further modulates glutathione metabolism and intracellular redox balance. This finding provides novel insights into the beneficial effects of MeSeCys on male reproductive cells, highlighting its potential as a nutritional supplement for male reproductive health.

Keywords: Se-methylselenocysteine; male reproduction; GC-1 cells; GC-2 cells; glutathione metabolism; mTOR pathway

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1. Introduction

Infertility is a matter of great concern that affects approximately 8 to 12% of couples globally, with male factor infertility accounting for 50% of cases [1]. Recent studies have shown that abnormal spermatogenesis is a major cause of male infertility, with endocrine disturbances, genetic abnormalities, immunological factors, and genetic causes as main factors [2]. Antioxidant supplementation is now recognized as a crucial intervention for subfertile men [3].

Se, a vital essential trace element, assumes a crucial part in maintaining the normal process of spermatogenesis and preserving male fertility [4,5]. It is worth noting that the bioavailability of Se within organisms is contingent upon its chemical species. Se species are categorized into inorganic Se (e.g., Se(VI) and Se(IV)) and organic Se (e.g., methylselenocysteine (MeSeCys), selenocysteine (SeCys), selenocystine ($SeCys_2$), and selenomethionine (SeMet)). Comparatively, organic Se merits better bioactivity and higher bioavailability [6]. Up to now, several Se species, such as Se (IV), OH-SeMet, and Se nanoparticles (SeNPs), have been investigated for their role in male reproduction improvement. For instance, Mojapelo et al. found that Se (IV) supplementation can boost serum glutathione peroxidase activity and luteinizing hormone and testosterone levels, thus improving the semen quality of Saanen goats [7]. OH-SeMet can enhance sperm quality, including higher sperm motility and sperm concentration [8]. Liu et al. investigated that Se nanoparticles (SeNPs) positively influenced the reproductive function of male Sprague-Dawley rats [9]. In addition, Liu et al. investigated and compared the effects of Se (IV) and SeMet on spermatogenesis. The results indicated that Se supplementation effectively regulated lipid peroxidation of germ cells by increasing glutathione peroxidase activity, and SeMet has a better effect than Se (IV) [10]. These findings provide a theoretical foundation for using Se nutritional supplements to improve the bioactivity of male reproductive cells.

Among different organic Se species, MeSeCys has been regarded as the major Se species in Se-enriched plants, including broccoli [11], garlic [12], and onion [13], and exhibits the most beneficial effects for humans [14]. In recent decades, most studies on MeSeCys' benefits have primarily concentrated on the antitumor, antioxidant, anti-inflammatory, and cognitive-improving properties. For instance, Ma et al. investigated the selective inhibitory effects of MeSeCys on cancer cell proliferation and found that MeSeCys increased the production of reactive oxygen species (ROS), thereby enhancing the susceptibility of lung carcinoma cells to chemotherapeutic agents [15]. Shin et al. revealed that a week-long pretreatment with MeSeCys markedly alleviated oxidative stress in rat lungs from γ-radiation through the nuclear factor Nrf2 signaling pathway [16]. Yang et al. found that MeSeCys has a superior anti-inflammatory effect and can suppress the development of *C. albicans* and the creation of hyphae in vitro [17]. Furthermore, MeSeCys was found to be quite effective in improving neuropathology and cognitive deficits in the Alzheimer's mice model by reducing synaptic and metabolic abnormalities [18]. Recent studies have begun to explore the beneficial effects of MeSeCys on male reproductive health, with numerous studies elucidating its protective and regulatory mechanisms in testicular function and sperm quality. For example, Mao et al. found that MeSeCys ameliorated the di(2-ethylhexyl) phthalate (DEHP)-induced ferroptosis in testicular Sertoli cells through the Nrf2/GPX4 axis [19]. Lu et al. confirmed that MeSeCys exerts a spermatogenic-promoting effect in both in vitro and in vivo models [20]. Furthermore, Che et al. demonstrated that MeSeCys protects against cadmium (Cd) -induced reproductive damage by mitigating oxidative stress, inhibiting the ERK/p38 MAPK pathway, and suppressing Cd-triggered cell cycle arrest, inflammation, and apoptosis [21]. As mentioned above, MeSeCys, as a major selenium species in Se-enriched plants, shows multiple biological functions. Therefore, it is crucial to further advance research into the effects of MeSeCys on improving the bioactivity of male reproductive cells, providing a basis for the development of MeSeCys-enriched foods.

Male germ cell lines GC-1 and GC-2 exhibit spermatocyte features and symbolize the early differentiation stages from type B spermatogonia to preleptotene spermatocytes [22]. Currently, the GC-1 and GC-2 cell lines are widely used to evaluate the effects of pharmacological agents and nutritional supplements on testicular cell function [23–25]. In this study, GC-1, as well as GC-2 cells, were selected as a cell model to explore the influence of

MeSeCys on male reproduction improvement. Several biological indicator assessments, such as the cell counting kit-8 (CCK-8) assay [26], 5-ethynyl-2′-deoxyuridine (EdU) proliferation assay, RNA-seq analysis [27], and the knockdown and overexpression of the target gene analysis, were conducted [28]. Simultaneously, the cellular uptake of MeSeCys was quantified using an inductively coupled plasma-mass spectrometry (ICP-MS) system [29]. By combining ICP-MS data with a series of biological indicator experiments, the effect and molecular mechanism of MeSeCys on promoting cell viability were systematically discussed. This study establishes an initial theoretical framework supporting the prospective use of MeSeCys-enriched foods as a beneficial dietary supplement.

2. Materials and Methods

2.1. Materials and Reagents

The materials and reagents used in this study are listed in the Supplementary Material.

2.2. Cell Viability Assay

GC-1 and GC-2 cells were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China), and detailed culture conditions are described in the Supplementary Material. Cell viability was evaluated by the CCK-8 assay. Cells were seeded in 96-well plates for 24 h and then treated with 3.125 to 100 μmol/L MeSeCys for 6, 12, and 24 h. The cells were then exposed to CCK-8 reagent at 37 °C for 40 min. Absorbance was subsequently assessed at 450 nm with a microplate reader (Thermo Scientific, Waltham, MA, USA).

2.3. Antioxidant Activity Analysis

To assess the protective effects of MeSeCys against H_2O_2-induced oxidative stress, cells were seeded in 96-well plates (1×10^4 cells/mL) for 24 h. After pretreatment with 75 μmol/L MeSeCys for 24 h, the cells were exposed to various concentrations of H_2O_2 (100, 200, 400, 800, 1600 μmol/L) for 6 h. Cell viability was determined by CCK-8 assay as described above.

2.4. Cell Proliferation Assay

Cell proliferation was quantified using the EdU assay. Cells were seeded in a 6-well plate for 24 h and then treated with 75 μmol/L MeSeCys for 24 h. The cells were stained according to the kit instructions and then observed using an immunofluorescence microscope (Leica, Wetzlar, Germany). The number of EdU-positive cells and total cells was counted using ImageJ software (version 2.1.0), and the proliferation rate was calculated as (EdU-positive cells/total cells) × 100%.

2.5. RNA-Seq Analysis

Cells treated with different conditions were collected, and RNA-Seq was conducted on an MGISEQ-2000 (Shenzhen, China), with three biological replicates allocated to each treatment group for the analysis. Raw sequencing data were normalized using the median of ratios method integrated in DESeq2 (v1.4.5), and differential gene expression analysis was subsequently conducted with the screening criteria set as adjusted *p*-value (*q* value) ≤ 0.05 and $|\log2(\text{fold change})| \geq 1$ for differentially expressed genes (DEGs). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, Gene Ontology (GO) annotation, and Gene Set Enrichment Analysis (GSEA) were conducted on Dr. Tom's platform (https://biosys.bgi.com (accessed on 15 January 2026)). PPI analysis was achieved via Metascape (https://metascape.org/ (accessed on 15 January 2026)) [30].

2.6. Analysis of Total Se

Cells treated with 75 μmol/L MeSeCys for 24 or 48 h were washed 5 times with PBS and trypsinized and resuspended in 1 mL PBS. Cell counts were determined using Countess II (ThermoFisher Scientific, Waltham, MA, USA). Cells were digested with nitric acid in DigiBlock (LabTech, Beijing, China) using the following procedure: the cells were digested at 85 °C for 3 h, followed by evaporation at 120 °C until the samples were nearly dry. The residue was diluted with 3 mL of deionized water for subsequent analysis by the ICP-MS Instrument (Agilent 7900, Santa Clara, CA, USA).

