IGF2 preserves osteosarcoma cell survival by creating an autophagic state of dormancy that protects cells against chemotherapeutic stress

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Abstract

Osteosarcoma (OS) is a malignant bone tumor in children and adolescents characterized by intrinsic therapeutic resistance. The insulin-like growth factor IGF2 is expressed at elevated levels in OS after treatment with chemotherapy, prompting an examination of its functional contributions to resistance. We found that continuous exposure to IGF2 or insulin in the absence of serum created a dormant growth state in OS cells that conferred resistance to various chemotherapeutic drugs in vitro. Mechanistic investigations revealed that this dormant state correlated with downregulation of downstream signaling by the IGF1 receptor, heightened cell survival, enhanced autophagy and the presence of extracellular glutamine. Notably, inhibiting autophagy or depleting glutamine was sufficient to increase chemotherapeutic sensitivity in OS xenografts in mice. Clinically, we confirmed that IGF expression levels were elevated in human OS specimens from patients who received chemotherapy. Together, our results suggest that activation of IGF or insulin signaling preserves the survival of OS cells under chemotherapeutic stress, providing a drug resistant population that may engender minimal residual disease. Attenuating this survival mechanism may help overcome therapeutic resistance in OS.
Introduction

Osteosarcoma (OS) is the most common nonhematogenous malignant bone tumor in children and adolescents. Death from OS is largely attributable to the unchecked survival of a chemoresistant cells and clinical outcomes have not changed substantially over the past 20 years (1,2,3). Characterization of the mechanisms of such chemoresistance will thus be a key to the development of novel therapeutic options.

The emergence of drug resistant cancer cells has been attributed to stochastic events mostly associated with genetic mutations (4, 5). However, the mutation-based mechanism is thought to occur over a relatively long time period and may therefore not account for the rapid emergence of latent drug-tolerant cells. Instead, nonmutational alterations after the onset of therapy induced by the modified microenvironment also can give rise to a drug-tolerant state in cancer cells (4, 6, 7). Also importantly, resistant subpopulations of cancer cells, possibly identical to cancer stem cells, may initially be present within tumors, and their subsequent enrichment by therapy may be responsible for the recurrence of disease (8–10). Since pre-existing resistant subpopulations, such as cancer stem cells, have not yet been fully ascertained in OS, elucidation of the molecular mechanisms underlying such therapy-induced alterations in cancer will be important for the development of new treatments.

We recently developed a mouse model of OS based on the overexpression of c-MYC in bone marrow stromal cells derived from Ink4a/Arf knockout mice. Injection of highly tumorigenic cells (designated AXT cells) into syngeneic mice results in the development of lethal OS with metastatic lesions that mimics human osteoblastic OS (11). We previously showed that cellular heterogeneity as well as therapeutic resistance in this model can be brought about by soluble factors released from the tumor microenvironment (12).

In the present study we identified insulin-like growth factor 2 (IGF2) as a soluble factor whose gene was expressed at an increased level in OS tumors after chemotherapy. Despite its function as a growth factor, IGF2 ensured cell survival with arresting cell cycle and conferred a drug-tolerant state in OS cells. These effects of IGF2 were mimicked by insulin. Our findings implicate IGF2 and possibly insulin signaling in the therapy resistance of OS.

Materials and Methods

Human OS samples

OS tissues were obtained from each patient at the time of biopsy (T1) and surgical resection after neoadjuvant chemotherapy (T2). All patients received basically same chemotherapy, and all samples were approved for analysis by the ethics committee of the

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Faculty of Medicine, Kyoto University. Characteristics of each patient in detail are described in Table 1.

Cell culture
AXT cells and human SAOS2 and U2OS (American Type Culture Collection (ATCC), Manassas, VA) were maintained in IMDM (Life Technologies, Carlsbad, CA) supplemented with 10% FBS. For continuous exposure to mouse or human IGF2 (R&D Systems, Minneapolis, MN) or human insulin (Sigma-Aldrich, St. Louis, MO), cells were incubated overnight in serum-containing medium, washed with serum-free medium and cultured in DMEM containing high glucose supplemented with 2 mM GlutaMAX (Life Technologies) and either 50 ng/ml IGF2 or 50 nM insulin. Medium was replaced three times a week, and the cells were subjected to analyses at 12 h after a medium change unless indicated otherwise.

