Oxytocin receptor is a promising therapeutic target of malignant mesothelioma

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Abstract
Malignant mesothelioma (MM) is one of the most aggressive tumors. We conducted bioinformatics analysis using Cancer Cell Line Encyclopedia (CCLE) datasets to identify new molecular markers in MM. Overexpression of oxytocin receptor (OXTR), which is a G-protein–coupled receptor for the hormone and neurotransmitter oxytocin, mRNA was distinctively identified in MM cell lines. Therefore, we assessed the role of OXTR and its clinical relevance in MM. Kaplan-Meier and Cox regression analyses were applied to assess the association between overall survival and OXTR mRNA expression using The Cancer Genome Atlas (TCGA) datasets. The function of OXTR and the efficacy of its antagonists were investigated in vitro and in vivo using MM cell lines. Consistent with the findings from CCLE datasets analysis, OXTR mRNA expression was highly increased in MM tissues compared with other cancer types in the TCGA datasets, and MM cases with high OXTR expression showed poor overall survival. Moreover, OXTR knockdown dramatically decreased MM cell proliferation in cells with high OXTR expression via tumor cell cycle disturbance, whereas oxytocin treatment significantly increased MM cell growth. OXTR antagonists, which have high selectivity for OXTR, inhibited the growth of MM cell lines with high OXTR expression, and oral administration of the OXTR antagonist, cligosiban, significantly suppressed MM tumor progression in a xenograft model. Our findings suggest that OXTR plays a crucial role in MM cell proliferation and is a promising therapeutic target that may broaden potential therapeutic options and could be a prognostic biomarker of MM.

KEYWORDS
G1 phase cell cycle checkpoints, G-protein–coupled receptors, malignant mesothelioma, oxytocin receptor, oxytocin receptor antagonists

Abbreviations: BAP1, BRCA1-associated protein-1; CCLE, Cancer Cell Line Encyclopedia; CDKN2A, cyclin-dependent kinase inhibitor 2A; FCS, fetal calf serum; IL, interleukin; LATS2, large tumor suppressor kinase 2; MM, malignant mesothelioma; NF2, neurofibromin 2; OXT, oxytocin; OXTR, oxytocin receptor; qRT-PCR, quantitative real-time polymerase chain reaction; SETD2, SET domain containing 2; TCGA, The Cancer Genome Atlas; TP53, tumor protein p53; YAP1, Yes-associated protein 1.

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INTRODUCTION

Malignant mesothelioma (MM) arises from mesothelial cells and is a highly aggressive neoplasm that is usually associated with long-term exposure to asbestos.\(^2\) The number of global annual deaths has been approximately 40,000 people in recent years, and the incidence of MM is still increasing in Eastern Europe and some Asian countries where asbestos has not been banned or where bans have been delayed.\(^1,3\) Most patients are diagnosed at advanced stages due to nonspecific and late symptoms, and the median survival time is 10-15 months after diagnosis.\(^4,5\) MM is intrinsically resistant to cytotoxic chemotherapy, and the response rate to a standard chemotherapy of cisplatin/carboplatin and pemetrexed for MM is 18.6%-41.3%.\(^6,7\) In October 2020, the Food and Drug Administration approved the combined use of nivolumab plus ipilimumab as a first-line treatment for adult patients with unresectable MM. Its therapeutic effect has been recognized in some patients, and the median progression-free survival and overall response rate were 6.8 months and 40%, respectively.\(^8-10\)

Molecular genetic analyses have identified several fundamental genetic alterations that are responsible for the MM development. Primal genetic alterations have been characterized as frequent inactivators of tumor suppressors, such as cyclin-dependent kinase inhibitor 2A (CDKN2A), BRCA1-associated protein-1 (BAP1), and neurofibromin 2 (NF2).\(^11,12\) Copy number loss and recurrent somatic mutations in these genes are related to cell cycle abnormalities, histone oncomodifications, and disorder of multiple signaling cascades, including the Hippo signaling and mammalian target of rapamycin pathways.\(^13,14\)

We previously identified frequent inactivation of the component molecules of the Hippo signaling pathway that control organ size through regulation of cell proliferation and apoptosis.\(^15-17\) Dysfunction of the Hippo signaling pathway constitutively activates the transcriptional coactivator, Yes-associated protein 1 (YAP1), and promotes the expression of several target genes;\(^18,19\) however, no targeted therapies exploiting these genetic alterations have emerged. Therefore, it is essential to establish new promising therapeutic strategies against MM.