2.7. Cell Transfection

SLC7A11 and LRP8 in GC-1 and GC-2 cells were transiently silenced by transfecting targeted siRNAs using CALNP™ RNAi in vitro (D-Nano, Beijing, China). The sequence of all siRNAs used is listed in Table S1. For SLC7A11 overexpression, SLC7A11 cDNA was subcloned into the pcDNA3.1-EGFP vector (Sangon Biotech, Shanghai, China). The SLC7A11 overexpression (pcDNA3.1-SLC7A11-EGFP) plasmid was transfected into both cells through Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. qRT-PCR Analysis

The mRNA expression levels of target genes were analyzed via qRT-PCR assay. Primer sequences for target genes and β-actin (internal control) are listed in Table S2.

2.9. Western Blot Analysis

The protein levels of GPX, SEPHS2, GCLC, GCLM, phosphorylated mTOR, total mTOR, and p-4EBP1 in GC-1 and GC-2 cells were analyzed by Western blot analysis. β-actin was used as the internal control, with the specific experimental procedure outlined in the supporting information.

2.10. Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical significance was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Prism 10.1.1 software served as the tool for data analysis, and results were considered statistically significant when $p < 0.05$.

3. Results

3.1. The Effect of Different Se Species on GC-1 and GC-2 Cells' Viability

The effects of five Se species (MeSeCys, $SeCys_2$, SeMet, SeCys, and Na_2SeO_3) on the cell viability were analyzed by CCK-8 assay following treatment durations of 6, 12, and 24 h. Notably, treatment with 50, 75, and 100 μmol/L MeSeCys significantly enhanced the viability of both cells after 24 h of exposure (Figure 1A,B). The maximum viability was observed at 75 μmol/L MeSeCys: 179.24% (GC-1) and 126.81% (GC-2) of the control group. In addition, treatment with 25 μmol/L $SeCys_2$ also enhanced GC-1 cell viability (129.59%) and GC-2 cell viability (119.15%) (Figure 1C,D). SeMet and SeCys only improved the viability of GC-1 cells (SeMet: 117.11% at 50 μmol/L; SeCys: 127.47% at 25 μmol/L) (Figure 1E,G). Furthermore, Na_2SeO_3 only increased the viability of GC-2 cells when the cells were treated with 6.25 μmol/L Na_2SeO_3 for 6 h (Figure 1J). Based on these results, 75 μmol/L for 24 h was selected for subsequent experiments.

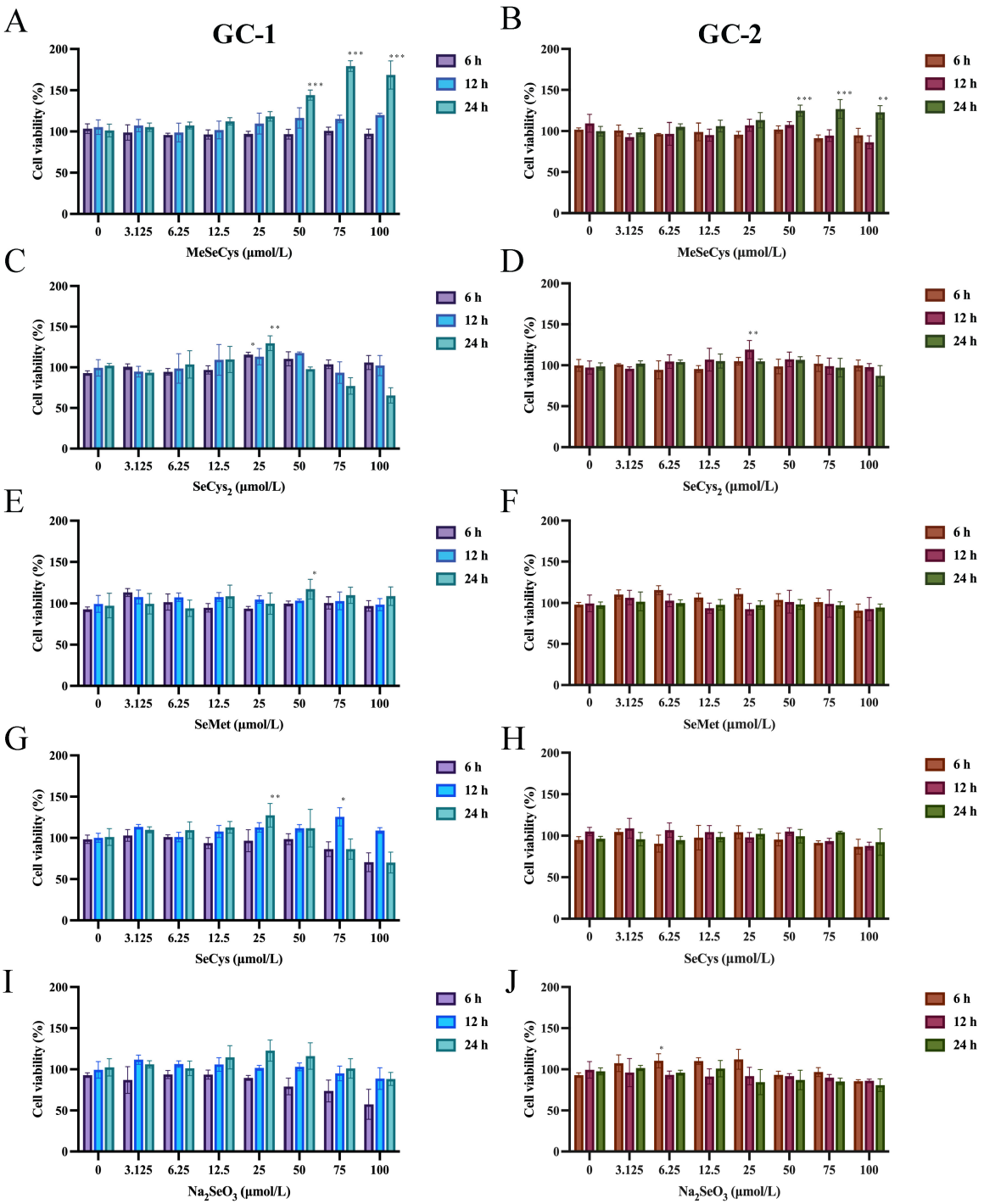

Figure 1. Effects of different Se species on the viability of GC-1 and GC-2 cells. Cells were treated with MeSeCys (**A**,**B**), $SeCys_2$ (**C**,**D**), SeMet (**E**,**F**), SeCys (**G**,**H**), and Na_2SeO_3 (**I**,**J**) at concentrations ranging from 3.125 to 100 μmol/L for 6 h, 12 h, and 24 h; data are presented as mean ± SD (n = 3). Statistically significant differences are represented as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2. The Effect of MeSeCys on the H_2O_2-Induced Cytotoxicity of GC-1 and GC-2 Cells

Subsequently, the protective effects of MeSeCys against H_2O_2-induced cytotoxicity were evaluated. As depicted in Figure 2A, H_2O_2 treatment (100–1600 μmol/L) dose-dependently reduced the GC-1 cell viability to 11.22–84.82%. Nevertheless, following 24 h pretreatment with MeSeCys, cell viability was significantly restored to 45.21–102.33%. Similarly, H_2O_2 exposure reduced GC-2 cells' viability to 4.67–84.55%, an effect that was attenuated by MeSeCys pretreatment, which enhanced viability to 14.15–96.18% (Figure 2B).