Cell proliferation assay
Cell viability was measured with Cell Titer Glo assay kit (Promega, Madison, WI). The detailed experimental scheme is shown in Supplementary Materials and Methods and Supplementary Fig. S1D.

Tumor xenograft model
All animal care and procedures were performed in accordance with the guidelines of Hoshi University and Keio University. For establishment of tumor xenografts, AXT cells (1 × 10⁶) were suspended in PBS and injected subcutaneously into 8-week-old female C57BL/6 mice (SLC, Shizuoka, Japan) on day 0. The schedule of administration of drugs is described in Supplementary Materials and Methods.

Reverse transcription (RT)-PCR
For patient samples total RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA) and treated with a DNase-one kit (QIAGEN) to remove genomic DNA. RT reaction was performed using 1 to 2 μg of total RNA with the SuperScript III first-strand synthesis system (Life Technologies) according to the manufacturer’s instructions. Real-time PCR was performed in triplicate using SYBR GREEN reagent (Applied Biosystems, Forester City, CA, USA). cDNA from human liver was obtained from Human MTC PanelI (Clontech, Mountain view, CA). HOS cells were obtained from ATCC. The abundance of target mRNAs was determined relative to that of ACTB mRNA. For mouse samples total RNA extraction, reverse transcription and real-time PCR analysis was performed as described previously (12). Sets of primers for real-time PCR are listed in Supplementary
RNA interference
AXT cells were subjected to reverse transfection for 24 h under serum-containing IMDM with Silencer Select siRNAs for Atg7 (s92536 or s92538; siRNAs 1 and 2, respectively) or with a control siRNA (Life Technologies), each at a concentration of 50 nM. The detailed experimental scheme is shown in Supplementary Materials and Methods and Supplementary Fig. S1E.

Flow cytometry
Cells were fixed for 1 day with ice-cold 70% ethanol, washed with PBS, and incubated for 1 h on ice with Alexa Fluor 647–conjugated Ki67 antibody (Cell Signaling Technology, Danvers, MA). Then cells were washed and suspended in PBS containing propidium iodide at 10 µg/ml and RNase A (Sigma-Aldrich). DNA damage was evaluated using Alexa Fluor 647–conjugated γH2AX antibody and an isotype control antibody according to the manufacturer’s instructions (Cell Signaling Technology). At least 10,000 live cells were analyzed by FACS Verse (BD Biosciences, San Jose, CA). Data were analyzed with Flowjo software (Tree Star, Ashland, OR).

Time-lapse imaging
AXT cells were cultured either in serum- or IGF2-containing medium for one or 8 days before analysis, respectively. Cells incubated in the IGF2-containing medium were also exposed to serum beginning at 17.75 h after the onset of analysis. Images were obtained as previously described (13).

Immunoblot analysis
Cells were lysed with Laemmli sample buffer (Bio-Rad, Hercules, CA). For preparation of a tumor homogenate, small fragments were suspended in the same buffer and disrupted using Biomasher (Nippi, Tokyo, Japan) and ultrasonic treatment. Immunoblot analysis was performed as previously described (11, 12). Actin or α-Tubulin was examined as a loading control.

Transmission electron microscopy
Sample preparation and acquisition of images was performed as described elsewhere (14).

Immunostaining
Immunohistochemical analysis was performed as previously described (11, 12) using
antibodies to GFP (Santa Cruz Biotechnology, Santa Cruz, CA) and LC3 (MBL, Nagoya, Japan). For immunofluorescence staining, cells were fixed with ice-cold acetone for 5 min for Ki67 and phospho-histone H2AX or 4% paraformaldehyde for 15 min for phospho-histone H3, respectively, washed three times with PBS, exposed to PBS containing 3% bovine serum albumin for 1 hour, and stained with antibodies to Ki67, phospho-histone H2AX or phospho-histone H3 (Cell Signaling Technology). Alexa Fluor 488, 555 or 594–conjugated secondary antibodies were purchased from Life Technologies. Nuclei were stained with TOTO3 (Life Technologies) or 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Stained cells were observed with a confocal microscope (LSM510; Zeiss, Göttingen, Germany) or a fluorescence microscope.