We analyzed genes that were highly expressed in MM cell lines compared with other types of cancer cell lines using the Cancer Cell Line Encyclopedia (CCLE) dataset to identify new therapeutic targets in MM. Oxytocin receptor (OXTR), which is a G-protein–coupled receptor, was identified as a gene that was markedly increased in MM cell lines. OXTR acts as a receptor for oxytocin (OXT), which is a peptide hormone released by the posterior pituitary that plays a role in milk secretion and uterine contraction during labor.\(^20\) An association between OXT-OXTR signaling and tumor development has been indicated in some cancer types.\(^21,22\) We further investigated the role of OXT-OXTR signaling in MM using knockdown experiments and OXTR antagonists, and cells at 10-15 passages were used for each assay.\(^15\) Five MM cell lines, including NCI-H28, NCI-H2052, NCI-H2373, NCI-H2452 and MSTO-211H, and MeT-5A (an immortalized mesothelial cell line), were purchased from the American Type Culture Collection. Cell line authentication of NCI-H2052, NCI-H2373, NCI-H2452, and MeT-5A cells was performed using short tandem repeat analysis. All MM cell lines and MeT-5A cells were maintained in RPMI-1640 culture medium (Sigma-Aldrich) containing 5% fetal calf serum (FCS) in an atmosphere of 5% CO\(_2\) and 95% air at 37°C. 293FT cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 5% FCS.

2.2 Short-hairpin RNA (shRNA) expression

For lentivirus production, 293FT cells were transfected with three plasmids: pMD2.G (Plasmid #12259; addgene), psPAX2 (Plasmid #12260; addgene), and shOXTR (using pLKO.1 puro [Plasmid #8453; addgene])/scramble shRNA (Plasmid #1864; addgene). Transfections were performed using X-tremeGENE HP DNA Transfection Reagent (#6366236001; Merck), and scrambled shRNA was used as control. The following shOXTR sequences were used: 5′-CCGGATCACGCTA GCTGTCTACATCCTCGAGGATGTAGACAGCTAGCGTGATTTTTTG-3′ (shOXTR #1), 5′-CCGGGTAAATCTCACTCCAGTATATTCTCGAGA ATATCTGGAGTGAAATTACTTTTG-3′ (shOXTR #1), and 5′-CCCGGACATCACCTTCGCTTCTACTCGAGTAGAAACCG GAAGGTGATGTCCTTTTTG-3′ (shOXTR #3). Three different shRNAs targeting OXTR were tested to minimize the possibility of off-target effects, and the two shRNAs with the most efficient knockdown (shOXTR #1 and #2) were selected for the experiments. Lentiviruses were harvested at 48 and 96 hours after transfection. The virus concentration protocol used was described elsewhere.\(^23-26\) Virus was frozen at ~80°C in appropriately sized aliquots for infection. Y-MESO-27, NCI-H2052, or NCI-H2373 cells were seeded on plastic dishes 24 hours prior to infection. When cells reached 30%-50% confluency, they were transduced with pLKO.1-control or pLKO1-OXTR-knockdown lentivirus in the presence of 8 µg/mL polybrene for 24 hours. The virus was removed after the infection and replaced with fresh cell culture medium. Transduced cells were selected using 2 µg/mL puromycin for 48 hours. Selected cells were then replated (Y-MESO-27 and NCI-H2052 at a density of 5.0 × 10^5 cells/well; NCI-H2373 at a density of 1.0 × 10^5 cells/well) in 100-mm dishes. After 36 hours, the cells were harvested for RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

2.3 In vivo xenograft mouse study

NCI-H2052 or NCI-H2373 cells were infected with lentiviral vectors encoding shOXTR #1 or scrambled shRNA. Six-to-eight-week-old female BALB/c nude (nu/nu) mice were purchased from Charles River Laboratories Japan, Inc. For subcutaneous tumor models, mice were injected in the left buttock with 1.5 × 10^6 shScramble or shOXTR
NCI-H2052/H2373 cells suspended in 200 μL of PBS. After 28 days, mice were euthanized with CO2, tumors were collected, and weights were measured.