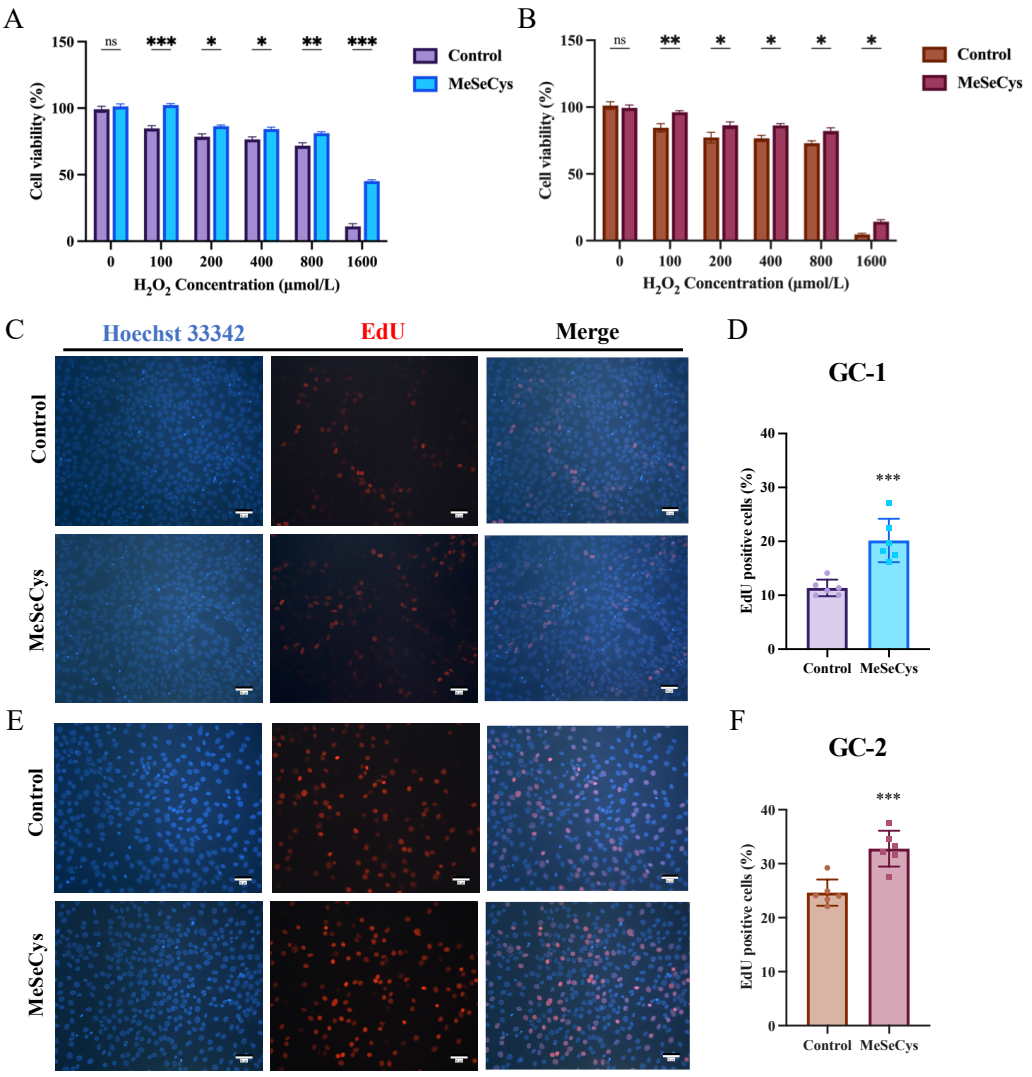

Figure 2. The effect of MeSeCys on the H_2O_2-induced cytotoxicity and cell proliferation rates of GC-1 (**A**) and GC-2 cells (**B**). Fluorescent staining of the EdU assay for detecting GC-1 (**C**) and GC-2 (**E**) cell proliferation rate. Percentage of EdU positive staining statistical analysis (**D**,**F**): the data are presented as mean ± SD (n = 3). Statistically significant differences are represented as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3. The Effect of MeSeCys on GC-1 and GC-2 Cell Proliferation

The cell proliferation was further analyzed using the EdU assay, and the results are shown in Figure 2C–F. 75 μmol/L MeSeCys treatment for 24 h increased the cell proliferation rates of GC-1 and GC-2 cells by 77.37% and 33.14%, which is consistent with the cell viability data shown in Figure 1A, B.

3.4. RNA-Seq Analysis

3.4.1. Statistics of DEGs

To investigate the underlying mechanisms of MeSeCys on male reproductive cells, we compared the gene expression profile of GC-1 and GC-2 cells exposed to MeSeCys with the control group by RNA-Seq analysis. Volcano plots were employed to elucidate the differences in gene expression, and the results were shown in Figure 3A,C. Firstly, in GC-1 cells, a total of 628 genes were significantly up-regulated, while 201 genes were significantly down-regulated (fold change > 2, $q < 0.05$). Conversely, in GC-2 cells, 324 genes

were notably up-regulated, and 57 genes were notably down-regulated (fold change > 2, $q < 0.05$).

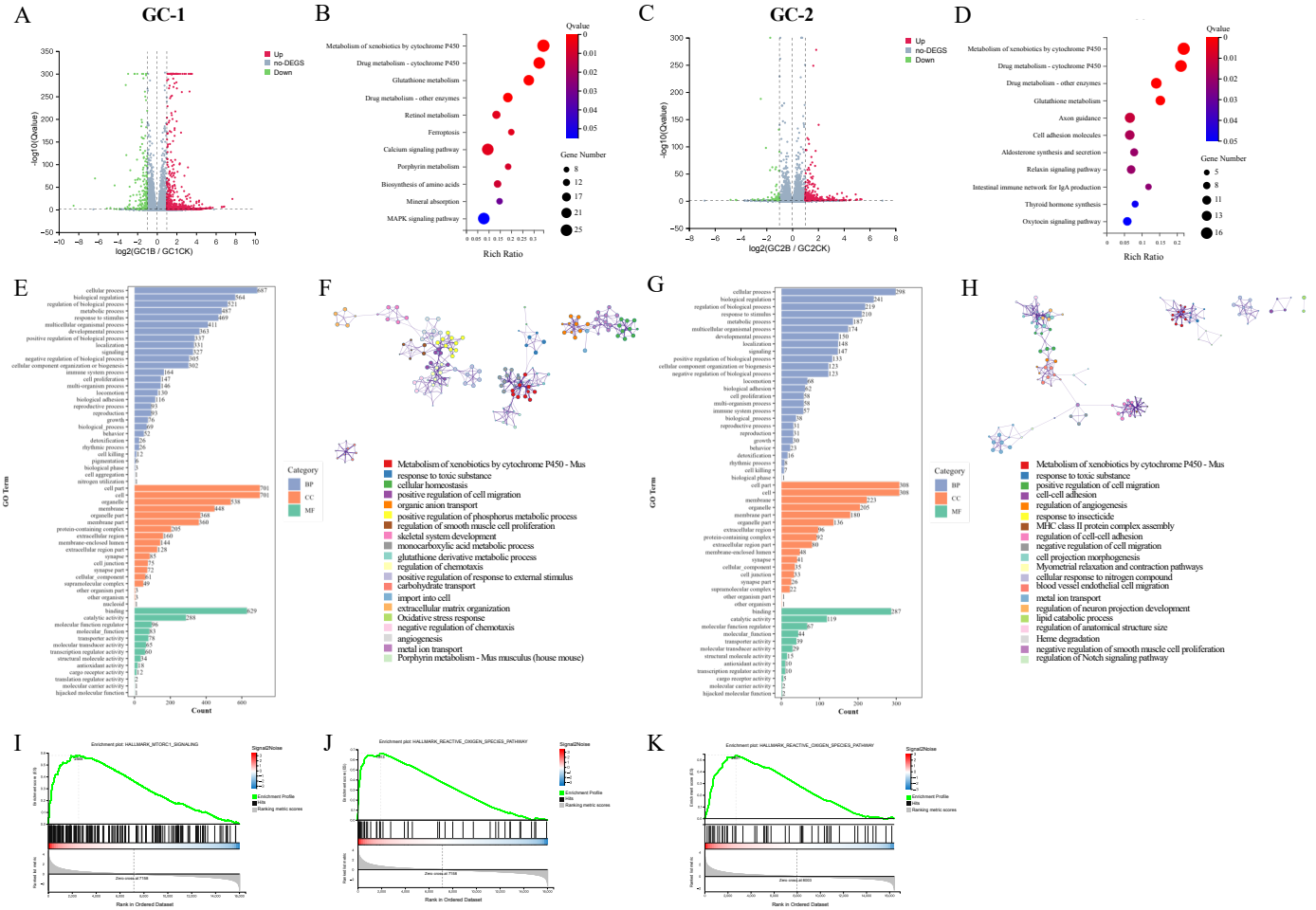

Figure 3. RNA-Seq analysis in GC-1 and GC-2 cells. Volcano map for DEGs in the control and MeSeCys treatment groups (**A**,**C**). KEGG pathway enrichment (**B**,**D**), GO annotation (**E**,**G**), PPI analysis (**F**,**H**), and GSEA (**I**–**K**) analyzed the DEGs between control and MeSeCys treatment groups.

3.4.2. KEGG Enrichment Analysis

In order to further identify key pathways influenced by MeSeCys in male reproductive cells, KEGG enrichment analysis was conducted on the previously identified DEGs. As shown in Figure 3B,D, the differential pathways in GC-1 cells were predominantly associated with glutathione metabolism, biosynthesis of amino acids, mineral absorption, and the MAPK signaling pathway. On the other hand, in GC-2 cells, the differential pathways were enriched in the glutathione metabolism, axon guidance, cell adhesion molecules, etc. It is noteworthy that glutathione metabolism was a common enriched pathway in both cell lines.

3.4.3. GO Annotation Analysis

Then, the DEGs identified above were analyzed by the GO annotation to elucidate the regulated function modules. Specifically, in both cells, the DEGs were predominantly enriched in categories such as “cellular process” and “biological regulation” at the biological process level, “cell part” and “cell” at the cellular components level, and “binding” and “catalytic activity” at the metabolic function level (Figure 3E,G). Compared with GC-2 cells, GC-1 cells had more DEGs across various GO categories.