**Measurement of the activity of Glutathione S-transferases (Gst)**

Gst activity was measured by Gst activity assay kit (Abcam, Cambridge, UK). Values were divided by each cell number and the activity per cell was calculated.

**Statistical analysis**

All assays were performed at least in triplicate and data are presented as means ± SD and analyzed by Student’s t test unless indicated otherwise.

**Results**

**IGF2 ensures survival of OS cells in the absence of serum**

Environmental soluble factors are known to alter the characteristics of cells. To clarify the mechanisms underlying emergence of chemoresistance of OS, the expression of various soluble factors was examined. After exclusion of highly expressed molecules without the treatment of chemotherapeutic agents, which were previously described in ref. (12), we found IGF2 as one of the candidates whose mRNA abundance was originally low but significantly upregulated after chemotherapy. The amount of Igf1 mRNA was unaffected (Fig. 1A, Supplementary Fig. S1A). This effect was even more pronounced in tumor cells sorted by GFP which was overexpressed in AXT cells but not observed in GFP-negative non-tumor cells. Serum IGF2 level was significantly increased in tumor bearing mice and further slight increase was induced by chemotherapy (Supplementary Fig. S1B). On the basis of these findings, we investigated the role of IGF2 in OS cells.

We first examined the effect of IGF2 on cell proliferation. IGF2 slightly stimulated proliferation in the presence of serum (Fig. 1B). Whereas withdrawal of serum results in cell cycle arrest followed by cell death, the addition of IGF2 was found to support AXT cell survival after serum withdrawal. Mitotic cells were scarcely observed in AXT cells cultured in the presence of IGF2 (Fig. 1C). Insulin and IGF1 share downstream
signaling pathways with IGF2 (15), and they also supported the survival of AXT cells after serum withdrawal (Fig. 1D, data not shown). In contrast, fibroblast growth factor 2 (FGF2) had no such effect. The human OS cell lines SAOS2 and U2OS survived in the absence of serum to a greater extent than did AXT cells. Although mitotic cells or many surviving cells were detected even under serum-free for 3 days or 10 days, respectively, in U2OS cells (Supplementary Fig. S1C), therefore, the effect of IGF2 was undervalued, the survival of both human cells was also significantly enhanced by the presence of IGF2 (Fig. 1E). These findings indicated that IGF2 and insulin ensure OS cell survival under serum free.

**Dormancy-like state induced by long-term exposure to IGF2 or insulin**

All AXT cells were dead under serum-free condition within 10 days, while flow cytometric analysis of live cells revealed that the G0-G1 fraction and the proportion of Ki67-negative (non-proliferating) cells was significantly increased, whereas the S and G2-M fractions were significantly decreased, for AXT cells maintained in IGF2-containing medium compared with those maintained in serum-containing medium (Fig. 2A, B, Supplementary Fig. S2A-C). AXT cells that had been arrested in IGF2 medium reentered the cell cycle in 24 hours after exposure to serum. Similar cell cycle arrest and downregulation of Ki67 expression were apparent both in AXT cells exposed to insulin as well as in SAOS2 cells exposed to IGF2 (Fig. 2C, D, Supplementary Fig. S2D-F).

To clarify the effect of IGF2 on cell cycle at the single-cell level, we performed time-lapse video microscopy. AXT cells cultured in serum-containing medium proliferated rapidly to achieve confluence within 1 day with a doubling time of 462 ± 30.3 min (n = 5) (Fig. 2E). In contrast, most cells cultured in the presence of IGF2 had not entered mitosis during observation for up to 19 h, although some cells entered the cell cycle. The arrested cells progressed into mitosis after subsequent exposure to serum (Fig. 2E, Supplementary Movie S1). The abundance of cyclins was downregulated in the IGF2-treated cells, however, this downregulation was reversed on exposure of the cell to serum (Fig. 2F). The proteasome inhibitor bortezomib also partially restored cyclin levels in IGF2-treated cells (Supplementary Fig. S2G), suggesting the involvement of proteasome mediated degradation. Similar to IGF2, insulin mediated the reversible downregulation of cyclin expression in AXT cells. Together, these findings suggested that continuous exposure to IGF2 or insulin in the absence of serum maintains survival under dormancy-like state of OS cells.

