Extensive protein expression changes induced by pamidronate in RAW 264.7 cells as determined by IP-HPLC

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Background. Bisphosphonate therapy has become a popular treatment for osteoporosis, Paget's disease, multiple myeloma, osteogenesis imperfecta, myocardial infarction, and cancer despite its serious side effects. Bisphosphonate-induced molecular signaling changes in cells are still not clearly elucidated. **Methods.** As bisphosphonates are primarily engulfed by macrophages, we treated RAW 264.7 cells (a murine macrophage cell line) with pamidronate and investigated global protein expressional changes in cells by immunoprecipitation high performance liquid chromatography (IP-HPLC) using 218 antisera. **Results.** Pamidronate upregulated proliferation-activating proteins associated with p53/Rb/E2F and Wnt/β-catenin pathways, but downregulated the downstream of RAS signaling, pAKT1/2/3, ERK-1, and p-ERK-1, and subsequently suppressed cMyc/MAX/MAD network. However, *in situ* proliferation index of pamidronate-treated RAW264.7 cells was slightly increased by 3.2% versus non-treated controls. Pamidronate-treated cells showed increase in the expressions of histone- and DNA methylation-related proteinsbut decrease of protein translation-related proteins. NFkB signaling was also suppressed as indicated by the down-regulations of p38 and p-p38 and the up-regulation of mTOR, while the protein expressions related to cellular protection, HSP-70, NRF2, JNK-1, and LC3 were upregulated. Consequently, pamidronate downregulated the protein expressions related to immediate inflammation,cellular differentiation, survival, angiogenesis, and osteoclastogenesis, but upregulated PARP-1 and FAS-mediated apoptosis proteins. These observations suggest pamidronate affects global protein expressions in RAW 264.7 cells by stimulating cellular proliferation, protection, and apoptosis but suppressing immediate inflammation, differentiation, osteoclastogenesis, and angiogenesis. Accordingly, pamidronate appears to affect macrophages in several ways eliciting not only itstherapeutic effects but also atypical epigenetic modification, protein translation, RAS and NFkB signalings. Therefore, our observations suggest pamidronate-induced protein expressions are dynamic, and the

affected proteins should be monitored by IP-HPLC to achieve the therapeutic goals during treatment.

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Short title: Pamidronate-induced protein expressions

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Abstract

Background.

Bisphosphonate therapy has become a popular treatment for osteoporosis, Paget's disease, multiple myeloma, osteogenesis imperfecta, myocardial infarction, and cancer despite its serious side effects. Bisphosphonate-induced molecular signaling changes in cells are still not clearly elucidated.

Methods.

As bisphosphonates are primarily engulfed by macrophages, we treated RAW 264.7 cells (a murine macrophage cell line) with pamidronate and investigated global protein expressional changes in cells by immunoprecipitation high performance liquid chromatography (IP-HPLC) using 218 antisera.

Results.

Pamidronate upregulated proliferation-activating proteins associated with p53/Rb/E2F and Wnt/β-catenin pathways, but downregulated the downstream of RAS signaling, pAKT1/2/3, ERK-1, and p-ERK-1, and subsequently suppressed cMyc/MAX/MAD network. However, *in situ* proliferation index of pamidronate-treated RAW264.7 cells was slightly increased by 3.2% versus non-treated controls. Pamidronate-treated cells showed increase in the expressions of histone- and DNA methylation-related proteins but decrease of protein translation-related proteins. NFkB signaling was also suppressed as indicated by the down-regulations of p38 and p-p38 and the up-regulation of mTOR, while the protein expressions related to cellular protection, HSP-70, NRF2, JNK-1, and LC3 were upregulated. Consequently, pamidronate downregulated the protein expressions related to immediate inflammation, cellular

differentiation, survival, angiogenesis, and osteoclastogenesis, but upregulated PARP-1 and FAS-mediated apoptosis proteins. These observations suggest pamidronate affects global protein expressions in RAW 264.7 cells by stimulating cellular proliferation, protection, and apoptosis but suppressing immediate inflammation, differentiation, osteoclastogenesis, and angiogenesis. Accordingly, pamidronate appears to affect macrophages in several ways eliciting not only its therapeutic effects but also atypical epigenetic modification, protein translation, RAS and NFkB signalings. Therefore, our observations suggest pamidronate-induced protein expressions are dynamic, and the affected proteins should be monitored by IP-HPLC to achieve the therapeutic goals during treatment.

Introduction

Bone undergoes constant remodeling maintained by a balance between osteoblasts and osteoclasts. Bisphosphonates inhibit the digestion of bone by causing osteoclasts to undergo apoptosis (*Ito et al. 2001*) and impair osteoclasts' ability to form a ruffled border (*Sato et al. 1991*), to adhere to the bone surface, and to synthesize protons necessary for bone resorption. Furthermore, bisphosphonates suppress osteoclast activity by decreasing osteoclast progenitor development and recruitment (*Cecchini et al. 1987; Endo et al. 1993*). These diphosphate analogs inhibit intermediate enzymes of mevalonate pathway and are used to treat osteoporosis and Paget's disease (historically osteitis deformans) (*Abelson 2008*). In osteoporosis and Paget's disease, IV zoledronic acid is the first-choice treatment for Paget disease because of its efficacy

and ease of administration (*Wat 2014*). The choice of zoledronic acid as the initial agent for most patients with active Paget disease is consistent with both the 2014 clinical practice guidelines of the Endocrine Society and the 2019 Paget's Association guidelines (*Singer et al. 2014*).

Bisphosphonates bind calcium and are readily deposited in bone. They also change bone ultrastructures, e.g., they obliterate Haversian canals and deposit irregular and thick reversal lines (*Acevedo et al. 2015; Carmagnola et al. 2013; Kim et al. 2017c; Lee 2013*). The common side effects of bisphosphonates include bone pain, low blood calcium levels, nausea, and dizziness. In addition, bisphosphonate-related osteonecrosis of the jaw (BRONJ) may develop in patients who have used bisphosphonates long term (*Marx et al. 2005; Ruggiero et al. 2004*). Total 37 BRONJ cases out of 1014 patients using bisphosphonates for osteoporosis treatment showed 62.6% were associated with intravenous and 37.4% with oral application (*Hansen et al. 2012*). The incidence of BRONJ is known to be low among patients treated with oral bisphosphonates (*Sarasquete et al. 2009*). The estimated prevalence of oral BRONJ was 0.05-0.07%. And the average oral bisphosphonate treatment duration was 43.1 months (range, 5-120 months) (*Hong et al. 2010*). Among the 320 osteoporotic patients who underwent tooth extraction, 11 developed BRONJ, reflecting an incidence rate of 3.44%. And the incidence of BRONJ increased with age, was greater in the mandible than the maxilla, and was associated with a duration of administration of more than 3 years (*Jeong et al. 2017; Marx et al. 2005; Ruggiero et al. 2004*). The pathophysiology of BRONJ is currently unclear. BRONJ has been attributed to infection (*Chirappapha et al. 2017; Choi et al. 2017; Park et al. 2009*), bisphosphonate-related osteonecrosis

(*Guimaraes et al. 2013*), quantitative reduction of the vascularization (*Kun-Darbois et al. 2018*), local immune dysfunction (*Hoefert et al. 2016b*), genetic predisposition like polymorphisms on CYP2C8 gene (*Sarasquete et al. 2009*), etc.

In addition, to the anti-osteoporotic effect of bisphosphonates, adjunctive bisphosphonate therapy appears to be effective at managing periodontitis (*Akram et al. 2017*), fibrous dysplasia (*Majoor et al. 2017*), and Gorham-Stout disease (*Hammer et al. 2005; Kim et al. 2015*). Therefore, it is believed bisphosphonates may have several systemic effects such as anti-inflammatory, anti-proliferative, and anti-angiogenesis effects (*Kamel et al. 2012; Ohlrich et al. 2016; Ribatti et al. 2008*). However, the biological effects of bisphosphonates in different cells have not been clearly elucidated at the molecular level.