To examine antitumor efficacy of cligosiban in vivo, 6- to 8-week-old female BALB/c nude (nu/nu) mice were injected in the left buttock with \(5.0 \times 10^6\) NCI-H2052 cells suspended in 200 μL of PBS. After subcutaneous administration, mice were randomized into two groups and treated with either vehicle (dimethyl sulfoxide) or 60 mg/kg cligosiban via oral administration every other day. One week after 10 doses of cligosiban, mice were euthanized with CO2, tumors were collected, and weights were measured.

All protocols for the mouse experiments were approved by the Nagoya University Institutional Animal Care and Use Committee.

### 2.4 Statistical analysis

Categorical variables were compared using Fisher’s exact test. Continuous variables were compared using the Mann-Whitney U test or unpaired t-test. Pearson’s correlation was applied to assess the linear association between two variables. Survival analysis was performed using an independent gene expression dataset of 85 MM cases from The Cancer Genome Atlas (TCGA; http://cancergeneme.nih.gov/) using Kaplan-Meier survival curves. Log-rank test was used to evaluate the statistical significance of differences of survival curves. Overall survival was defined as the time from the initial pathologic diagnosis to the date of death or last follow-up, at which point the data were censored. Cox proportional hazards model analyses were performed to adjust for covariates of statistical significance in the univariate analysis, including sex, age, histology, stage, and OXTR mRNA expression, and to estimate the relative hazard of mortality over the follow-up period. Statistical analyses were conducted using IBM SPSS version 27 software and JMP pro 15 software. A two-sided statistical significance level of 0.05 was used for all statistical analyses.

Detailed methods for transfection with siRNA, RNA isolation, qRT-PCR analysis, cell cycle analysis, cell proliferation analysis, colony formation assay, wound-healing assay, and drug sensitivity assay are described in the supplementary materials (Appendix S1).

## 3 RESULTS

### 3.1 OXTR is highly expressed in MM and associated with poor prognosis

We analyzed genes that were highly expressed in MM cell lines \((n = 11)\) compared with other types of cancer cell lines \((n = 1008)\) using the CCLE datasets. We examined the expression ratio of each gene in MM cell lines compared with other cancer cell lines and found that the ratio of OXTR was the highest in the CCLE datasets (Table S1). OXTR mRNA expression was significantly higher in MM cell lines than that in other cancer cell lines \((P < .001, \text{Mann-Whitney } U \text{ test})\) (Figure 1A) and was the highest in 25 tumor types (Figure 1B).

Moreover, we examined OXTR mRNA expression using the TCGA dataset to determine the clinical significance of OXTR expression in MM. The level of OXTR mRNA expression was significantly higher in MM tissue than in almost all normal tissues, except the mammary gland, and was the highest in a total of 37 tumor types, in accordance with the results of the CCLE data analysis (Figure S1A). OXTR mRNA expression in approximately 33.3% of MM cases was 100 times higher than that of the lowest case (Figure 1C). Therefore, we divided the 87 cases into three groups according to OXTR mRNA expression level: low \((n = 29)\), moderate \((n = 29)\), and high \((n = 29)\) groups. Clinical and molecular biological characteristics of the 87 MM cases from TCGA datasets are summarized in Figure 1C and Table S2. Although no statistical differences were found between clinical features (sex, age, histology, and stage) and OXTR mRNA expression, biphasic types were less common in the low-OXTR group, and both cases of sarcomatoid type belonged to the high-OXTR group (Figure 1C and Table S2).

Analysis of the molecular biological characteristics, which included frequently inactivated driver genes in MM such as BAP1, NF2, tumor protein p53 (TP53), large tumor suppressor kinase 2 (LATS2), and SET domain containing 2 (SETD2), revealed that OXTR mRNA expression was statistically higher in cases with NF2 inactivation than in those with intact NF2 (Figure 1C and Figure S1B). Kaplan-Meier analysis revealed that the overall survival curves were clearly divided according to OXTR expression levels, and high expression was significantly associated with worse overall survival \((P < .001, \text{log-rank test})\) (Figure 1D). Univariate Cox regression analysis revealed that histological classification and OXTR mRNA expression were statistically associated with overall survival, and multivariable Cox regression analysis indicated that OXTR mRNA expression was the strongest independent predictor of overall survival (hazard ratio for death, 2.2; 95% confidence interval, 1.60-3.07; \(P < .001\)) (Table 1).