**Long-term exposure leads to attenuation of responsiveness to IGF2 in AXT cells**

To gain insight into the mechanism underlying the dormancy-like state, we examined
signaling downstream of the IGF1 receptor (IGF1R). Binding of ligands to IGF1R triggers activation of signaling by the PI3K–Akt pathway (15). Removal of serum resulted in the dephosphorylation of Akt as well as of p70S6K and S6 in 2 h (Fig. 3A). Exposure of the serum-deprived cells to IGF2 induced a transient increase in the phosphorylation levels of these proteins, which had started to decline by 3 h. To determine whether the transient nature of these effects of IGF2 was due to a loss of activity, we refreshed the IGF2-containing medium for the final 30 min of each incubation. However, such replenishment of IGF2 did not restore the phosphorylation levels of Akt and, more clearly, p70S6K and S6 at 12 h (Fig. 3A). This attenuation of responsiveness was also apparent in cells cultured for 3 days under the presence of IGF2 (Fig. 3B). These cells fully responded to IGF2 24 h after the final supplement of IGF2. However, this activation was rapidly downregulated and subsequent replenishment could not lead to the same level of activation of p70S6K and S6 as the initial response (Fig. 3B). Notably, downregulation of signaling apparent in cells cultured in the presence of IGF2 or insulin for 7 days was fully reversed by exposure of the cells to serum (Fig. 3C). We obtained similar results with the human OS cell lines SAOS2 and U2OS (Fig. 3D, E). A low level of signal activation was still apparent in AXT cells exposed to IGF2 or insulin for 7 days. Inhibition of Akt activity resulted in the decrease of cell viability to a greater extent than in the presence of serum, suggesting that this low level of activation is critical to support cell survival (Supplementary Fig. S3A). IGF1R appeared to be constitutively activated in AXT cells cultured in the presence of IGF2 (Supplementary Fig. S3B). These findings suggested that the dormancy-like state elicited by continuous exposure of OS cells to IGF2 is partially attributable to downregulation of survival signaling caused by attenuation of responsiveness of IGF1R signaling pathways to IGF2.

**Dormancy-like state confers resistance to anticancer drugs**

Given that slowly cycling cells are resistant to anticancer agents (16), we examined whether the dormancy-like state alters the sensitivity of OS cells to such agents. Whereas exposure of AXT cells to IGF2 in the presence of serum did not affect the sensitivity of the cells to adriamycin, that in the absence of serum reduced the sensitivity compared with that apparent in the presence of serum (Fig. 4A, B). The same reduction of sensitivity could also be observed concerning cisplatin and methotrexate. Treatment of AXT cells with insulin conferred a similar level of drug resistance (Fig. 4C, Supplementary Fig. S4A, B). Similar results were also obtained with the human OS cell lines SAOS2 and U2OS exposed to IGF2 (Fig. 4D).

To elucidate the mechanisms responsible for the reduced sensitivity, we examined the amount of phosphorylated histone H2AX (γ-H2AX), a marker of DNA damage (17,
The proportion of AXT cells found to be highly positive for γ-H2AX increased to 65.7% and 52.4% after treatment with adriamycin or cisplatin, respectively, in serum-containing medium. In contrast, the corresponding values for cells cultured in the presence of IGF2 were only 38.6% and 31.5%, respectively. Conversely, the proportion of γ-H2AX–negative cells was higher for AXT cells cultured with IGF2 than for those cultured with serum (Fig. 4E, Supplementary Fig. S4C, D). In addition, we found significant up-regulation of Glutathione S transferases and their enzymatic activity (Fig. 4F, G), suggesting that detoxication process is enhanced under IGF2 mediated dormancy-like state compared to in the presence of serum (19).

These findings suggested that the dormancy-like state of OS cells by long-term exposure to IGF2 renders the cells resistant to cytotoxicity induced by chemotherapeutic drugs.