3.4.4. PPI Analysis

PPI network analysis further showed that DEGs in GC-1 cells were associated with "cellular homeostasis", "glutathione derivative metabolic process", and "import into cell" (Figure 3F). In GC-2 cells, DEGs were linked to "positive regulation of cell migration", "cell-cell adhesion", and "metal ion transport" (Figure 3H). These observations suggest that MeSeCys could play a role in modulating the intracellular absorption and metabolic pathways of MeSeCys in both GC-1 and GC-2 cells.

3.4.5. GSEA

For a more in-depth exploration of the possible molecular mechanisms, GSEA was conducted. As shown in Table S3, GSEA identified 19 pathways associated with MeSeCys-mediated regulation of GC-1 cell viability (NOM $p < 0.05$). Notably, the MTORC1 signaling (Figure 3I) and reactive oxygen species pathway (Figure 3J) were implicated in the MeSeCys-induced enhancement of GC-1 cells' viability. In addition, GSEA identified 15 pathways associated with MeSeCys-mediated regulation of GC-2 cell viability (NOM $p < 0.05$) (Table S4). Similar to GC-1 cells, the reactive oxygen species pathway (Figure 3K) was also enriched in GC-2 cells.

3.5. Potential Mechanisms Underlying MeSeCys-Mediated Enhancement of GC-1 and GC-2 Cell Viability

3.5.1. Uptake of MeSeCys by Male Reproductive Cells

The cellular uptake of Se is a crucial factor in determining its role in organisms [31]. For the purpose of investigating MeSeCys cellular uptake, ICP-MS analysis was performed on cells that had been exposed to 75 μmol/L MeSeCys for durations of 24 h and 48 h. As depicted in Figure 4A, after 24 h of MeSeCys treatment, the total Se content in GC-1 cells increased from 12.40 to 32.98 fg/cell compared to the control group, and after 48 h of treatment, the total Se content further increased to 53.54 fg/cell (Figure 4A). Similarly, the total Se content in GC-2 cells increased from 13.98 to 23.03 fg/cell after 24 h of MeSeCys treatment and subsequently increased to 53.29 fg/cell after 48 h (Figure 4C). These results indicated that MeSeCys was effectively taken up by male reproductive cells and further regulates cell viability.

It is essential to investigate the channel protein associated with MeSeCys uptake. The qRT-PCR analysis results showed the significant upregulation of mRNA levels for *Slc7a11* and *Lrp8* (Figure 4B,D), which are two membrane proteins previously identified as mediators of cellular Se uptake [32]. Hence, it was hypothesized that SLC7A11 and LRP8 might play a role in the cellular uptake of MeSeCys. Consequently, both cell lines with SLC7A11 and LRP8 knockdown were constructed. Three distinct siRNA sequences were designed for the target genes, and subsequent qRT-PCR analysis identified that the most efficient siRNAs were SLC7A11-SiRNA-2 and LRP8-SiRNA-1 (Figure S1). The results revealed that the knockdown of either SLC7A11 or LRP8 significantly reduced the MeSeCys uptake in both cells (Figure 4E–H). Specifically, in GC-1 cells, the knockdown of the SLC7A11 gene reduced the intracellular Se content from 33.96 fg/cell to 14.73 fg/cell ($p < 0.001$), whereas the knockdown of the LRP8 gene decreased the Se content from 31.27 fg/cell to 19.65 fg/cell ($p < 0.05$). Similarly, in GC-2 cells, SLC7A11 gene knockdown reduced intracellular Se levels from 29.67 fg/cell to 17.45 fg/cell ($p < 0.01$), while LRP8 gene knockdown decreased Se content from 27.87 fg/cell to 18.54 fg/cell ($p < 0.05$). Notably, SLC7A11 knockdown resulted in a more substantial decrease in Se uptake compared to LRP8 in both cell lines. Additionally, SLC7A11 knockdown abolished MeSeCys-induced cell viability enhancement (Figure 4I,K), whereas LRP8 knockdown had no significant effect. Thus, SLC7A11 was identified as a key channel protein for MeSeCys uptake and

the regulation of cell viability. Subsequently, overexpression experiments were further conducted, and the results indicated that the overexpression of SLC7A11 in both cells markedly increased the uptake of MeSeCys (Figure 4J,L).

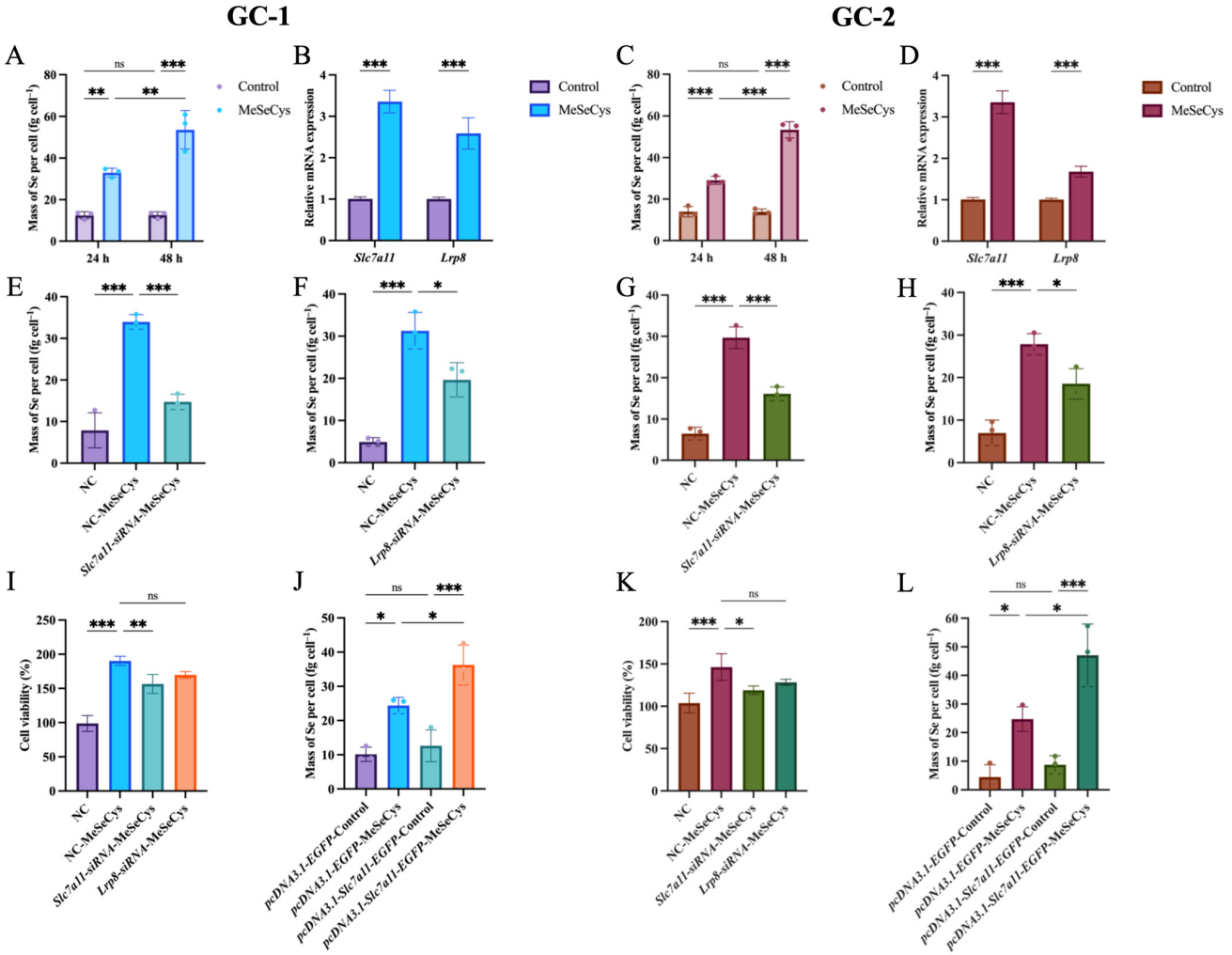

Figure 4. Uptake of MeSeCys by GC-1 and GC-2 cells. Intracellular Se content of GC-1 (**A**) and GC-2 (**C**) cells with or without 75 μmol/L MeSeCys treatment for 24 and 48 h. The mRNA expression level of *Slc7a11* and *Lrp8* was detected by qRT-PCR (**B**,**D**). Intracellular Se content (**E**–**H**,**J**,**L**) and cell viability (**I**,**K**) of cells under siRNA or overexpressing plasmid treatment. All data are presented in the form of mean ± SD (n = 3). Statistically significant differences are represented as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant ($p > 0.05$).