Pamidronate (pamidronate disodium or pamidronate disodium pentahydrate) is a nitrogen-containing bisphosphonate and used to prevent bone loss due to steroid use like glucocorticoid-induced low bone mineral density in children (*Jayasena et al. 2015*) or to inhibit calcium release from bone by impairing osteoclast-mediated bone resorption (*Miyazaki et al. 2011*), pamidronate is frequently used to treat high calcium levels (*Polyzos et al. 2011*). In addition, it has also been used as an experimental treatment for osteogenesis imperfecta and been studied for the treatment of complex regional pain syndrome (*Chevreau et al. 2017*).

Immunoprecipitation high-performance liquid chromatography (IP-HPLC) had been used previously by several authors to detect organic compounds including peptides quantitatively, but the technique used was complicated and of limited applicability (*Clarke et al. 1998; Luo et al. 2013*). Recently, a new IP-HPLC protocol was developed

to determine protein expression levels in different biological fluids, such as blood serum, urine, saliva (*Kim & Lee 2015*), inflammatory exudates (*Kim et al. 2017a; b; 2018*), and different protein extracts from cells (*Kim et al. 2019; Yoon et al. 2018b*), liver (*Yoon et al. 2018a*), and cancer tissues (*Kim et al. 2017d*). The IP-HPLC is comparable to enzyme-linked immunosorbent assay (ELISA). The former uses protein A/G agarose beads in buffer solution and UV spectroscopy to determine protein concentrations, whereas the latter uses fluorescence-conjugated antibodies fixed in plastic wells and fluoroscopy. Furthermore, multiple trials have shown that IP-HPLC can be used to rapidly determine multiple protein levels accurately (< ±5% standard deviation) and reproducibly. In the previous study (*Yoon et al. 2018b*), 64 proteins were assessed by IP-HPLC 4-8 times repeatedly and their results showed low error range < ±5% standard deviation (shown in the raw data sheets of supplementary dataset 5).

When pamidronate is injected into blood vessels, it immediately chelates Ca^{++} (*Ebetino et al. 2011; Fernandez et al. 2002*) and is bound to serum albumin (90% of tiludronate) (*Sansom et al. 1995*), and subsequently recognized by macrophages, which suggests its various pharmacologic effects may be associated with the cellular functions of pamidronate-laden macrophages. Therefore, the present *in vitro* study was undertaken to investigate the effects of pamidronate on protein expressions in RAW 264.7 macrophages by IP-HPLC.

Materials & Methods

RAW264.7 cell culture with pamidronate treatment

RAW 264.7 cells, an immortalized murine macrophage cell line (ATCC, USA), were cultured as previously described (*Yoon et al. 2018b*). About 70% confluent RAW 264.7 cells grown on Petri dish surfaces were treated with 6.5 μM disodium pamidronate (similar to the therapeutic dose, 1.5 mg/kg) (Sigma, USA) for 12, 24, or 48 h; control cells were treated with 1 mL of normal saline. Cultured cells were harvested with protein lysis buffer (PRO-PREP™, iNtRON Biotechnology INC, Korea) and immediately preserved at -70°C until required.

In situ proliferation index of RAW 264.7 cells after 24 h of pamidronate treatment

RAW 264.7 cell proliferations were directly observed on plastic surfaces of Petri dishes after treatment with pamidronate at 6.5 μM for 12, 24, or 48 h, and compared with non-treated controls. When cells formed clusters containing 20-30 cells after 24 h of pamidronate treatment, ten representative histological images (taken at areas photographed before pamidronate treatment) were obtained using an inverted microscope (DP-73, Olympus Co., Japan). Cell numbers were obtained using the iSolution Lite program (IMT i-Solution Inc., Canada), proliferation indices were calculated by dividing increases in cell numbers after 24 h and 48 h of culture by initial cell numbers and compared between pamidronate treatment groups and non-treated controls.

Immunoprecipitation high-performance liquid chromatography (IP-HPLC)

Protein extracts (about 100μg) were subjected to immunoprecipitation using a protein A/G agarose column (Amicogen, Korea). Protein A/G agarose columns were

separately pre-incubated with 1 μg of 218 different antisera (147 monoclonal antibodies and 71 polyclonal antibodies (36 antibodies were purified with affinity columns) specific to target amino acid motifs (product companies were listed in Table 1); for proliferation-related proteins (n=11), cMyc/MAX/MAD signaling proteins (n=3(1)), p53/Rb/E2F signaling proteins (n=4(2)), Wnt/β-catenin signaling proteins (n=6), epigenetic modification-related proteins (n=7), protein translation-related proteins (n=5), growth factor-related proteins (n=18), RAS signaling proteins (n=22), NFkB signaling proteins (n=12(6)), up-regulated inflammatory proteins (n=17), down-regulated inflammatory proteins (n=27(1)), p53-mediated apoptosis-related proteins (n=15(2)), FAS-mediated apoptosis-related proteins (n=5(3)), cell survival-related proteins (n=5(11), protection-related proteins (n=12(13)), differentiation-related proteins (n=11(11)), oncogenesis-related proteins (n=10(10)), angiogenesis-related proteins (n=14(9)), osteogenesis-related proteins (n=11(4)), and control housekeeping proteins (n=3) (numbers in parenthesis indicate number of overlapping antibodies, Table 1).

Briefly, each protein sample (about 100μg) was mixed with 5 mL of binding buffer (150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA, 0.2mM sodium vanadate, 0.2mM PMSF, 0.5% NP-40, and mixture of protein inhibitors (Sigma, USA)) and incubated with protein A/G agarose beads (200 μL, Amicogen, Korea) bound with objective antibody on a rotating stirrer for 1 hour at 4°C. After washing beads with PBS (phosphate buffered saline solution), target proteins were eluted using 150μL of IgG elution buffer (Pierce, USA). Immunoprecipitated proteins were analyzed using a HPLC unit (1100 series, Agilent, USA) equipped with a reverse phase column and a micro-analytical detector system (SG Highteco, Korea), using 0.15M NaCl/20% acetonitrile

192 solution at 0.4 mL/min for 30 min, and proteins were detected using a UV spectrometer
193 at 280 nm. Control and experimental samples were run sequentially to allow
194 comparisons. For IP-HPLC, whole protein peak areas (mAU*s) were mathematically
195 calculated with analytical algorithm (see Supplementary data 1) by subtracting negative
196 control antibody peak areas, and protein expression levels (mAU) were compared and
197 normalized using the square roots of protein peak areas. Analyses were repeated two to
198 six times to achieve mean standard deviations of ≤ ±5% (RAW data, Supplementary
199 data 2). Objective protein expression level (%) between experiment and control groups
200 were calculated and results were analyzed using the chi-squared test program (*Kim et*
201 *al. 2019; Yoon et al. 2018a; b*).
202 The housekeeping proteins *β*-actin, *α*-tubulin, and glyceraldehyde 3-phosphate
203 dehydrogenase (GAPDH) were also used as internal controls. Expressional changes of
204 housekeeping proteins were adjusted to < ±5% using a proportional basal line
205 algorithm. Protein expressional changes of ≤ ±5%, ±5-10%, ±10-20%, and ≥±20%
206 change were defined as minimal, slight, meaningful, or marked, respectively.
207 When the IP-HPLC results were compared with the western blot data of cytoplasmic
208 housekeeping protein (β-actin), the former exhibiting minute error ranges less than ±5%
209 and could be analyzed statistically, while the latter showed a large error range of more
210 than 20%, and thus it was almost impossible to analyze them statistically (see
211 Supplementary data 3). Therefore, the present study utilized IP-HPLC to statically
212 analyze global protein expression changes in pamidronate-treated RAW 264.7 cells
213 rather than Western blot method.
214

Statistical analysis

Proportional data (%) of experimental and control groups were plotted, and analyses were repeated two to six times until standard deviations were ≤±5%. Results were analyzed by measuring standard error ($s = \pm\sqrt{\frac{\sigma^2}{n}}$). The expressions of control housekeeping proteins, that is, *β*-actin, *α*-tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) non-responsive (≤5%) to 12, 24, or 48 h of pamidronate treatment.