### 3.2 Analysis of OXTR function in MM

We analyzed OXTR mRNA expression across our 14 MM cell lines compared with MeT-5A by qRT-PCR, and found that the expression level was >30 times higher in seven out of 14 MM cell lines compared with MeT-5A cells (Figure 2A). Therefore, we classified the seven MM cell lines as the high-OXTR group and the other seven as the low-OXTR group. We previously analyzed frequently inactivated driver genes in MM cell lines, including BAP1, NF2, TP53, and LATS2, and found that most of the MM cell lines with NF2 inactivation were in the OXTR-high group, while other alterations of driver genes were not associated with OXTR expression levels (Table S3). These results were consistent with the findings from the analysis of TCGA dataset (Figure S1B). To investigate whether NF2 affects OXTR expression, we conducted NF2 knockout using two NF2-intact MM
Multivariable analysis explored the functional relevance of the negative regulation of two to three times higher cell lines with low expression. Additional OXT treatment moderately increased proliferation of MM cell lines. First, we conducted an OXT administration experiment on MM cell lines to determine whether OXT-OXTR signaling was involved in MM cell proliferation. We conducted lentiviral shRNA knockdown in the three MM cell lines with high OXTR expression, whereas no significant difference was noted in MeT-5A and NCI-H2452 cells compared with the siRNA control. These results indicated that OXT-OXTR signaling promotes proliferation of MM cell lines with high OXTR expression. Notably, OXTR knockdown significantly reduced proliferation in the three MM cell lines with high OXTR expression, whereas no significant difference was noted in MeT-5A and NCI-H2452 cells compared with the siRNA control. These results indicated that OXT-OXTR signaling promotes proliferation of MM cell lines with high OXTR expression.

### TABLE 1 Univariate and multivariable analysis of overall survival in MM cases from TCGA datasets (n = 85)

| Variable                          | Univariate analysis | Multivariable analysis |
|----------------------------------|---------------------|------------------------|
|                                  | HR  | 95% CI  | P   | HR  | 95% CI  | P   |
| Sex                              |     |         |     |     |         |     |
| Female                           | Reference |      |     | Reference |      |     |
| Male                             | 0.89 | 0.49-1.59 | 0.689 | 0.92 | 0.48-1.77 | 0.801 |
| Age                              |     |         |     |     |         |     |
| ≤65 years                        | Reference |      |     | Reference |      |     |
| >65 years                        | 1.39 | 0.87-2.23 | 0.171 | 1.13 | 0.67-1.92 | 0.641 |
| Histology                        |     |         |     |     |         |     |
| Epithelioid                      | Reference |      |     | Reference |      |     |
| Sarcomatoid or biphasic          | 2.03 | 1.21-3.41 | 0.008 | 2.03 | 1.13-3.66 | 0.018 |
| Stage                            |     |         |     |     |         |     |
| I                                |     |         |     |     |         |     |
| II                               | 0.63 | 0.26-1.51 | 0.301 | 0.99 | 0.40-2.44 | 0.979 |
| III                              | 0.76 | 0.36-1.59 | 0.462 | 0.88 | 0.39-1.95 | 0.747 |
| IV                               | 0.71 | 0.30-1.67 | 0.429 | 0.88 | 0.34-2.33 | 0.804 |
| OXTR mRNA expression<sup>a</sup> | 2.25 | 1.66-3.05 | <0.001 | 2.2 | 1.60-3.07 | <0.001 |

Abbreviations: CI, confidence interval; HR, hazard ratio; MM, malignant mesothelioma; OXTR, oxytocin receptor; TCGA, The Cancer Genome Atlas.

<sup>a</sup>Two cases with unknown prognosis were excluded from 87 MM cases of TCGA datasets.

<sup>b</sup>OXTR mRNA was converted by log<sub>2</sub> and used for analysis as continuous variables.

<sup>c</sup>P-values were calculated by Wald test.