3.5.2. Selenocompound Metabolism in GC-1 and GC-2 Cells

Previous studies have demonstrated that after entering the organisms, MeSeCys undergoes metabolism and transformation, followed by the biosynthesis of various selenoproteins, thereby exerting antioxidant, anticancer, and reproductive detoxification effects [33]. Therefore, the expression of genes related to selenocompound metabolism was assessed by qRT-PCR methods (Figure 5A,B). After MeSeCys treatment, the mRNA expression levels of major members of the selenocompound family, including *Selenom*, *Selenop*, *Selenow*, and *Selenos*, were dramatically up-regulated in both GC-1 and GC-2 cells. Notably, the upregulation of mRNA expression levels was more pronounced in GC-1 cells. Furthermore, the protein levels of selenophosphate synthetase 2 (SEPHS2) and glutathione peroxidase 4 (GPX4) were evaluated. As illustrated in Figure 5C–H, SEPHS2 and GPX4 pro-

tein levels were significantly increased in MeSeCys-treated GC-1 and GC-2 cells, indicating enhanced selenocompound metabolism.

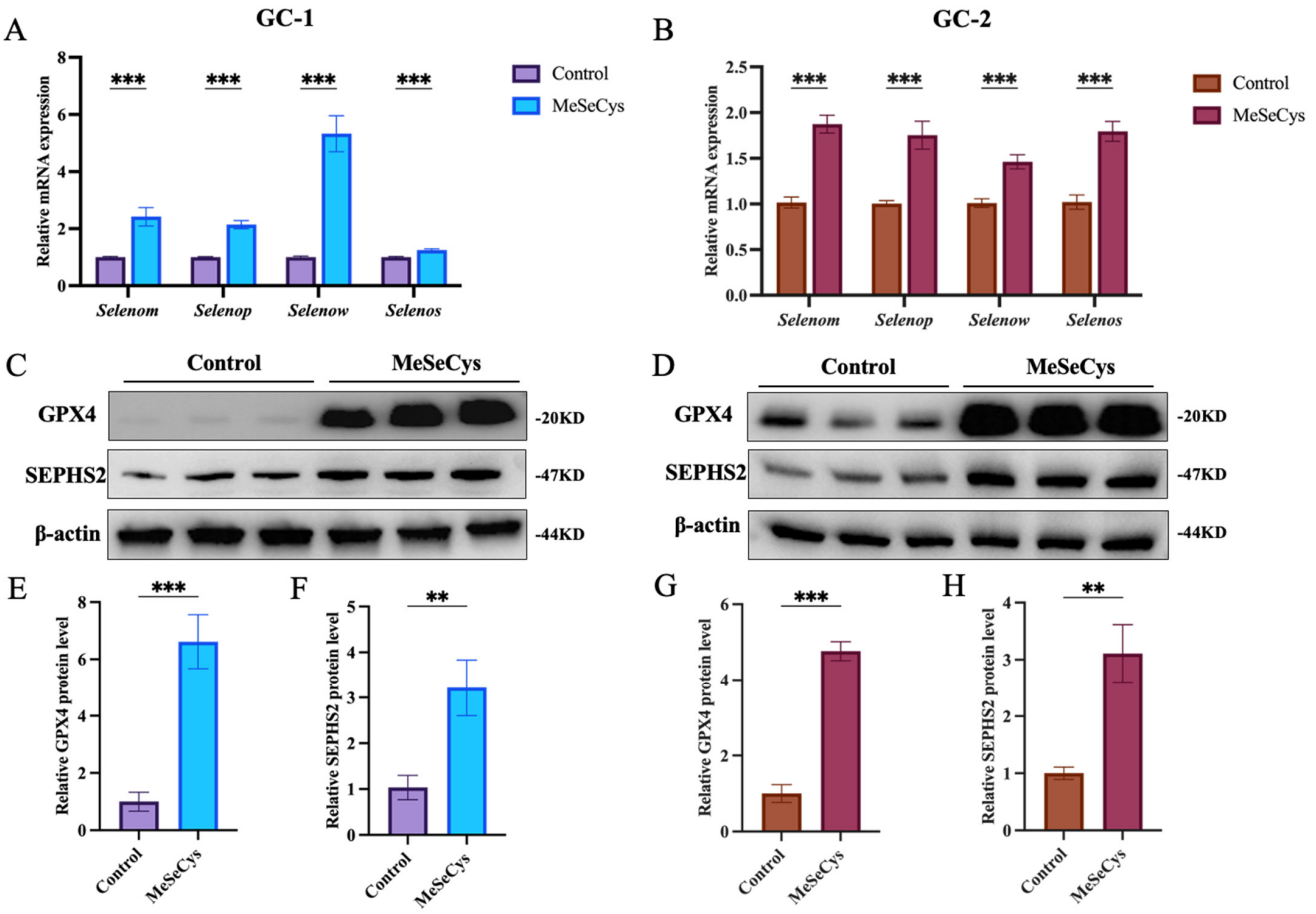

Figure 5. MeSeCys regulates selenocompound metabolism. The mRNA expression level of selenocompound metabolism-related genes in GC-1 (**A**) and GC-2 (**B**) cells. The protein expression level of GPX4 and SEPHS2 in GC-1 (**C**–**F**) and GC-2 (**D**–**H**) cells. Data are presented as mean ± SD (n = 3). Statistically significant differences are represented as ** $p < 0.01$; *** $p < 0.001$.

3.5.3. Glutathione Metabolism in Male Reproductive Cells

Previous results showed GPX4, a key selenoprotein involved in glutathione metabolism, was upregulated after MeSeCys treatment [19]. According to the RNA-Seq analysis results, it suggested that the enhancement of cell viability in GC-1 and GC-2 cells induced by MeSeCys was associated with the glutathione metabolic pathway. Subsequently, intracellular GSH levels, which are beneficial for maintaining normal germ cell function, were measured in the control group and MeSeCys group (Figure 6A,F). In GC-1 cells, the GSH levels increased from 0.46 mmol/10^4 cells to 0.64 mmol/10^4 cells, marking a 41.51% increase, whereas in GC-2 cells, GSH levels rose from 0.52 mmol/10^4 cells to 0.71 mmol/10^4 cells, indicating a 35.91% increase. Additionally, the expression of key genes in the glutathione signaling pathway was examined, revealing significant upregulation in the mRNA levels of *Slc3a2*, *Slc1a5*, *Gclc*, *Gclm*, *Gss*, *Gsta4*, *Gstm1*, and *Gstp1* (Figure 6B). Furthermore, the protein levels of key components in the glutathione signaling pathway were assessed, with GCLC and GCLM showing a significant increase in the MeSeCys group, and the upregulation of these two proteins was more pronounced in GC-1 cells. (Figure 6C–E,H–J). Moreover, BSO, a glutathione synthesis inhibitor, was found to counteract the MeSeCys-induced increase in GC-1 and GC-2 cell viability (Figure 6L), further highlighting the key role of the glutathione metabolism in the regulation of cell viability by MeSeCys in both cells.