Results

In situ proliferation index of RAW 264.7 cells after 24 h of pamidronate treatment

Both 6.5 μM pamidronate-treated RAW 264.7 cells and non-treated controls proliferated on Petri dishes and formed large cell clusters after 24 h of culture (Figs. 1 A-F). The *in situ* proliferation index of pamidronate-treated RAW 264.7 cells was 73.1 ± 2.32 % at 24 h, 74.7 ± 2.8 % at 48 h, and that of non-treated RAW 264.7 cells was 69.9 ± 2.46 % by the *in situ* proliferation assay (Fig. 1 G). These results indicate pamidronate slightly elevated mitosis of RAW 264.7 cells, murine macrophages, by 3.2% in 24 h and 4.8% in 48 h of culture.

Effects of pamidronate on the expressions of proliferation-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with 6.5 μM pamidronate for 12, 24, or 48 h exhibited increases in levels of proliferation-activating proteins, Ki-67 (by 12.6%), proliferation cell

239 nuclear antigen (PCNA, 4%), cyclin dependent kinase 4 (CDK4, 10.1%), mitosis phase
240 promoting factor (MPF) recognized by a mitosis-specific monoclonal antibody (MPM-2 ,
241 10.7%), polo-like kinase 4 (PLK4, 7.3%), cyclin D2 (4.9%), and lamin A/C (8.7%) and
242 also increases in proliferation-inhibiting proteins, p14 (21.1%), p15/16 (14%), p27
243 (18.7%) levels versus non-treated controls. These expressional changes of proliferation-
244 activating proteins became noticeable after 24 and 48 h of pamidronate treatment but
245 remained at < ±15%, but the proliferative activity of RAW 264.7 cells was limited by the
246 increase of protein expressions of proliferation-inhibiting proteins (Figs. 2 A and B).
247 These results suggest pamidronate might have a mild proliferative effect on RAW 264.7
248 cells.
249
250 ***Effects of pamidronate on the expressions of cMyc/MAX/MAD network proteins in***
251 ***RAW 264.7 cells***
252 The expressions of cMyc and MAX decreased by 12.6% and 7.9%, respectively,
253 after 12 h of pamidronate treatment and consistently decreased by 7.4% and 1.8%,
254 respectively, at 48 h versus non-treated controls, whereas MAD-1 expression
255 decreased by a maximum of 15.7% after 12 h of treatment and slightly increased by
256 1.7% at 48 h. On the other hand, p27 expression increased by 18.7% after 48 h of
257 treatment (Figs. 2 C and D). These results indicate pamidronate suppressed
258 cMyc/MAX/MAD network expressions and resulted low level of Myc-Max heterodimers
259 which are strongly binding to E-box (CACGTG). These expressional changes of
260 cMyc/MAX/MAD network proteins may negatively contribute to the proliferative effect of
261 pamidronate on RAW 264.7 cells.

Effects of pamidronate on the expressions of p53/Rb/E2F signaling proteins in RAW 264.7 cells

Pamidronate increased the expression of p53 in RAW 264.7 cells by 22.2% at 12 h but its increase was diminished by 8.7% at 48 h versus non-treated controls, and decreased the expression of negative regulator of p53, MDM2, by 4.3% at 12 h. Rb-1 expression was also slightly increased by 7.9%, 7.3%, 15.8% at 12, 24, and 48 h, respectively. Notably, the expression of CDK4, activator of Rb-1 was increased by 16.6% at 12 h, although p21, CDK inhibitor was also increased by 11% at 12 h concurrent with the elevation of p53 expression. Resultantly, the expression of the objective transcription factor, E2F-1, increased by 12.8% at 24 h and by 9.1% at 48 h (Figs. 2 E and F). This up-regulation of p53/Rb/E2F signaling by pamidronate may indicate the increase in the level of Rb-1 phosphorylation and positively affect RAW 264.7 cell proliferation.

Effects of pamidronate on the expressions of Wnt/β-catenin signaling proteins in RAW 264.7 cells

The expressions of Wnt1, β-catenin, and adenomatous polyposis coli (APC) in RAW 264.7 cells were increased by 25.2%, 12.9%, and 8.7%, respectively, by pamidronate at 24 h versus non-treated controls, while the expression of E-cadherin was reduced by 13.8% coincident with slight increase of snail expression by 2.2% at 48 h. Resultantly, the expression of the objective transcription factor T-cell factor 1 (TCF-1) was increased by 9.3% at 12 h and by 13.3% at 48 h (Figs. 2 G and H). These findings regarding the

up-regulation of Wnt/β-catenin signaling and downregulation of E-cadherin by pamidronate may have significantly increased RAW 264.7 proliferation.

Effects of pamidronate on the expressions of epigenetic modification-related proteins in RAW 264.7 cells

Histone H1 expression increased in pamidronate treated cells to 131.3% at 24 h and to 122.3% at 48 h versus non-treated controls. Regarding histone modification, the expression of lysine-specific demethylase 4D (KDM4D) was 5% lower at 24 h, but that of histone deacetylase 10 (HDAC10) showed little change. With respect to DNA modification, DNA (cytosine-5)-methyltransferase 1 (DNMT1) expression was 10.4% higher at 48 h and those of DNA methyltransferase 1-associated protein 1 (DMAP1) and methyl-CpG binding domain 4 (MBD4) were 18.2% and 15.9% higher at 24 h, respectively, and were maintained at 8.6% and 21% higher at 48 h (Figs. 3 A and B). These results suggest pamidronate increased histone and DNA methylation and subsequently hindered DNA transcription in RAW 264.7 cells, and that this epigenetic effect of pamidronate might be related to the down-regulation of various proteins.

Effects of pamidronate on the expressions of translation-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate showed gradual reductions in protein translation-related protein levels versus non-treated controls. Although deoxyhypusine hydroxylase (DOHH) expression slightly increased by 17% and 5.4% after 24 and 48 h of treatment, respectively, deoxyhypusine synthase (DHS) expression was consistently

reduced by 18.8% and 16.8%, respectively, at these times. The protein expressions of objective factors of protein translation, that is, eukaryotic translation initiation factor 5A-1 (eIF5A-1) and eIF5A-2, were also reduced by 2.9% and 3.2% at 48 h, respectively, while that of eukaryotic translation initiation factor 2-α kinase 3 (eIF2AK3; an inactivator of eIF2) was increased by 6.8% at 24 h (Figs. 3 C and D). We considered that the pamidronate-induced reductions in the expressions of translation-related proteins might cause global inactivation of cellular signaling. However, changes in the levels of these protein levels which are normally abundant in cells tended to remain at < ±15% after 48 h of pamidronate treatment.

Effects of pamidronate on the expressions of growth factor-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate for 48 h showed increases in the expressions of growth hormone (by GH, 13.5%), growth hormone-releasing hormone (GHRH, 6.6%), platelet-derived growth factor-A (PDGF-A, 13.2%), insulin-like growth factor-1 (IGF-1, 12.8%), IGF-2 receptor (IGFIIR, 22.7%), epidermal growth factor receptor (ErbB-1, HER1, 19.2%), HER2 (receptor tyrosine-protein kinase ErbB-2 , 12%), transforming growth factor-β1 (TGF-β1, 16.4%), TGF-β2 (27.7%), TGF-β3 (20.7%), SMAD4 (18.4%), fibroblast growth factor-7 (FGF-7 known as a keratinocyte growth factor, 20.7%), and estrogen receptor β (ERβ, 14%) over 48 h versus non-treated controls whereas the expressions of FGF-1, FGF-2, and CTGF decreased by 14%, 13.9%, and 9.6%, respectively. The expressions of other growth factor-related proteins, including those of hepatocyte growth factor α (HGFα) and Met, changed

minimally (by ±5%) like the expressions of housekeeping proteins (Figs. 3 E and F). These results indicate pamidronate influenced the expressions of many growth factors necessary for the growth and differentiation of RAW 264.7 cells, that is, it increases the expressions of GH, GHRH, PDGF-A, IGF-1, IGFIIR, HER1, HER2, TGF-β1, TGF-β2, TGF- β3, SMAD4, FGF-7, and ERβ, while reduces the expressions of extracellular matrix maturation, that is, FGF-1, FGF-2, and CTGF.