Survival in IGF2 medium is dependent on glutamine and autophagy

To gain further insight into the mechanism underlying the dormancy-like state, we examined the effects of various agents. Consequently, those related to glutamine metabolism or autophagy showed similar or greater cytotoxic effects on AXT cells cultured in the presence of IGF2 or insulin than on those maintained in the presence of serum (Fig. 5A, B, Supplementary Fig.S5), implicating these processes in the dormancy-like state. Depletion of glutamine from IGF2- or insulin-containing medium resulted in total suppression of AXT cell survival (Fig. 5C).

The conversion of LC3I to LC3II is indicative of an increase in autophagy flux (20, 21). The accumulation of LC3II in AXT cells cultured in the presence of serum suggests the constitutive activation of autophagy. The ratio of LC3II to LC3I appeared even greater in AXT cells maintained in IGF2-containing medium than in those cultured in the presence of serum (Fig. 5D), suggestive of a more pronounced increase in autophagy flux. Treatment of chloroquine resulted in the accumulation of LC3II by blocking autophagy in its final step (22). Electron microscopy revealed the presence of a greater number of vacuoles, including autophagosomes with a double-membrane structure, in the cytoplasm of AXT cells cultured with IGF2 compared with those maintained in the presence of serum (Fig. 5E). Consistent with these findings, knockdown of Atg7, an E1-like enzyme required for autophagy (21, 23), not only reduced the abundance of LC3II (suggestive of inhibition of autophagy) (Fig. 5F), but also attenuated the viability of AXT cells maintained in the presence of IGF2 or insulin (Fig. 5G, H). Thus, the maintenance of OS cell survival by IGF2 or insulin appears to be dependent on enhancement of autophagy.
Suppression of autophagy and depletion of glutamine enhance the antitumor activity of chemotherapeutic agents in vivo

We investigated whether the suppression of autophagy flux or depletion of glutamine might have an antitumor effect in vivo. Chloroquine, bafilomycin A or L-asparaginase enhanced the antitumor activity of the combination of adriamycin, ifosfamide, and methotrexate (Fig. 6A). Treatment with L-asparaginase, which hydrolyzes asparagine and glutamine to produce aspartate and glutamate, respectively (24), reduced the serum concentration of glutamine and increased those of glutamate and aspartate (Supplementary Fig. S6A). The chemotherapeutic drugs alone increased the expression of LC3 in some tumors (Fig. 6B, C), suggesting that autophagy flux could be increased by chemotherapy. Additional treatment with chloroquine or bafilomycin A had a more pronounced effect on LC3 accumulation in tumors compared with chemotherapy alone (Fig. 6B,C), likely reflecting the effective inhibition of autophagy (22). Combined treatment with chemotherapy and these agents not only resulted in a reduction in tumor size but also induced severe damage to OS cells and a consequent loss of solidity within tumors compared with the effects of chemotherapy alone (Fig. 6C, Supplementary Fig. S6B). Collectively, these findings suggested that a combination of agents targeted to the dormancy-like cells and conventional chemotherapy is a potential therapeutic option for OS.

Increased expression levels of IGFs after chemotherapy in human OS

We finally analyzed 7 paired pre- and post-chemotherapy human OS samples to examine the correlation between the expression levels of IGFs and chemotherapy (Fig. 6D, Table 1). IGF1 expression tends to be upregulated after chemotherapy in all tumor samples. In contrast, IGF2 expression basically varied between samples and both down- and upregulation were observed after chemotherapy. However, notably, in OS-1,2,4,5 and 6 with favorable prognosis, IGF2 level was decreased after chemotherapy, otherwise the cases (OS-3,7) did not achieve successful clinical course. In addition, a case (OS-7) whose expression level of IGF2 was markedly upregulated after chemotherapy exhibited poor response to chemotherapy.

Discussion

We have here shown that the expression level of Igf2 was elevated in OS cells damaged by chemotherapy. Although serum free condition induced cell death, presence of IGF2 or insulin could guarantee survival of AXT cells, which, consequently, caused dormancy-like state, as evidenced by reduced expression of Ki67, down-regulation of cyclins, and decreased activation level of survival signaling. Importantly, subsequent exposure to
serum released the cells from the dormancy-like state.