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Cell lines with low OXTR expression (Y-MESO-30 and NCI-H2452). Both MM cell lines transfected with NF2 knockdown siRNA showed two to three times higher OXTR expression than those transfected with siRNA control (Figure 2B), indicating that NF2 is involved in the negative regulation of OXTR expression in MM. Next, we explored the functional relevance of OXTR in MM cell lines. First, we conducted an OXT administration experiment on MM cell lines to determine whether OXT-OXTR signaling was involved in MM cell proliferation. Additional OXT treatment moderately increased proliferation of three MM cell lines with high OXTR expression (NCI-H2052, Y-MESO-27, and NCI-H2373), but no significant difference was found in the proliferation of MeT-5A cells and one MM cell line with low OXTR expression (NCI-H2452) (Figure 2C). Then, we conducted transient OXTR knockdown with synthetic oligonucleotides and obtained efficient knockdown in these cell lines (Figure 2D).
OXTR knockdown increased the $G_1$ phase fraction and decreased the $G_2/M$ phase fraction in the three MM cell lines, indicating that OXTR downregulation disturbed tumor cell cycle mainly due to $G_1$ cell cycle arrest (Figure 3B). In addition, OXTR knockdown increased the sub-$G_1$ fraction in the three MM cell lines (Figure 3B). Based on the results of the cell cycle analyses, we investigated the expression levels of representative cell cycle–related genes, such as cyclins and cyclin-dependent kinases. In the three MM cell
The oxytocin–oxytocin receptor (OXT–OXTR) signaling promotes proliferation of malignant mesothelioma (MM) cell lines with high OXT expression. A, qRT-PCR analysis of OXTR in 14 MM cell lines and MeT-5A (immortalized mesothelial cell line). OXTR mRNA expression of MeT-5A was used as a control (=1.0). GAPDH expression was used as a control. B, Neurofibromin 2 (NF2) knockdown using siRNA in Y-MESO-30 and NCI-H2452 was confirmed by qRT-PCR analysis. A significant increase in OXTR expression was observed in MM cell lines with NF2 intact (Y-MESO-30 and NCI-H2452) treated with NF2 siRNA compared with the siRNA control. C, Effect of OXT on cell proliferation in MeT-5A and four MM cell lines (NCI-H2452, NCI-H2052, Y-MESO-27, and NCI-H2373). Three days after treatment with 1 nM OXT, cell proliferation was analyzed in MM cell lines and MeT-5A cells by WST-1 colorimetric assay. D, OXTR knockdown using siRNA in MeT-5A and five MM cell lines (NCI-H2452, NCI-H2052, Y-MESO-27, and NCI-H2373). A significant reduction in cell proliferation was observed in MM cell lines with high OXTR expression (NCI-H2052, Y-MESO-27, and NCI-H2373) treated with OXTR siRNA. Data represent mean and standard deviation of triplicate samples from a representative experiment. All P-values were calculated using two-sided unpaired t-test.

OXTR knockdown significantly reduced the expression levels of CCNE2, CDK2, CDK1, and AURKA, but did not decrease those of CCNE1 (Figure 3C), suggesting that CCNE2, CDK2, and CDK1 downregulation induced G1 cell cycle arrest, and CDK1 and AURKA downregulations were associated with decreased G2/M phase fraction.28-30 Furthermore, we found a weak positive correlation between CCNE2 or CDK2 and OXTR mRNA expression and moderate positive correlations between CDK1 or AURKA and OXTR mRNA expression in 87 MM cases in the TCGA dataset (Figure 3D). These results indicate that downstream signals from OXTR could promote MM cell proliferation through cell cycle regulation. Moreover, we investigated the role of OXTR in MM colony formation ability and found that OXTR knockdown significantly suppressed colony formation in cell lines with high OXTR expression (Figure 4A). In addition, wound-healing assay showed that OXTR knockdown significantly decreased the migration of MM cells with high OXTR expression (Figure 4B), suggesting that increased OXTR expression in MM could facilitate tumor progression. To assess the suppressive effect involved in MM progression by OXTR knockdown in vivo, we injected NCI-H2052/NCI-H2373 cells transfected with shOXTR lentivirus or empty lentivirus into the subcutaneous space of nude mice. Four weeks after transplantation, the tumor weights of both OXTR knockdown groups were significantly reduced compared with the control groups (NCI-H2052: 37.0 vs. 14.0 mg, P = .001; NCI-H2373: 63.3 vs. 14.3 mg, P = .004) (Figure 4C). These results indicate that OXTR knockdown dramatically suppresses MM tumor progression and that OXTR could be a new promising therapeutic target in MM.