Effects of pamidronate on the expressions of RAS signaling proteins in RAW 264.7 cells

Although many RAS upstream signaling proteins were upregulated by pamidronate, RAS downstream effector proteins were significantly downregulated. The increase in the expressions of KRAS (by 16.8%), NRAS (7.7%), HRAS (12.6%), phosphatidylinositol 3-kinase (PI3K, 12.3%), Jun N-terminal protein kinase-1 (JNK-1, 12.4%), mammalian target of rapamycin (mTOR, 14.2%), phosphatase and tensin homolog (PTEN, 11.2%), RAF-B (serine/threonine-protein kinase B-Raf, 18%), Rab 1 (GTPase, 10.5%), neurofibromin 1 (NF-1, 16%), protein kinase C (PKC, 10%), p-PKC (14%), son of sevenless homolog 1 (SOS-1, 22.7%), SOS-2 (14.1%), and signal transducer and activator of transcription-3 (STAT3, 7.2%) were found over 48 h of treatment versus non-treated controls, while RAS downstream expressions of pAKT1/2/3, 5' AMP-activated protein kinase (AMPK), extracellular signal–regulated kinase 1 (ERK-1), and p-ERK-1 were decreased by 8.8%, 2.9%, 7.9%, and 8%, respectively. And the expressions of A-kinase anchoring protein (AKAP) and caveolin-1 were also reduced by 16.6% and 15.4%, respectively (Figs. 3 G and H). These results

indicate pamidronate significantly reduced the expressions of the downstream effector proteins, ERK-1 and p-ERK-1, albeit many upstream proteins (KRAS, NRAS, HRAS, PI3K, JNK-1, mTOR, PTEN, RAF-B, Rab 1, NF-1, PKC, p-PKC, SOS-1, SOS-2, and STAT3, and thus, suggest RAS signaling (a major signal for cellular growth) was gradually attenuated in RAW 264.7 macrophages.

Effects of pamidronate on the expressions of NFkB signaling proteins in RAW 264.7 cells

Pamidronate had different effects on the expressions of NFkB signaling proteins in RAW 264.7 cells. The expressions of NFkB upstream signaling proteins were increased by pamidronate, that is; nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB, by 11%), PTEN (11.2%), mTOR (14.2%), peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α, 10.4%), and nuclear factor (erythroid-derived 2)-like 2 (NRF2, 12.1%), while the expressions of NFkB downstream effector proteins, pAKT1/2/3, growth arrest and DNA damage 45 (GADD45), GADD153, p38, p-p38, steroid receptor coactivator-1 (SRC1), and multi-drug resistance (MDR) were reduced by 8.8%, 13.4%, 17.3%, 10.2%, 10.2%, 15.5%, and 19.1%, respectively. The expression of ikappaB kinase (IKK) expressions was decreased by 15.5% after 48 h of treatment, and those of tumor necrosis factor α (TNFα) and activating transcription factor 6 (ATF6) only decreased by < 5%. These results indicate the expressions of many proteins that enhance NFkB signaling tended to be downregulated by treatment with pamidronate for 48 h, that is, pAKT1/2/3, GADD45, GADD153, p38, p-p38, SRC1, MDR, and more, proteins suppressing NFkB signaling tended to be upregulated by

pamidronate, that is, PTEN, mTOR, PGC-1α, and NRF2 (Figs. 4 A and B). These results indicate pamidronate effectively suppressed NFkB signaling in RAW 264.7 cells.

Effects of pamidronate on the expressions of upregulated inflammatory proteins in RAW 264.7 cells

The proteins upregulated by pamidronate were; CD3 (a T cell co-receptor constituting T cell receptor (TCR) complex, by 21.6%), CD4 (a co-receptor of the T cell receptor (TCR), 12%), neural cell adhesion molecule (NCAM, CD56, detecting natural killer cells, gamma delta (γδ) T cells, activated CD8+ T cells, and dendritic cells, 32.8%), CD80 found on the surface of dendritic cells, B cells, monocytes and antigen-presenting cells (23.2%), programmed cell death protein 1 (Pdcd-1/1, CD279, 18.9%), IL-8 (a chemoattractant cytokine, 12.8%), IL-12 (a T cell-stimulating factor, 17.5%), MMP-3 stromelysin-1 involved in wound repair, progression of atherosclerosis, and tumor initiation (17.7%), MMP-9 (a regulating factor for neutrophil migration, angiogenesis, and wound repair, 13.3%), MMP-12 (a macrophage metalloelastase contributing to elastin degradation, 10.4%), cathepsin C (a lysosomal exo-cysteine protease degrading various extracellular matrix components, 21.6%), C-X-C chemokine receptor type 4 (CXCR4, 17%), monocyte chemotactic protein-1 (MCP-1, an eotaxin, 33.5%), cyclooxygenase 2 (COX2, an important mediator of inflammation, 23.7%), versican (a large extracellular matrix proteoglycan that is involved with tissue homeostasis and inflammation, 26.5%), and kininogen (a constituent of the blood coagulation system as well as the kinin-kallikrein system, 13%) (Figs. 4 C and D).

These results indicate pamidronate stimulated cell-mediated immunity and chronic inflammation by upregulation of CD3, CD4, NCAM, CD80, Pdcd-1/1, IL-8, IL-12, MMP-3, MMP-9, MMP-12, cathepsin C, CXCR4, MCP-1, COX2, versican, and kininogen in RAW 264.7 cells.

Effects of pamidronate on the expressions of downregulated inflammatory proteins in RAW 264.7 cells

Proteins downregulated were tumor necrosis factor α (TNFα, by 11.6%), IL-1α (a "dual-function cytokine", which means it plays a role in the nucleus by affecting transcription, apart from its extracellular receptor-mediated effects as a classical cytokine, 24.1%), IL-6 (an important mediator of fever and of the acute phase response, 8.6%), IL-10 (an anti-inflammatory cytokine, 20.2%), IL-28 which play a role in the adaptive immune response (14.2%), B-lymphocyte antigen CD20 (6%), CD28 which is necessary for T cell activation and survival (6.8%), PECAM-1 (CD31, a role for leukocyte transmigration, angiogenesis, and integrin activation, 11.3%), CD34 (a transmembrane phosphoglycoprotein protein which is expressed in early hematopoietic and vascular-associated tissues, 25.4%), CD40 (a costimulatory protein found on antigen presenting cells, 8.5%), intercellular adhesion molecule 1 (ICAM-1, CD54, 19.8%), CD68 (a marker for the various cells of the macrophage lineage, 21.6%), CD99 (MIC2, a heavily O-glycosylated transmembrane protein which is expressed in all leukocytes, 7.6%), vascular cell adhesion molecule-1 (VCAM, CD106, a role in leukocyte-endothelial cell signal transduction, 12.7%), cathepsin G (an important role in eliminating intracellular pathogens and breaking down tissues at inflammatory sites,

7.8%), cathepsin K (a lysosomal cysteine protease involved in bone remodeling and resorption, 27.9%), COX1 (prostaglandin G/H synthase 1 involved in cell signaling and maintaining tissue homeostasis, 11.6%), lysozyme (17.1%), macrophage colony-stimulating factor (M-CSF, 16.1%), MMP-1 (an interstitial collagenase, 23.3%), MMP-2 (a role for lymphangiogenesis, 22%), MMP-10 (stromelysin-2, 14.1%), leukotriene A4 hydrolase (LTA4H, 4.9%), cathelicidin antimicrobial peptides LL-37 (an antimicrobial peptide, 23.6%), α1-antitrypsin (a protease inhibitor, 22.1%), β-defensin 1 (a microbicidal and cytotoxic peptide, 7.4%), β-defensin 2 (a microbicidal and cytotoxic peptide, 4.8%), and β-defensin 3 (a microbicidal and cytotoxic peptide, 7.6%) over 48 h of pamidronate treatment (Figs. 4 E and F).