Components of the microenvironment have increasingly been implicated in the maintenance of normal tissue stem cells in a dormant state (25, 26). AXT cells were established from bone marrow stromal cells of Ink4a/Arf knockout mice and harbor a mutant form (R267C) of p53 (data not shown). Although the stress-activated protein kinase p38 or changes in the activity of related signaling molecules have been suggested to regulate quiescence in cancer cells (27, 28), the mechanisms underlying the induction of quiescence in the cells whose machinery for arresting cell cycle is almost impaired like AXT remain to be elucidated.

Growth factor signaling networks incorporate negative feedback system to maintain homeostasis (29). Previous reports suggest that activation of the IGF / insulin signaling is limited by feedback inhibition which is mediated by PI3K-AKT-mTOR pathway. Activated S6K phosphorylates IRS proteins, which induces degradation of IRS proteins, reduction of their interaction with IGF1R and insulin receptor or the inhibition of their downstream signaling. Negative feedback from downstream effectors has also been implicated in the insulin resistance that results in impaired glucose tolerance in diabetes (30-32). Similar negative feedback might contribute to the dormant OS cells under continuous exposure to IGF2 or insulin. The constitutive activation of IGF1R in AXT cells exposed to IGF2 might partially support this notion.

A very recent report suggests the direct implication of IGF2 in the emergence of therapeutic resistance in cancer (33). Overexpression of IGFs and IGF receptors is commonly observed in human OS and is implicated in disease pathogenesis (34–37). Due to the difficulty in obtaining sufficient numbers of pre- and post-chemotherapy human samples, we have not reached definite conclusion. However, analysis of 7 paired specimens suggests the possibility that IGF-signaling can modulate therapeutic sensitivity in human OS. Activation of IGF signaling in regions that are separated from the vasculature might allow the survival of stressed tumor cells until resuming proliferation after "angiogenic switch" (38). Such "niches" or "hotbeds" of surviving tumor cells might thus engender minimal residual disease (Supplementary Fig. S6C).

The survival of OS cells supported by IGF2 or insulin was found to be dependent on enhanced autophagy flux and glutamine availability. Both intra- and extra-cellular increased glutamine levels were suggested to be linked to upregulation of autophagy flux and protection from cell death (39, 40). Quiescent hematopoietic stem cells have also been suggested to be dependent on autophagy flux for their survival (41, 42). In addition, inhibition of metabolic processes or autophagy flux in quiescent fibroblasts or therapy-induced senescent lymphoma cells resulted in cell death (43, 44). Therefore, intracellular events underlying IGF2- or insulin-mediated dormancy in OS cells might be
common to quiescence induced in other cell types by other stimuli. Targeting of IGF- or insulin-mediated dormant cancer cells combined with conventional therapies might thus provide a firmer basis for the development of new options to overcome therapeutic resistance in individuals with OS.

**Acknowledgments**

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References

Figure Legends

Figure 1. IGF2 ensures OS cell survival under serum-free condition.
(A) Real-time PCR analysis of Igf2 expression in AXT cell-derived subcutaneous tumors in mice subjected to chemotherapy. Data are presented as box-and-whisker plots for the indicated numbers of tumors. N.S., not significant.
(B) (left and middle) Cell viability of AXT cells cultured in medium containing serum either alone or together with mouse IGF2, or in serum-free medium with or without IGF2. Each ratio relative to the value for day 0 was calculated. (right) Number of viable AXT cells cultured under serum free with or without IGF2 for indicated days was counted. Live cells were evaluated by trypan blue exclusion.
(C) (upper) Morphology of AXT cells maintained in the presence of serum or cultured in IGF2-containing medium for 6 days. Cells enclosed by circles are mitotic cells. (lower) AXT cells cultured in the presence of serum or IGF2-containing medium for 5 days were stained with a phospho-histone H3 antibody and DAPI.
(D) Viability of AXT cells cultured in serum-free medium with or without FGF2 (20 ng/ml) or insulin.
(E) Viability of SAOS2 or U2OS cells cultured in serum-free medium with or without human IGF2.