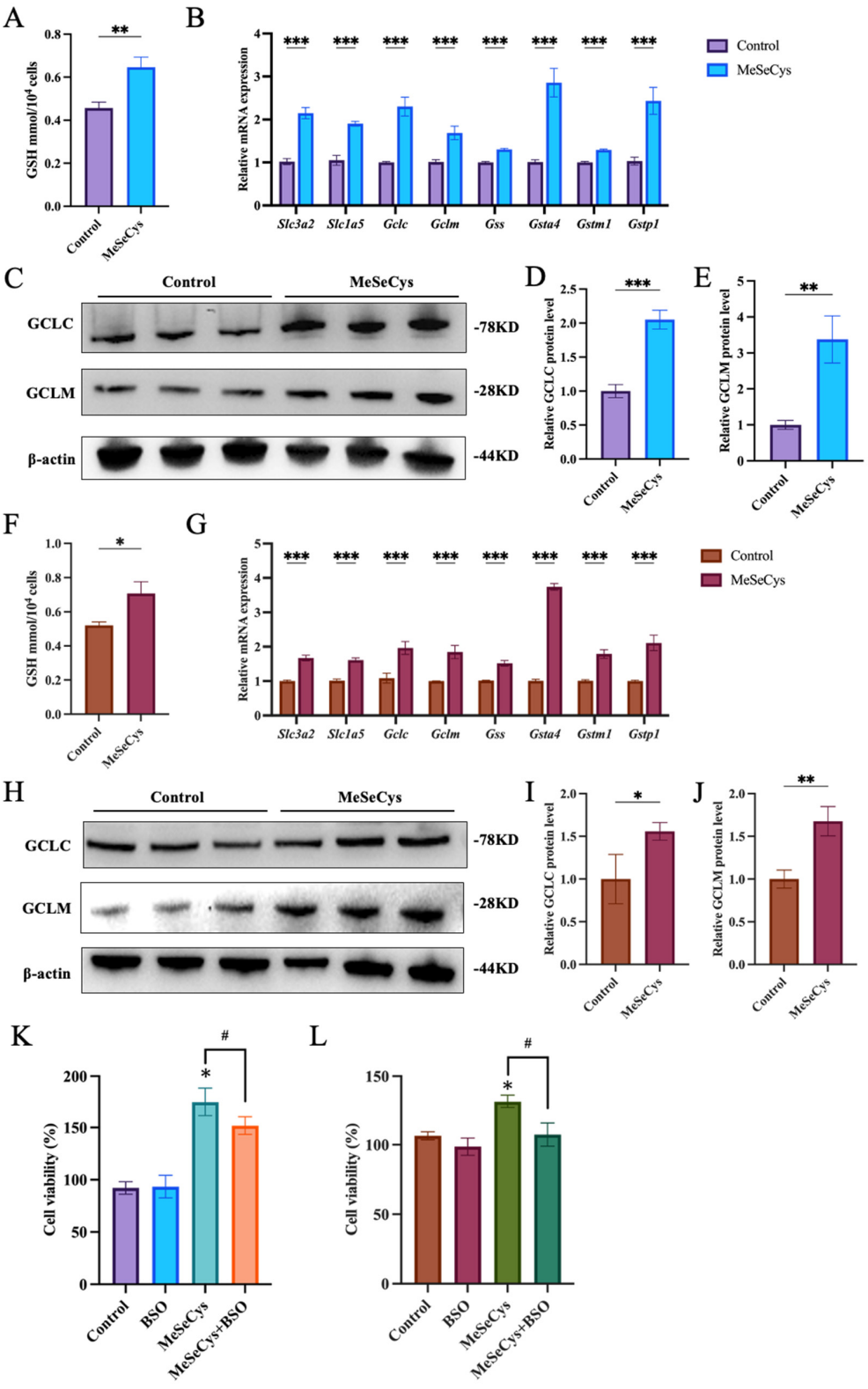

Figure 6. MeSeCys activates glutathione metabolism. Determination of intracellular GSH content in GC-1 (**A**) and GC-2 (**F**) cells. The mRNA expression level of glutathione metabolism-related genes (**B**,**G**). The protein expression level of GCLC and GCLM was determined by Western blotting (**C**–**E**,**H**–**J**). Cytotoxicity testing of the cells treated with MeSeCys alone or in combination (**K**,**L**). Data are presented as mean ± SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$.

3.5.4. MeSeCys Specifically Activates the mTOR Pathway in GC-1 Cells

Previous studies have demonstrated that the activation of the mTOR signaling pathway positively influences cell viability, especially in relation to cell proliferation and cell growth [34]. The GSEA results indicated that MeSeCys may selectively regulate the mTOR signaling pathway in GC-1 cells compared to GC-2 cells. (Figure 3C). Therefore, the potential regulation of the mTOR signaling pathway by MeSeCys in GC-1 cells and GC-1 cells was further examined. As depicted in Figure 7A, the mRNA expression levels of key genes within the mTOR signaling pathway, including *Atf4*, *Rps6*, and *Eif4ebp1*, were significantly up-regulated in GC-1 cells treated with MeSeCys.

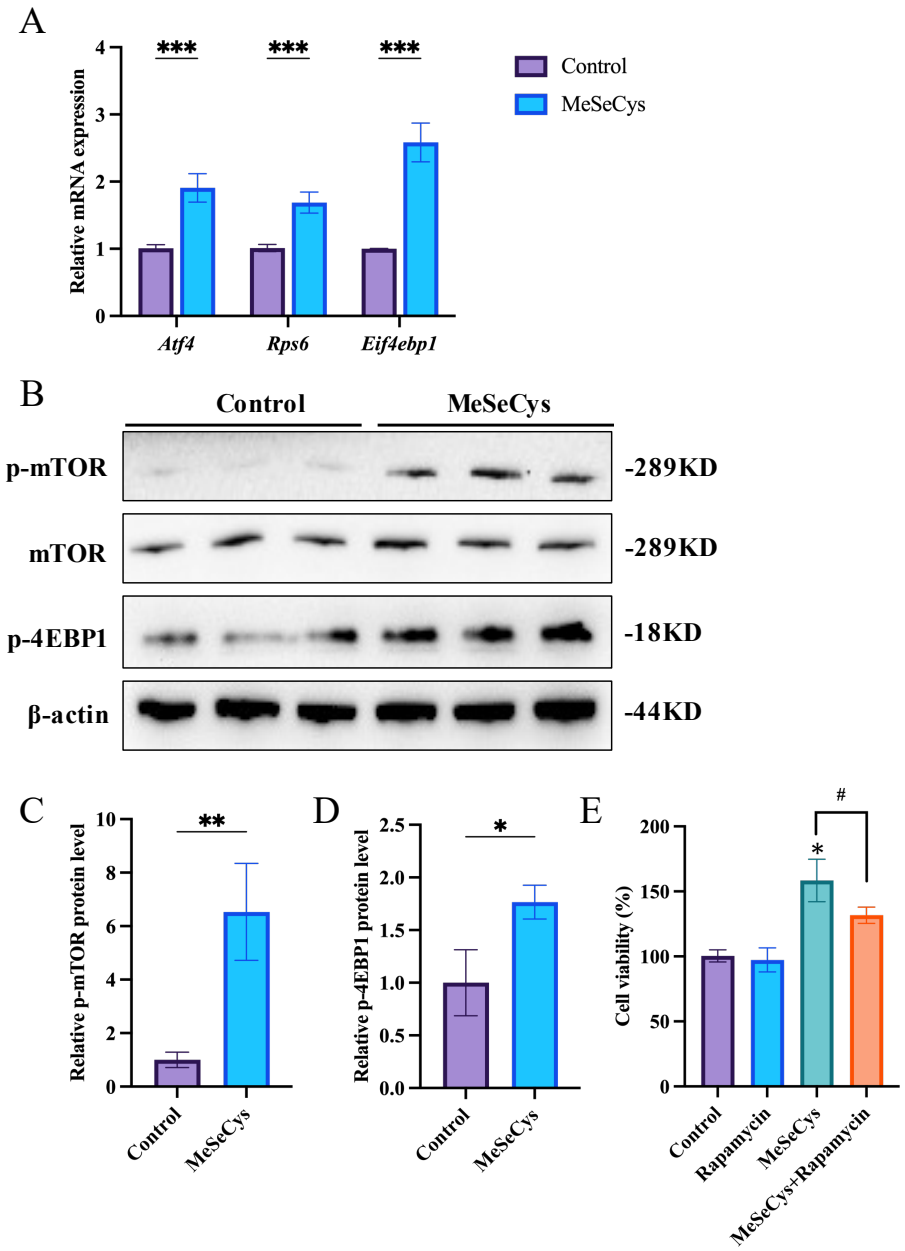

Figure 7. MeSeCys activates the mTOR pathway in GC-1 cells. The mRNA expression level of the mTOR signaling pathway-related genes (**A**). The protein expression level of phosphorylated mTOR, total mTOR, and p-4EBP1 (**B**–**D**). Cell viability after treatment with MeSeCys alone or in combination with rapamycin (**E**). Data are presented as mean ± SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$.

Subsequently, the key proteins associated with the mTOR signaling pathway were assessed. As a central activating molecule in the mTOR signaling pathway, p-mTOR (phosphorylated mTOR) is critical for transmitting upstream growth signals to downstream effectors, thereby regulating cell proliferation, metabolism, and survival [34]. As shown in Figure 7B–D, the results demonstrated that the ratios of p-mTOR to mTOR in GC-1 cells were significantly increased in the MeSeCys treatment group. Particularly, the ratios of p-mTOR to mTOR remained unchanged in GC-2 cells treated with MeSeCys (Figure S2), indicating that the mTOR signaling pathway is not activated by MeSeCys in GC-2 cells. Moreover, the expression level of p-4EBP1 protein was significantly higher in GC-1 cells treated with MeSeCys compared to the control group. Additionally, the introduction of rapamycin, an mTOR inhibitor, resulted in a significant decrease in cell viability, indicating that the mTOR signaling pathway was involved in the regulation of GC-1 cell viability by MeSeCys (Figure 7E), confirming the specific role of the mTOR pathway in GC-1 cells.