These results indicate pamidronate inhibited innate immunity, immediate inflammatory rection, and wound repair processes by downregulation of TNFα, IL-1α, IL-6, IL-10, IL-28, CD20, CD28, PECAM-1, CD34, CD40, CD68, CD99, VCAM, cathepsin G, cathepsin K, COX1, lysozyme, M-CSF, MMP-1, MMP-2, MMP-10, LTA4H, LL-37, α1-antitrypsin, β-defensin 1, β-defensin 2, and β-defensin 3 in RAW 264.7 cells.

Effects of pamidronate on the expressions of p53-mediated apoptosis-related proteins in RAW 264.7 cells

Pamidronate affected the expressions of p53-mediated apoptosis-related proteins, particularly p53 protein, which was increased by 14.5% after treatment for 24 h, while the expression of E3 ubiquitin-protein ligase MDM2 was decreased by 4.3% at 12 h versus non-treated controls. After treatment for 48 h, the expressions of pro-apoptotic proteins, Bcl-2-associated death promoter (BAD), Bcl-2 homologous antagonist/killer

(BAK), pro-apoptotic member of the Bcl-2 protein family NOXA, apoptosis regulator BAX, and apoptosis inducing factor (AIF) were decreased by 12.4%, 12.2%, 26.6%, 23.5%, and 16%, respectively, but the expressions of p53 upregulated modulator of apoptosis (PUMA) and apoptotic protease activating factor 1 (APAF-1) were increased by 12.4% and 5.4%. The expressions of apoptosis executor proteins, caspase 9, c-caspase 9, caspase 3, c-caspase 3, and poly [ADP-ribose] polymerase 1 (PARP-1) increased by 36.2%, 20.9%, 27.5%, 14.6%, and 26.5% at 48 h, whereas that of cleaved PARP-1 (c-PARP-1) was reduced by 18.2% at 24 h. On the other hand, the expression of the anti-apoptosis protein, BCL2 gradually decreased by 12.9% at 48 h (Figs. 5 A and B). These results indicate pamidronate induced PARP-1/caspase 9/caspase 3-mediated apoptosis independently of p53/BAX and AIF signalings and in RAW 264.7 cells, which suggests pamidronate might induce PARP-1-mediated non-apoptotic cell death.

Effects of pamidronate on the expressions of FAS-mediated apoptosis-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate showed increases in the expressions of FAS-mediated apoptosis-related proteins as compared with non-treated controls. After treatment with pamidronate for 48 h, the expressions of death receptors on cell surfaces, that is, of FAS, FAS ligand (FASL), and FAS-associated protein with death domain (FADD), were increased by 4.6%, 15.3%, and 24.4%, respectively, and those of caspase 8, caspase 3, and c-caspase 3 were also increased by 30.8%, 27.5%, and 14.6%, respectively. On the other hand, the expressions of FLICE-like inhibitory protein (FLIP) and BH3 interacting-domain death agonist (BID) were minimally changed (<

±5%) (Figs. 5 C and D). These findings indicate pamidronate might induce apoptosis via caspase 8 and 3 through FASL/FAS/FADD signaling in RAW 264.7 cells.

Effects of pamidronate on the expressions of cell survival-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate showed variable changes in the expressions of cell survival-related proteins as compared with non-treated controls. The expressions of PTEN, telomerase reverse transcriptase (TERT), NRF2, PGC-1α, PKC, p-PKC, and focal adhesion kinase (FAK) were increased by 11.2%, 8.6%, 12.1%, 10.4%, 10%, 14%, and 13.7%, respectively, after 48 h of pamidronate treatment, while those of pAKT1/2/3, survivin, BCL2, p38, p-p38, and SP-1 were reduced by 9.1%, 10.9%, 12.9%, 10.2%, 10.2%, and 9.6%, respectively. On the other hand, the expressions of SP-3, AMPK, and ATF6 hardly changed (< ±5%) (Figs. 5 E and F). These results suggested cell survival was enhanced by the up-regulations of NRF2/PGC-1α and PKC/FAK signaling, which are features of mitochondrial biogenesis and the signal transduction cascade, respectively, but reduced by the down-regulations of AKT/survivin/BCL2 and p38/SP-1 signalings, which are features of cell exposure to stressors, such as oxidative damage. These results suggest that pamidronate increases energy metabolism and signal transduction in RAW 264.7 cells, but that the abilities of their cells to overcome different cytological stressors is relatively poor.

Effects of pamidronate on the expressions of cell protection-related proteins in RAW 264.7 cells

The expressions of cell protection-related proteins in RAW 264.7 cells were increased by pamidronate; heat shock protein-70 (HSP-70) by 21.7% at 12 h, 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-2 (PLC-β2) by 30.6% at 48 h, PI3K by 12.3% at 48 h, master regulator of mitochondrial biogenesis PGC-1α by 10.4% at 12 h, mitogen-activated protein kinase JNK-1 by 12.4% at 48 h, PKC by 10% at 48 h, p-PKC by 14% at 12 h, focal adhesion kinase (FAK) by 13.7% at 24 h, mucin 1 by 3.3% at 48 h, and mucin 4 by 10.8% at 24 h versus non-treated controls, whereas the expressions of HSP-27, HSP-90, and 5'AMP-activated protein kinase (AMPK) were decreased by pamidronate; by 3.6% at 48 h, by 14.5% at 12 h, and by 2.9% at 12 h, respectively.

The expressions of anti-oxidative proteins in RAW 264.7 cells were increased by pamidronate; detoxifying enzyme glutathione S-transferase ω 1 (GSTO1) by 9.9% at 24 h, autophagy substrate LC3 by 30.9% at 12 h, NRF2 by 12.1% at 24 h, while the expressions of Cu-Zn superoxide dismutase-1 (SOD-1), nitric oxide synthase 1 (NOS1), heme oxygenase 1 (HO-1), and endoplasmic reticulum (ER) stress-regulated transmembrane transcription factor ATF6 were decreased by pamidronate; by 13% at 24 h, by 19.2% at 48 h, by 5.5% at 48 h, and by 5.5% at 48 h, respectively. And sodium-dependent vitamin C transporter 2 (SVCT2) and cross-linking enzyme transglutaminase 2 (TGase-2) were decreased by 11.1% and 14.6% at 12 h, respectively, but gradually increased by 8.1% and 17.6% at 48 h, respectively (Figs. 6 A and B).

Although RAW 264.7 cells treated with pamidronate appeared to be silent, as they exhibited reduced NFkB signaling and had low levels of antioxidant-related proteins, SOD-1, NOS1, and HO-1 in their cytoplasms, they had higher levels of the cell

protection-related proteins, HSP-70, PLC-β2, PI3K, PGC-1α, JNK-1, PKC, p-PKC, FAK, and mucin 1 and 4 than non-treated controls. These observations suggest the expressions of cellular protection-related proteins, such as those involved in detoxification and autophagy, are upregulated by pamidronate in RAW 264.7 cells despite reduced RAS and NFkB signalings.

Effects of pamidronate on the expressions of differentiation-related proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate for 48 h showed increases in the expressions of the differentiation-related proteins p63 (25.4%), vimentin (39.8%), transglutaminase 2 (TGase-2, 17.6%), PLC-β2 (30.6%), PI3K (12.3%), PKC (10%), p-PKC (14%), FAK (13.7%), integrin α5 (21%), neural cell adhesion molecule (NCAM, CD56, 32.8%), cysteine rich protein-1 (CyRP-1, 16.3%), AP-1 complex subunit mu-1 (AP1M1, 12.8%), transcription factor SP-3 (5.3%), sonic hedgehog (SHH, 22%), and S-100 (27.1%) as compared with non-treated controls, but reductions in the expressions of the differentiation-related proteins, caveolin-1 (15.4%), α-actin (10.8%), intercellular adhesion molecule 1 (ICAM-1, CD54, 19.8%), homing cell adhesion molecule (HCAM, CD44, 27.5%), platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31, 11.3%), receptor for sonic hedgehog PTCH-1 (12.9%), transcription factor SP-1 (9.6%), and cystatin A (28.9%) (Figs. 6 C and D).