Figure 2. Dormancy-like state induced under the presence of IGF2.
(A) Representative flow cytometric analysis of AXT cells maintained in serum-containing medium, cultured in IGF2-containing medium for 10 days, or exposed to IGF2 for 9 days and then to serum for 1 day. Ki67-negative population is enclosed by a polygon.
(B) Quantitation of the Ki67-negative population for cells analyzed as in (A).
(C) Representative flow cytometric analysis of SAOS2 cells maintained in serum-containing medium or cultured in IGF2-containing medium for 4 days.
(D) Quantitation of the Ki67-negative population for cells analyzed as in (C).
(E) Time-lapse video microscopy of AXT cell proliferation at the indicated times for cells either maintained in the presence of serum (upper) or cultured with IGF2 for 8 days (lower). Representative cells traced until mitosis are indicated by arrows.
(F) Immunoblot analysis of cyclin expression in AXT cells cultured with IGF2 for 7 days or with IGF2 for 6 days and then with serum for 1 day.

Figure 3. Immunoblot analysis of signaling molecules downstream of IGF1R.
(A) AXT cells were either maintained in serum-containing medium or deprived of serum for 2 h and then exposed to IGF2 for the indicated times either without or with medium replenishment for the final 30 min of incubation. (B) AXT cells were cultured with IGF2
for 3 days and then exposed to IGF2 for the indicated times. 0h indicates 24h after the final medium replacement. Cells were also subjected to medium replenishment at 3, 6 and 12h and incubation for 1h prior to protein collection.

(C) AXT cells were either cultured with IGF2 or insulin (Ins) for 7 days or exposed to IGF2 or insulin for 6 days and then incubated with serum for 1 day.

(D) SAOS2 or U2OS cells were either maintained in serum-containing medium or deprived of serum for 3 h and then exposed to IGF2 for the indicated times.

(E) SAOS2 or U2OS cells were either cultured with IGF2 for 10 or 6 days, respectively, or exposed to IGF2 for 9 or 5 days and then incubated with serum for 1 day.

**Figure 4. Long-term exposure of OS cells to IGF2 confers resistance to anticancer drugs.**

(A) Viable AXT cells were assessed after exposure for 2 days to the indicated concentrations of Adriamycin (ADR) in medium containing serum or both serum and IGF2 (left) or in medium containing serum or IGF2 (right).

(B) Number of viable AXT cells evaluated by trypan blue exclusion was counted after exposure to ADR for 2 days in medium containing serum or IGF2.

(C) Viable AXT cells were assessed after exposure for 2 days to the indicated concentrations of ADR in medium containing serum or insulin.

(D) Viable SAOS2 or U2OS cells were evaluated after exposure for 2 days to the indicated concentrations of ADR in medium containing serum or IGF2.

(E) Quantitation of AXT cells highly positive or negative for γ-H2AX evaluated by flow cytometric analyses after culture in the presence of serum or IGF2 for 7 days and then incubated in the additional presence of ADR (100 ng/ml) or cisplatin (CDDP) (100 ng/ml) for 24 h.

(F) Real-time PCR analysis of Gstm expression in AXT cells cultured in the presence of serum or IGF2-containing medium for 6 days.

(G) Gst activity of AXT cells cultured in the presence of serum or IGF2-containing medium for 5 days.

**Figure 5. Survival in the dormancy-like state is dependent on glutamine and autophagy.**

(A,B) Viability of AXT cells was assessed after exposure for 2 days to the indicated concentrations of L-asparaginase or chloroquine in medium containing IGF2, insulin, or serum.

(C) Viability of AXT cells was assayed at the indicated times after culture in the absence or presence of IGF2 or insulin and with or without glutamine. Data are presented in
arbitrary units (A.U.).

(D) Immunoblot analysis of LC3 in AXT cells cultured in the presence of serum or IGF2 for 12 days. Cells were also exposed to 50 µM chloroquine (CQ) for the final 24 h before analysis for the indication of LC3II.

(E) Representative electron microscopy of AXT cells cultured in the presence of serum or IGF2 for 12 days. The boxed areas in the upper panels are shown at higher magnification in the lower panels. Arrows indicate vacuoles.