3.3 | OXTR antagonists inhibit MM progression

Our results led us to hypothesize that OXTR antagonists could have antitumor effects against MM cells with high OXTR expression. We used four OXTR antagonists (Table S4) to examine whether they could suppress MM cell proliferation in NCI-H2052, Y-MESO-27, and NCI-H2373 MM cell lines with high OXTR expression. All four OXTR antagonists showed inhibitory effects against MM cell proliferation (Figure 5A). Interestingly, the IC50 of the antagonists for the three MM cell lines showed a tendency toward a positive correlation with their inhibition constant (Ki) for OXTR (Figure 5A and Table S4). We selected two OXTR antagonists, atosiban (Ki for OXTR = 397 nM) and cligosiban (Ki for OXTR = 9.5 nM), to further investigate the cytostatic activity of OXTR antagonists. The IC50s of both OXTR antagonists were analyzed in MeT-5A cells, five MM cell lines with low OXTR expression, and five MM cell lines with high OXTR expression. As expected, cligosiban showed stronger cytostatic activity for MM cell lines than atosiban, and the average IC50 for the five MM cell lines with high OXTR expression was significantly lower than that of the five MM cell lines with low OXTR expression (30.2 vs. 63.1 μM; P = .008, Mann-Whitney U test) (Figure S3A, B, and Figure 5B). In addition, the IC50 of cligosiban was the highest for MeT-5A compared with all MM cell lines (Figure 5B). We examined the effect of cligosiban combined with cisplatin (DDDP), which is a standard chemotherapy agent in MM treatment. Cligosiban showed significant additive effects in combination with CDDP in the three MM cell lines with high OXTR expression, namely NCI-H2052, Y-MESO-27, and NCI-H2373 (Figure 5C). The clinical efficacy of cligosiban with oral administration was investigated in some clinical trials for men with premature ejaculation, and the rate of serious adverse events was extremely low.31,32 Therefore, we further investigated the antitumor effects of orally administered cligosiban on MM using a xenograft model of NCI-H2052 (Figure 5D). Interestingly, the tumor weights after 10 doses of orally administered cligosiban were significantly reduced compared with those of the control group (29.0 vs. 56.8 mg; P = .021) (Figure 5E). Additionally, to determine whether cligosiban affected OXTR expression in MM cells, we repeated the experiment and found that there was no difference of OXTR expression between the treated and control groups (Figure S4A and B), suggesting that the antitumor effect of cligosiban is mainly exerted through the blockade of OXT to OXTR, but the OXTR expression is not affected. These results indicate that OXTR antagonists, which have high selectivity for OXTR, inhibit MM tumor progression and are new promising antitumor agents.

4 | DISCUSSION

To the best of our knowledge, this is the first study to show that OXTR expression is remarkably upregulated in MM compared with other tumor types and cases with high expression have worse clinical outcomes. OXTR knockdown dramatically suppressed cell
Oxytocin receptor (OXTR) knockdown disturbs tumor cell cycle in malignant mesothelioma (MM) cell lines with high OXTR expression. A, Cell proliferation analyses in MM cell lines with high OXTR expression (NCI-H2052, Y-MESO-27, and NCI-H2373) transfected with OXTR-knockdown lentivirus or control. *P < .05 (shOXTR vs. control). B, Flow cytometric analyses in NCI-H2052, Y-MESO-27, and NCI-H2373 cells 5 d after transfection with OXTR-knockdown lentivirus or control. C, qRT-PCR analyses of cell cycle–related genes in three MM cell lines after infection with OXTR-knockdown lentivirus or control. GAPDH expression was used as a control. D, Correlation of mRNA expression levels of OXTR and cell cycle–related genes (CDK1, CDK2, CCNE1, CCNE2, and AURKA) in 87 MM tissues from The Cancer Genome Atlas (TCGA) datasets. Pearson correlation coefficient and P-values were calculated. All statistical tests were two-sided. A and C, Data represent mean and standard deviation of triplicate samples from a representative experiment. All P-values were calculated using two-sided unpaired t-test.