The proteins essential for the differentiation, migration, adhesion, and endocytosis of RAW 264.7 cells, that is, p63, vimentin, TGase-2, PLC-β2, PI3K, PKC, p-PKC, FAK, integrin α5, NCAM (CD56), CyRP-1, SP-3, SHH, and S-100 were upregulated by

treatment with pamidronate for 48 h, whereas some proteins required for further differentiation into active macrophages or dendritic cells, that is, caveolin-1, α-actin, ICAM-1 (CD54), HCAM (CD44), PECAM-1 (CD31), PTCH-1, SP-1, and cystatin A, were downregulated. These observations suggest pamidronate-treated RAW 264.7 cells maintain major signal transduction organelles for cellular proliferation and protection but are defective in terms of advanced cytological differentiation due to reductions in the expressions of caveolin-1, α-actin, PTCH-1, and SP-1.

Effects of pamidronate on the expressions of oncogenic proteins in RAW 264.7 cells

RAW 264.7 cells treated with pamidronate showed increases in the expressions of the oncogenic proteins, carcinoembryonic antigen (CEA, 21.2%), conserved regulatory molecule 14-3-3 (29.5%), deleted in malignant brain tumors 1 protein (DMBT1, 22.8%), telomerase reverse transcriptase (TERT, 8.6%), transmembrane subunit containing three EGF-like domains mucin 4 (10.8%), and serine protease inhibitor maspin (22.7%) as compared with non-treated controls, and also increases in the expressions of the tumor suppressor proteins, phosphatase and tensin homolog (PTEN, 11.2%), mTOR (14.2%), GTPase-activating protein neurofibromin 1 (NF-1, 16%), breast cancer type 1 susceptibility protein (BRCA 1, 16%), BRCA 2 (6.3%), and methyl-CpG binding protein-4 (MBD4, 21%). Whereas the expressions of strong oncogenic proteins, BCL2, SP-1, proto-oncogene serine/threonine-protein kinase PIM-1, and Yes-associated protein (YAP) were reduced by 12.9%, 9.6%, 17.4%, and 34.3%, respectively, after treatment

559 for 48 h. In addition, the expression of tumor suppressor protein ATM was also
560 diminished by 5.2% after treatment for 12 h (Figs. 6 E and F).
561 Concomitant increases in the expressions of oncogenic proteins and tumor
562 suppressor proteins indicate RAW 264.7 cells were stimulated by pamidronate and
563 reacted by initiating oncogenic signaling for cellular proliferation, survival, and
564 apoptosis.
565
566 ***Effects of pamidronate on the expressions of angiogenesis-related proteins in***
567 ***RAW 264.7 cells***
568 RAW 264.7 cells treated with pamidronate showed rapid reductions in the
569 expressions of angiogenesis-related proteins, as follows, HIF-1α (12%), angiogenin
570 (13.2%), vascular endothelial growth factor A (VEGF-A, 14.7%), VEGFR2 (12.5%), p-
571 VEGFR2 (22.1%), von Willebrand factor (vWF, 16%), capillary morphogenesis protein 2
572 (CMG2, 18.5%), COX1 (11.6%), FGF-1 (14%), FGF-2 (13.9%), MMP-2 (22%), MMP-10
573 (14.1%), plasminogen activator inhibitor-1 (PAI-1, 12.4%), PECAM-1 (CD31, 11.3%),
574 and vascular cell adhesion molecule-1 (VCAM-1, CD106, 12.7%) after treatment with
575 pamidronate for 48 h versus non-treated controls. The expressions of endothelin 1 (21-
576 amino acid vasoconstricting peptide, ET-1) and PDGF-A were increased by 18.6% and
577 13.2%, respectively, whereas the expressions of VEGF-C, lymphatic vessel endothelial
578 hyaluronan receptor 1 (LYVE-1), Fms-related tyrosine kinase 4 (FLT-4), and
579 plasminogen barely changed (< ±5%) (Figs. 7 A and B).
580 Among the angiogenesis-related proteins, the expressions of the blood vessel-
581 forming proteins, angiogenin, VEGF-A, VEGFR2, vWF, and CMG2 were markedly

582 reduced by pamidronate, while those of the lymphatic vessel-forming proteins, VEGF-C
583 and LYVE-1 tended to increase slightly (< 5%). Pamidronate also reduced the
584 expressions of the extracellular matrix proteins, FGF-1, FGF-2, MMP-2, and MMP-10,
585 which are required for *de novo* angiogenesis and wound healing. These results suggest
586 pamidronate significantly suppresses the expressions of angiogenesis-related proteins
587 in RAW 264.7 cells, and that it might be able to potently inhibit blood vessel formation *in*
588 *vivo*.
589
590 ***Effects of pamidronate on the expressions of osteogenesis-related proteins in***
591 ***RAW 264.7 cells***
592 Treatment with pamidronate for 48 h decreased the expressions of the
593 osteogenesis-related proteins; osteoprotegerin (OPG, 30.7%), osterix (4.5%),
594 mammalian Runt-related transcription factor 2 (RUNX2, 23.8%), osteocalcin (16.2%),
595 and connective tissue growth factor (CTGF, 9.6%) and those of the osteoclastogenesis-
596 related proteins; receptor activator of nuclear factor kappa-B ligand (RANKL, 31.6%),
597 cathepsin K (27.9%), and HSP-90 (9.1%) versus non-treated controls. On the other
598 hand, the expressions of osteopontin and TGF-β1 were increased by pamidronate by
599 18.8% and 16.4% and the expressions of bone morphogenetic protein-2 (BMP-2, 8.3%),
600 BMP-3 which negatively regulates bone density (16.8%), BMP-4 (6.8%), osteonectin
601 (4.6%), and alkaline phosphatase (ALP, 5.3%), tended to be increased (Figs. 7 C and
602 D).
603 The expressions of the major osteoblast differentiation proteins; OPG, osteocalcin,
604 and RUNX2, and of the osteoclast differentiation proteins; RANKL, HSP-90, and

605 cathepsin K, were markedly reduced by 48 h of pamidronate treatment, whereas the
606 expressions of the bone matrix proteins, osteopontin, BMP-2, BMP-4, osteonectin, and
607 ALP tended to increase. In particular, the expressions of BMP-3 (an antagonist to other
608 BMP's in the differentiation of osteogenic progenitors) and TGF-β1 (an inhibitor of
609 osteoclast activity) were markedly increased by pamidronate treatment. These results
610 suggest pamidronate-treated RAW 264.7 cells are hardly differentiated into osteoclasts
611 and give sparse influence on adjacent osteoblastic cells by expression of bone matrix
612 proteins.
613
614 ***Global protein expressions in pamidronate-induced RAW 264.7 cells***
615 Global protein expression changes of representative proteins (n=73) from above 19
616 different protein signaling pathways are illustrated as a star plot in Fig. 8. Although
617 pamidronate is low molecular weight entity, it was found to widely affect the expressions
618 of proteins in different signaling pathways in RAW 264.7 cells. In particular, pamidronate
619 inactivated epigenetic modification and protein translation and subsequently down-
620 regulated the expressions of some proteins required for the proliferation, differentiation,
621 protection, and survival of RAW 264.7 cells.
622 The increases observed in the expressions of proliferation-related proteins were
623 presumably related to the up-regulations of p53/Rb/E2F and Wnt/β-catenin signaling by
624 pamidronate albeit suppression of cMyc/MAX/MAD network signaling. The suppression
625 of RAS signaling induced by pAKT1/2/3, ERK-1, and p-ERK-1 down-regulations was
626 followed by cMyc/MAX/MAD network down-regulation and by a subsequent inhibition in
627 RAW 264.7 cell proliferation. Furthermore, the marked suppression of NFkB signaling

appeared to be associated with elevation of PARP-1- and FAS-mediated apoptosis and reduction of cellular differentiation, survival, immediate inflammatory reaction, and wound repair.