4. Discussion

Infertility is defined as the inability to conceive after 12 months of consistent and unprotected sexual intercourse [35]. This condition represents a significant global health concern, impacting 15% of couples, with male-related infertility being a contributing factor in nearly half of these instances [1]. Current research indicates that male infertility is closely related to oxidative stress, inflammation, and cell apoptosis [36]. Nutritional interventions, particularly Se supplementation, can enhance male reproduction. For instance, Se has been shown to ameliorate reproductive function in rats exposed to arsenic, an effect attributed to its ability to suppress inflammatory responses, oxidative stress, and caspase-3 activation [37]. In addition, in Cd-exposed rats, the combined Se and zinc treatment resulted in a more pronounced reduction in Cd levels in plasma and testes and enhanced protection by fully restoring sperm motility and the testicular antioxidant status [38]. Se is an essential nutrient for humans. In organisms, Se is metabolized into 25 known selenoproteins, which serve diverse biological functions, including antioxidant effects, anticancer activities, and the enhancement of fertility and reproduction [39]. MeSeCys, a major organic Se form in selenium-enriched crops, exhibits superior antioxidant capacity and bioavailability [40]. However, the direct positive effects of MeSeCys on the bioactivity of male reproductive cells remain unclear. This study aims to elucidate the effects of MeSeCys on GC-1 and GC-2 cells and elucidate the underlying molecular pathways.

It is well established that the bioactivity of Se is highly dependent on its chemical form. Previous research demonstrated that certain Se species, such as Na_2SeO_3 [7], OH-SeMet [8], and Se nanoparticles [9], can directly enhance male reproductive capacity by enhancing antioxidant status and reducing lipid peroxidation and apoptosis. Of the five typical Se forms (MeSeCys, $SeCys_2$, SeMet, SeCys, and Na_2SeO_3), MeSeCys exerted the most significant influence on improving the viability of GC-1 and GC-2 cells. In detail, the viability of GC-1 cells rose to 179.24%, and that of GC-2 cells increased to 126.81%. In addition, MeSeCys also demonstrates protective effects on the viability of cells exposed to H_2O_2 and stimulates cell proliferation, with a more pronounced positive effect on GC-1 cells. Over the past few decades, research on the benefits of MeSeCys has mainly focused on its antitumor properties, antioxidant capacity, anti-inflammatory actions, and improvement of cognitive deficits. Notably, this study, together with the previous research conducted by our research group [20,21], collectively reveals that MeSeCys also exerts a positive effect on improving the bioactivity of male reproductive cells.

Notably, after MeSeCys treatment, GC-1 cells showed higher viability than GC-2 cells. Thus, it is crucial to elucidate the mechanisms by which MeSeCys enhances GC-1 and GC-2 cell viability, including the common mechanism and the specific mechanism that accounts

for the higher increase in GC-1 cell viability. Subsequent RNA-Seq and bioinformatics analysis suggested that Se absorption, glutathione metabolism, biosynthesis of amino acids, as well as the mTOR signaling pathway, are involved in MeSeCys-mediated regulation of GC-1 cell viability. In addition, Se absorption and glutathione metabolism also likely contribute to GC-2 cell viability regulation, representing common pathways through which MeSeCys modulates mouse germ cell function. Given that amino acid biosynthesis is crucial for mTORC1 activation [41], thereby influencing cellular responses to growth factors and nutrients [42], it is speculated that the mTOR pathway may specifically regulate GC-1 cell viability. Thus, we will discuss the common and distinct pathways through which MeSeCys regulates the viability of these two cell lines separately.

4.1. Common Mechanisms in GC-1 and GC-2 Cells

4.1.1. Cellular Uptake via SLC7A11 and LRP8

Firstly, the uptake of MeSeCys by male reproductive cells was analyzed. ICP-MS analysis of intracellular Se content indicated that MeSeCys is effectively absorbed by cells. Notably, after 24 h of MeSeCys treatment, GC-1 cells had higher Se content than GC-2 cells, aligning with the earlier findings of more significant cell viability enhancement in GC-1 cells. Based on the previous studies, only two membrane proteins, SLC7A11 and LRP8, have been reported to mediate cellular Se uptake [32]. SLC7A11 serves as a dual-channel protein for the cellular uptake of selenite and cystine, which are substrates for glutathione metabolism. It transports selenite, which is reduced to selenide outside the cell, into the cell [43]. On the other hand, LRP8, a cell membrane receptor for the Se-transport protein SELENOP, mediates Se uptake via endocytosis. Studies have shown that when LRP8 is knocked out, selenocysteine enters cells through the SLC7A11 channel [44]. However, the cellular uptake pathway of MeSeCys remains unclear. Given the upregulated expression of *Slc7a11* and *Lrp8* mRNAs in the RNA-Seq and qRT-PCR results, it is speculated that they may be associated with MeSeCys cellular uptake. When SLC7A11 or LRP8 was knocked down, Se uptake by both cell lines decreased dramatically. Cell viability tests showed that SLC7A11 knockdown reduced MeSeCys-induced viability increases in GC-1 and GC-2 cells, while LRP8 knockdown had no such effect. Thus, SLC7A11 likely plays a key role in MeSeCys uptake and cell viability regulation. Moreover, when SLC7A11 expression in GC-1 and GC-2 cells was upregulated to 30–40 times, Se uptake by these cells significantly increased. This study first confirms that SLC7A11 is the key channel protein for MeSeCys to enter cells.

4.1.2. Regulation of Selenocompound Metabolism

Notably, once taken up by cells, Se predominantly exerts its biological effects through metabolic processes such as the synthesis of selenoproteins. Research has demonstrated that after MeSeCys is absorbed by cells, it undergoes further intracellular metabolism [45]. Hence, it was assumed that after MeSeCys entered the GC-1 and GC-2 cells, it might participate in the metabolism of intracellular selenocompound. qRT-PCR analysis revealed that several members of the testis-enriched selenoprotein family, including *Selenom*, *Selenop*, *Selenow*, and *Selenos*, were upregulated. These selenoproteins have key roles in various biological processes, such as Se metabolism, antioxidant defense, and maintaining organismal health [46]. Additionally, the protein levels of SEPHS2, which is directly involved in selenoprotein synthesis [47], and GPX4, a selenoenzyme crucial for cellular signaling and homeostasis, were significantly upregulated. Consequently, this study demonstrates that upon uptake by GC-1 and GC-2 cells, MeSeCys undergoes intracellular metabolism of selenocompounds.

4.1.3. Activation of Glutathione Metabolism

On the other hand, GPX4 is associated with glutathione metabolism and exhibits the highest activity and content in the testes, maintaining the structural integrity and normal function of cells [48,49]. Several studies have shown that MeSeCys can enhance GPX activity, thereby exerting a protective effect associated with glutathione metabolism in a variety of disease models [10,50]. Thereby, the high expression of GPX4 in GC-1 and GC-2 cells exposed to MeSeCys suggested that glutathione metabolism may be linked to enhanced cell viability. Subsequent investigations probed whether MeSeCys modulates GC-1 and GC-2 cell viability by influencing this glutathione metabolic equilibrium. The results showed that MeSeCys treatment elevated intracellular GSH levels in both cells. Specifically, in GC-1 cells, GSH levels rose from 0.46 to 0.64 mmol/10^4 cells, a 41.51% increase, compared to a 35.91% increase in GC-2 cells (from 0.52 to 0.71 mmol/10^4 cells). The more pronounced GSH increase in GC-1 cells matches their greater cell viability boost. The expression of membrane proteins *Slc3a2* and *Slc1a5* increased, which transport GSH biosynthesis precursors. Additionally, genes directly involved in GSH synthesis (*Gclc*, *Gclm*, *Gss*) and those involved in GSH's redox regulation (*Gsta4*, *Gstm1*, *Gstp1*) were also upregulated. Additionally, protein levels of GCLC and GCLM increased following MeSeCys treatment, and BSO, an inhibitor of glutathione synthesis, was found to counteract the MeSeCys-induced increase in the viability of GC-1 and GC-2 cells. These results indicate that MeSeCys, by supplying a highly bioavailable Se source, directly participates in the synthesis and activation of glutathione peroxidase, regulates the glutathione antioxidant metabolic network, and enhances the viability of male reproductive cells.