(F) Immunoblot analysis of Atg7 and LC3 in AXT cells at 4 days after transfection with control (siCont) or Atg7-targeted (si1, si2) siRNAs.

(G, H) Viable AXT cells transfected with each siRNA were assessed after culture in the presence of IGF2 (G) or insulin (H) for 3 or 5 days. Assays were performed in quadruplicate and each ratio relative to the value for day 0 was calculated.

**Figure 6. Chloroquine, bafilomycin A and L-asparaginase enhance antitumor activity of conventional chemotherapy in vivo.**

(A) Weight of AXT cell-derived tumors for mice subjected to chemotherapy (Chemo) either alone or together with chloroquine (CQ), L-asparaginase or bafilomycin A. Data are presented as box-and-whisker plots for 12 tumors.

(B) Immunoblot analysis of LC3 in individual tumors treated with chemotherapy either alone or together with CQ or bafilomycin A as in (A). Numbers indicate the individual tumor.

(C) Serial sections of tumors from mice treated with chemotherapy either alone or together with CQ as in (A) were subjected both to hematoxylin-eosin (H&E) staining and to immunohistochemical analysis for GFP and LC3. Boxed regions in the H&E panels on the left are shown at higher magnification in the corresponding panels on the right.

(D) Real-time PCR analysis of **IGF1** and 2 expressions in paired pre-(blue) and post-(orange) chemotherapy human OS samples. Values relative to respective positive control (a liver sample or HOS cells) are also shown.
Table 1. Characteristics of human osteosarcoma patients

<table>
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<td>132</td>
<td>CDF</td>
</tr>
<tr>
<td>OS-2</td>
<td>6</td>
<td>(-)</td>
<td>female</td>
<td>fibula</td>
<td>126</td>
<td>CDF</td>
</tr>
<tr>
<td>OS-3</td>
<td>20</td>
<td>Lung</td>
<td>male</td>
<td>tibia</td>
<td>42</td>
<td>DOD</td>
</tr>
<tr>
<td>OS-4</td>
<td>17</td>
<td>(-)</td>
<td>male</td>
<td>tibia</td>
<td>116</td>
<td>CDF</td>
</tr>
<tr>
<td>OS-5</td>
<td>15</td>
<td>(-)</td>
<td>male</td>
<td>femur</td>
<td>95</td>
<td>CDF</td>
</tr>
<tr>
<td>OS-6</td>
<td>22</td>
<td>(-)</td>
<td>male</td>
<td>femur</td>
<td>89</td>
<td>CDF</td>
</tr>
<tr>
<td>OS-7</td>
<td>15</td>
<td>(-)</td>
<td>female</td>
<td>femur</td>
<td>31</td>
<td>DOD</td>
</tr>
</tbody>
</table>

Abbreviations: CDF, continuously disease-free; DOD, died of disease
A

Whole tumor

GFP(+) cells

GFP(-) cells

B

Cell viability (fold)

Time (days)

10% FBS+ IGF2

10% FBS

IGF2

Serum free

C

10% FBS

IGF2

D

Cell viability (fold)

Time (days)

Insulin

FGF2

Serum free

E

SAOS2

U2OS

Cell viability (fold)

Time (days)

p-Histone H3 / DAPI

p-Histone H3 / DAPI
Shimizu T et al. Figure 3
OS-1 OS-2 OS-3 OS-4 OS-5 OS-6 OS-7 liver

Human osteosarcoma samples (relative to a liver sample)

Pre-chemotherapy (T1)

Post-chemotherapy (T2)

IGF1

Chemo Chemo + CQ

LC3I

LC3II

Actin

Shimizu T et al. Figure 6

A

B

C

D

Tumor weight (g)

Chemo CQ L-asparaginase bafilomycin A

n=12

P=0.02

P=0.045

P=0.012

Chemo Chemo + bafilomycin A

OS-OS-OS

Stroma

OS

OS

IGF1

IGF2

Pre-chemotherapy (T1) Post-chemotherapy (T2)
IGF2 preserves osteosarcoma cell survival by creating an autophagic state of dormancy that protects cells against chemotherapeutic stress


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