In normal tissue, OXTR is predominantly expressed in the mammary gland and uterine myometrium at the end of pregnancy and plays important roles in the induction of milk ejection and uterine contraction. OxTR is also present in the central nervous system and modulates various behaviors. Thus, OXT secretion levels are known to be important in men and women. At the end of pregnancy, OXT expression is strikingly increased in the uterine myometrium for contraction, while OXT secretion levels do not significantly change. Similarly, our analysis showed that OXT expression was significantly increased in MM, while endogenous OXT expression in MM was not higher than that in other cancer types (Figure SSA–C). Thus, we hypothesized that OXT upregulation plays crucial roles in MM progression without increasing OXT secretion. Several studies have reported that OXT was associated with tumor progression in some cancer types, such as small cell lung carcinoma and prostate cancer. However, the molecular mechanism of OXTR downstream in tumor promotion is not well understood. We showed that OXTR knockdown significantly suppressed proliferation of MM cell lines through an increase in the G1 phase of the cell cycle. Furthermore, cell cycle arrest was caused by decreased expression levels of cyclin, cyclin-dependent kinases, and serine/threonine protein kinases, such as CCNE2, CDK1, CDK2, and AURKA. In particular, CDK1 and AURKA were previously reported as potential therapeutic target genes in MM. To further support this, expression levels of the four genes were positively correlated with OXTR expression in the TCGA dataset. These results indicate that OXTR upregulation promotes MM progression through cell cycle acceleration, although further studies are required to identify the mechanism involved in the downregulation of the four genes caused by OXTR knockdown.

In this study, OXTR antagonists were shown to have antitumor effects against MM cells with high OXTR expression, although the
(A) Absorbance of WST-1 (Relative to 0 μM) for Cligosiban, OT-R antagonist 1, L368,899 hydrochloride, and Atosiban.

(B) IC50 (Cligosiban) and MeT-SA for different cell lines.

(C) Absorbance of WST-1 (Relative to siControl) for Control, CDDP, Cligosiban, CDDP+Cligosiban, CDDP, Cligosiban, CDDP+Cligosiban, and Control, CDDP, Cligosiban, CDDP+Cligosiban, CDDP, Cligosiban, CDDP+Cligosiban for NCI-H2052, Y-MESO-27, and NCI-H2373.

(D) BALB/c- nu mice were inoculated subcutaneously with 5.0 x 10^6 cells, administered 60 mg/kg cligosiban every other day (10 times in total), and sacrificed after 1 week.

(E) Tumor weight (mg) comparison between control and cligosiban.
IC$_{50}$ values of these drugs were relatively high. Atosiban has been clinically used in European countries as a treatment for preterm birth, and its antitumor effects were reported in the human prostate cancer cell line DU145 although the selectivity for OXTR was relatively lower than the other antagonists (Table S4). Our analysis showed that the IC$_{50}$ values of OXTR antagonists for MM cell lines tended to positively correlate with the Ki for OXTR. OXTR is known to be structurally similar to vasopressin receptors; therefore, the selectivity for OXTR could be an important factor to use OXTR antagonists as antitumor agents. Developing new agents with a high selectivity for OXTR is essential to obtain OXTR antagonists with high antitumor effects against MM. However, the selectivity of cligosiban for OXTR is ~100 times greater than for the vasopressin receptor, and the Ki is less than one-fortieth that of atosiban (Table S4). A phase Ib, randomized clinical trial using cligosiban was recently conducted in men with premature ejaculation and reported extremely low rate of adverse events. Therefore, it may be possible to further increase the dose when we will clinically use cligosiban as an antitumor agent against MM. In our xenograft model, a total of 10 doses of cligosiban (60 mg/kg) was administered every other day via oral administration (control group, n = 4; cligosiban group: n = 4). DMSO or cligosiban were administered 10 times in total. E. Tumor weights were measured 1 wk after the last administration of DMSO or cligosiban. Horizontal lines indicate mean and standard deviation. C and E, All P-values were calculated by two-sided unpaired t-test.

In conclusion, we identified high OXTR expression in MM, which was associated with poor overall survival. OXTR knockdown and administration of OXTR antagonists in vitro and in vivo experiments showed significant suppression of MM cell proliferation. These results indicate that OXTR could be a promising therapeutic target and a prognostic biomarker, enabling a personalized approach to treatment of MM.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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