4.2. Cell-Specific Mechanism: mTOR Pathway Activation in GC-1 Cells

Furthermore, MeSeCys, a redox-active Se compound, exerts synergistic anticancer effects with sorafenib by activating the mTOR signaling pathway [51]. Consequently, the hypothesis that MeSeCys might specifically regulate GC-1 cell viability through the mTOR pathway was explored. Ribosomal protein s6 (*Rps6*), as well as eukaryotic translation initiation factor 4e-binding protein 1 (*Eif4ebp1*), were upregulated after MeSeCys treatment, which jointly participate in protein synthesis, cell growth, and cell proliferation [34]. Previous studies have shown that mTORC1 regulates intracellular glutathione levels by mediating the SLC7A11 through ATF4 to control cystine uptake [52], and *Atf4* mRNA expression was also upregulated by MeSeCys in GC-1 cells. Moreover, the p-mTOR/mTOR ratio and p-4EBP1 protein expression increased significantly in GC-1 cells. However, in GC-2 cells, no alteration in the p-mTOR/mTOR ratio was observed, indicating that the mTOR signaling pathway is specific to MeSeCys regulation of GC-1 cell viability. Also, cell viability decreased after adding rapamycin (an mTOR inhibitor), indicating that the mTOR signaling pathway plays a role in the MeSeCys-induced regulation of GC-1 cell viability. However, the above phenomenon was not observed in GC-2 cells, which is presumably due to the fact that GC-1 cells are at the mitotic and proliferative stage, whereas GC-2 cells are at the meiotic stage. As the mTOR signaling pathway serves as a central regulator of cell growth and proliferation, GC-1 cells may inherently exhibit a higher baseline sensitivity to mTOR signaling.

Based on the aforementioned evidence, it is hypothesized that MeSeCys primarily enhances the viability of male reproductive cells through the following pathways (Figure 8). Both GC-1 and GC-2 cells take up MeSeCys through the SLC7A11 or LRP8 channel. After entry, MeSeCys not only upregulates the synthesis of selenoproteins but also enhances GSH production via the glutathione metabolism pathway, thereby improving cell viability and protecting cells from H_2O_2-induced injury. Importantly, in GC-1 cells, MeSeCys specifically activates the mTOR pathway, which increases *Aft4* expression and further

regulates intracellular GSH content and redox balance, leading to a greater improvement in the viability of GC-1 cells compared to GC-2 cells. This study offers fresh views on the molecular mechanisms behind MeSeCys' benefits to male germ cells, emphasizing its positive impact on early spermatogenesis and highlighting its potential as a nutritional supplement for men's reproductive health.

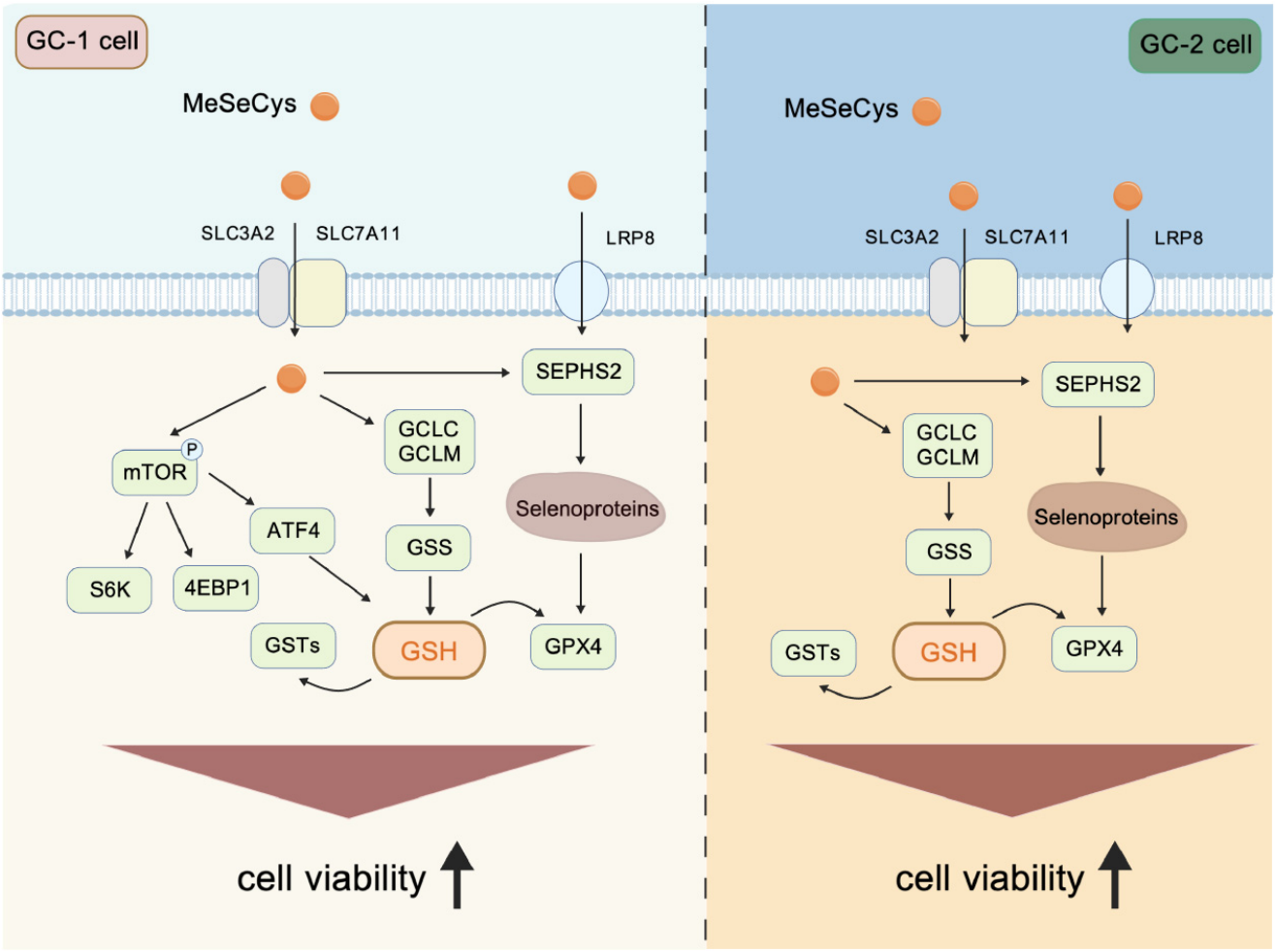

Figure 8. Potential mechanism of MeSeCys enhancing the viability of GC-1 and GC-2 cells. Black arrows indicate the direction of molecular signaling or metabolic pathways.

However, while this study has made some progress in exploring the benefits of MeSeCys to male germ cells, it also has certain limitations. For instance, future research should explore MeSeCys' effects on male reproduction at the single-cell level, which can reveal individual cell heterogeneity, unique gene expression, and cell-to-cell communication in cell populations, offering deeper insights into the complex mechanisms of MeSeCys in male reproduction. Additionally, although it was found that MeSeCys regulates the viability of reproductive cells through selenocompound metabolism, glutathione metabolism, and the mTOR signaling pathway, whether there are other underlying mechanisms remains an open question for future research. Moreover, while MeSeCys can promote the proliferation of normal spermatogenic cells, excessive proliferation may lead to abnormal spermatocyte development, thus necessitating strict optimization and control of the optimal concentration of MeSeCys. Furthermore, future studies should evaluate the efficacy and safe therapeutic concentrations of MeSeCys in animal models and clinical trials and investigate whether the uptake and signaling activation of MeSeCys act specifically on germ cells or also affect

other somatic tissues so as to clarify its potential translational value in the treatment of male infertility in humans.

5. Conclusions

This study provides strong evidence that MeSeCys significantly enhances cell viability and boosts the resistance to H_2O_2-induced oxidative damage in GC-1 and GC-2 cells, which serve as models for investigating male germ cell function. In this study, it was observed that in both cells, MeSeCys entered via the SLC7A11 or LRP8 transporter and functioned by upregulating selenocompound metabolism and activating glutathione metabolic pathways. Notably, in GC-1 cells, MeSeCys specifically regulated cell viability through the mTOR signaling pathway, further modulating intracellular redox balance. The findings from this in vitro experiment uncover the molecular mechanisms by which MeSeCys exerts beneficial effects on male germ cells and also point to its potential as a nutritional supplement for supporting male reproductive health, with further in vivo and clinical investigations required to validate these promising preliminary observations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox15020270/s1, Figure S1: qRT-PCR analysis of related genes in GC-1 and GC-2 cells treated with siRNAs or overexpression plasmids. Relative mRNA expression of GC-1 and GC-2 cells treated with SLC7A11 (**A**,**C**) or LRP8 (**B**,**D**) siRNAs. Relative mRNA expression of GC-1 and GC-2 cells treated with SLC7A11 overexpression plasmids (**E**,**F**). Figure S2: The mTOR signaling pathway is not activated by MeSeCys in GC-2 cells. The protein expression level of phosphorylated mTOR and total mTOR was determined by Western blotting (**A**,**B**). Table S1: Sequence of siRNAs. Table S2: Sequence of primers used in qRT-PCR. Table S3: GSEA analysis of GC-1 cells. Table S4: GSEA analysis of GC-2 cells.

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