Overall changes in protein expressions induced by pamidronate affected the differentiation of RAW 264.7 cells and resulted in the productions of immature and/or inactive macrophages expressing lower levels of M2 macrophage differentiation proteins (wound healing proteins, TNFα, IL-1α, IL-6, IL-10, PECAM-1, CD99, VCAM, cathepsin G, cathepsin K, COX1, lysozyme, M-CSF, MMP-1, MMP-2, MMP-10, LL-37, α1-antitrypsin, β-defensin 1, β-defensin 2, and β-defensin 3), angiogenesis-related proteins (HIF-1α, angiogenin, VEGF-A, VEGFR2, p-VEGFR2, vWF, CMG2, COX1, FGF-1, FGF-2, MMP-2, MMP-10, PAI-1, PECAM-1, and VCAM-1), and osteoclast/osteoblast differentiation proteins (OPG, osterix, RUNX2, osteocalcin, and CTGF) and those of the osteoclastogenesis-related proteins (RANKL, cathepsin K, and HSP-90) than non-treated controls. Thus, pamidronate-treated RAW 264.7 cells simultaneously exhibited anti-inflammatory, anti-angiogenesis, and bone resorption inhibitive effects. However, the essential protein expression changes for cell proliferation, RAS signaling, and NFkB signaling rarely exceeded ±20%, which suggests pamidronate-treated cells exhibit relatively benign nature and be under homeostatic control.

Highly up- and down-regulated proteins by pamidronate in RAW 264.7 cells

In dot graphs plotted with highly up- and down-regulated proteins (> ± 10%, n=155, Fig. 9), pamidronate-treated RAW 264.7 cells showed reactive upregulation (10-30%) of

some proteins for cellular proliferation (CDK4, E2F-1, and TCF-1), protection (HSP-70, LC3, PLC-β2, and p-PKC), differentiation (vimentin, NCAM, p63, S-100, and SHH), RAS signaling proteins (KRAS, HRAS, SOS1, SOS2, RAF-B, JNK-1, and Rab), NFkB signaling proteins (NFkB, mTOR, NRF2, PGC-1α, and PTEN), and oncogenic proteins (DMBT1, 14-3-3, and CEA) versus non-treated controls, and were tended to be proliferative but their cellular activities became abortive by the downregulation (10-20%) of some essential proteins (p14, p15/16, cMyc, MAX, MAD-1, E-cadherin, FGF-1, FGF-2, CTGF, AKAP, caveolin-1, MDR, IKK, GADD45, GADD153, SRC1, and p-p38) and by increases in the expression of histone and DNA methylation-related proteins (histone H1, MBD4, and DMAP1) and by decreases in the expressions of protein translation-related proteins (eIF2AK3 and DHS) (Fig. 9 A and C).

On the other hand, pamidronate-treated RAW 264.7 cells appeared to be degenerated by marked downregulation (10-30%) of M2 macrophage differentiation-related inflammatory proteins (M-CSF, lysozyme, α1-antitrypsin, CD34, cathepsin K, and MMP-2) and survival-related proteins (BCL2, survivin, SP-1, and p-p38) and by marked upregulation (10-40%) of apoptosis-related proteins (caspase 9, c-caspase 9, caspase 3, c-caspase 3, PARP-1, p53, and PUMA) versus non-treated controls. Subsequently, the major protein expressions for angiogenesis (VEGF-A, p-VEGFR2, angiogenin, HIF-1α, VCAM-1, FGF-1, FGF-2, PECAM-1, MMP-2, and MMP-10) and osteoclastogenesis (OPG, RANKL, cathepsin K, RUNX2, osteocalcin, and HSP-90) were dramatically suppressed (10-40%) by pamidronate (Fig. 9 A-C).

Discussion

674

675 Pamidronate is a nitrogen-containing, synthetic bisphosphonate, and its phosphate
676 groups are believed to interfere with phosphorylation processes or interact with proteins
677 in cells (*Chen et al. 2012; Nishida et al. 2003; Stefanucci et al. 2015*). Pamidronate is
678 not sequestered as a waste material but relatively well adapted in cells, and thus, it is
679 presumed pamidronate is maintained as a metabolite and influences not only the
680 intracellular mevalonate pathway and protein isoprenylation but also signaling
681 molecules and genetic materials (*Henneman et al. 2011; Iguchi et al. 2010; Kaiser et al.*
682 *2013; Tatsuda et al. 2010*). It has been shown pamidronate has considerable impact on
683 cells such as macrophages, osteoclasts, and endothelial cells, and that its long-time
684 usage is associated with the risk of BRONJ (*Hoefert et al. 2015; Sharma et al. 2016;*
685 *Zhang et al. 2013*). In the present study, we assessed the effects of a therapeutic dose
686 of pamidronate on the expressions of proteins in RAW 264.7 cells by IP-HPLC. As RAW
687 264.7 cells are derived from murine macrophages, and their immunological roles to
688 dialysed coffee extract were assessed by IP-HPLC (*Yoon et al. 2018b*), and this study
689 also explored RAW 264.7 cells for their macrophage roles to pamidronate.
690 Pamidronate-induced proliferation of RAW 264.7 cells was examined by counting
691 cell numbers directly on Petri dishes, and protein expressional changes were
692 determined by IP-HPLC. The *in situ* proliferation index of pamidronate-treated RAW
693 264.7 cells over 24 h was 73.1 ± 2.32 %, whereas that of non-treated cells was 69.9 ±
694 2.46 %, thus the pamidronate-induced increase was 3.2%. Furthermore, this increase in
695 *in situ* proliferation index matched the pamidronate-induced increases in the
696 expressions of different proliferation-related proteins as determined by IP-HPLC. These

data suggest pamidronate can slightly activate mitosis of murine macrophages, RAW 264.7 cells.

When we explored cellular mechanism responsible for altering protein expressions in RAW 264.7 cells, we noticed that the epigenetic environment was generally inactivated by pamidronate due to the up-regulations of DMNT1, MBD4, and DMAP1 and the down-regulation of KDM3D, which would tend to increase histone and DNA methylation levels. Protein translation was also inactivated by a marked reduction in DHS expression and an increase in eIF2AK3 (an inactivator of eIF2) expression versus non-treated controls. We suggest the concurrent inactivations of epigenetic modification and protein translation by pamidronate may have reduced global RAW 264.7 cell activity.

Pamidronate-treated RAW 264.7 cells showed a marked reduction in cMyc/MAX/MAD network signaling during culture, and this was followed by the up-regulation of p27 (a negative regulator of G1 progression) by 16.7% at 48 h. Whereas p53/Rb/E2F signaling was enhanced by the up-regulations of p53, Rb-1, and CDK4 resulted in an increase in the expression of the objective transcription factor, E2F-1. Also, Wnt/β-catenin signaling was also enhanced by the up-regulations of Wnt-1, β-catenin, and snail, which led to the up-regulation of the objective transcription factor, TCF-1. As a result, the expressions of the proliferation-activating proteins Ki-67, PCNA, MPM2, CDK4, cyclin D2, and lamin A/C, were increased by pamidronate, and concurrently the expressions of the proliferation-inhibiting proteins p14, p15/16, p21, and p27 were compensatory increased during 48 h of pamidronate treatment. These results indicate pamidronate-treated RAW 264.7 cells were partly activated and

proliferative due to increased p53/Rb/E2F and Wnt/β-catenin signaling despite a marked reduction in cMyc/MAX/MAD network signaling.

Pamidronate-treated RAW 264.7 cells showed increases in the expressions of some growth factors and associated proteins, such as IGF-1, IGFIIR, GH, HER1, HER2, TGF-β1, - β2, - β3, SMAD4, and ERβ, and subsequently, the expressions of upstream RAS signaling proteins including KRAS, HRAS, SOS-1, SOS-2, PI3K, JNK-1, and RAF-B were increased. However, downstreams of AKT and ERK signaling were reduced by the down-regulations of pAKT1/2/3, ERK-1, and p-ERK-1 and by the up-regulations of PTEN and mTOR. Consequently, RAS signaling was attenuated by the down-regulations of pAKT1/2/3, ERK-1, and p-ERK-1 in pamidronate-treated RAW 264.7 cells. Therefore, it appeared the pamidronate-induced negative regulation of RAS signaling might significantly reduce the expression of cMyc/MAX/MAD-1 network proteins.

Although the expressions of NFkB, NRF2, PGC-1α, PTEN, and mTOR tended to increase (< 10%) after pamidronate treatment, the expressions of p38, p-p38, GADD45, GADD153, ATF6, MDR, and SRC-1 were reduced after 48 h of treatment. In addition, the expressions of the reactive oxygen and nitrogen species-related proteins SOD-1, NOS1, and HO-1 were consistently reduced by pamidronate. These observations indicate NFkB signaling was reduced due to pamidronate-induced suppression of the downstream effector protein p38 (p-p38) in RAW 264.7 cells, and that treated cells were less reactive to oxidative or endoplasmic reticulum stress than non-treated controls.

Although pamidronate suppressed RAS and NFkB signalings simultaneously, RAW 264.7 cells expressed higher levels of the protection-related proteins HSP-70, JNK-1,

743 PLC-β2, LC3, and FAK, the cell survival-related proteins TERT, NRF2, PGC-1α, p-PKC,
744 and FAK, and the oncogenesis-related proteins CEA, 14-3-3, and DMBT1 than non-
745 treated controls. In particular, increases in the expressions of HSP-70 (protects against
746 thermal and oxidative stress), JNK-1 (a mitogen-activated protein kinase responsible to
747 different stress stimuli), LC3 (autophagosome biogenesis protein), NRF2 (transcription
748 factor for many antioxidant genes), 14-3-3 (a regulator of diverse signaling proteins),
749 DMBT1 (a glycoprotein containing multiple cysteine-rich domains that interact with
750 tumor cells), and TERT (an RNA-dependent polymerase that lengthens telomeres in
751 DNA strands) indicated pamidronate stressed RAW 264.7 cells and stimulated them to
752 respond by expressing protection- and oncogenesis-related proteins.
753 Macrophages constitute a component of the front line of host defense and mediate
754 innate immune responses by triggering; the productions of cytokines, chemokines, and
755 cytotoxic molecules, the mobilizations of cells such as neutrophils and other leukocytes,
756 the phagocytosis of pathogens and their delivery to lysosomes for degradation, and the
757 induction of autophagy (*Zhang et al. 2016*). Many authors have reported macrophage
758 functions are reduced after pamidronate treatment *in vitro* and *in vivo* (*Escudero &*
759 *Mandalunis 2012; Hoefert et al. 2015; Hoefert et al. 2016a; Mian et al. 1994*). In the
760 present study, although the general cytodifferentiation proteins, p63, vimentin, PLC-β2,
761 PI3K, PKC, FAK, integrin α5, SHH, and S-100 were upregulated by pamidronate, the
762 M2 macrophage differentiation-related proteins, TNFα, lysozyme, cathepsin G,
763 cathepsin K, M-CSF, ICAM-1, and α1-antitrypsin were consistently downregulated,
764 which suggested pamidronate prevented the differentiation of RAW 264.7 cells into

765 active M2 macrophages, and resulted retarded wound healing after pamidronate
766 treatment *in vivo* (*Ariza Jimenez et al. 2018; Chen et al. 2018*).
767 Pamidronate-treated RAW 264.7 cells also showed increases in the expressions of
768 the apoptosis executor proteins, caspase 8, caspase 3, and c-caspase 3, which are
769 activated by the FAS-mediated apoptosis signaling cascade, and that the expressions
770 of caspase 9 and c-caspase 9 were also increased by p53 upregulated modulator of
771 apoptosis (PUMA) and APAF-1 even though the expressions of the upstream p53-
772 mediated apoptosis signaling proteins, BAD, BAK, BAX, NOXA, and BCL2 were
773 suppressed. In addition, the expression of PARP-1 was increased by pamidronate
774 whereas the expression of cleaved PARP-1 (c-PARP-1) was decreased. These results
775 suggest pamidronate-treated RAW 264.7 cells underwent FAS/caspase 3/PARP-1-
776 mediated apoptosis, that is, parthanatos, due to the accumulation of polymeric
777 adenosine diphosphate ribose (poly (ADP-ribose) or PAR) caused by severe DNA
778 damage. Actually, pamidronate-treated RAW 264.7 cells were continuously proliferative
779 as evidenced by the up-regulations of p53/Rb/E2F and Wnt/β-catenin signaling, though
780 they only showed a slight increase in cell numbers after 24 h of pamidronate treatment
781 versus non-treated controls, which suggests some cells unable to differentiate into
782 mature macrophages may have succumbed to FAS-mediated or PARP-1-associated
783 apoptosis.
784 Pamidronate reduced the expressions of the osteoclastogenesis-related proteins,
785 RANKL and cathepsin K in RAW 264.7 cells, indicating it inhibited osteoclast
786 differentiation, which is in-line with the reported disappearance of osteoclasts in
787 bisphosphonate-treated animals (*Kameka et al. 2014; Kawata et al. 2004; Mayahara &*

788 *Sasaki 2003*) and has implications regarding the effects of pamidronate effects on
789 osteolytic diseases such as including osteoporosis, fibrous dysplasia, Paget's disease,
790 and Gorham's disease (*Hammer et al. 2005; Kravets 2018; Saraff et al. 2018*), etc.
791 Pamidronate also downregulated the osteoblast differentiation proteins OPG,
792 RUNX2, osterix, and osteocalcin but slightly induced the expressions of bone matrix
793 proteins such as osteopontin, BMP-2, BMP-4, osteonectin, and ALP together with BMP-
794 3 which negatively regulates bone density. These findings may be relevant to the
795 osteoinductive effects of low-dose bisphosphonate reported in chronic periodontitis and
796 after dental implantation (*Alqhtani et al. 2017; Ata-Ali et al. 2016; Bhavsar et al. 2016;*
797 *Khojasteh et al. 2019*). However, pamidronate-treated RAW 264.7 cells may negatively
798 regulate cytodifferentiation to osteoblasts *in vivo* and their abnormal bone production
799 can contribute to the disruption of Haversian system canaliculi, which leads osteocyte
800 death and increases the risk of osteonecrotic infections like BRONJ (*Acevedo et al.*
801 *2015; Favia et al. 2009; Park et al. 2009*).
802 Interestingly, pamidronate altered expressions of inflammatory proteins in RAW
803 264.7 cells both positively and negatively. The expressions of inflammatory proteins that
804 participate in immediate inflammatory reaction, e.g., TNFα, IL-1, lysozyme, CD68, LL-
805 37, and β-defensin-1, -2, -3, were markedly reduced, whereas those that participate in
806 delayed inflammatory reaction, e.g., CD3, CD80, Pdcd-1/1, IL-12, and MCP-1, were
807 elevated. The inhibition of immediate inflammatory reaction results the failure of innate
808 immunity, and is relevant to severe necrotic infection of BRONJ involved with reduction
809 of granulation tissue (*Burr & Allen 2009; Carmagnola et al. 2013; Marx & Tursun 2012;*
810 *Ziebart et al. 2011*). Actually, pamidronate markedly suppressed the expressions of the

angiogenesis-related proteins, HIF-1α, VEGF-A, VERFR2, p-VEGFR2, vWF, CMG2, FGF-1, FGF-2, MMP-2, MMP-10, COX-1, PAI-1, VCAM-1, and PECAM-1 in RAW 264.7 cells versus non-treated controls but had relatively little effect on the expressions of the lymphatic vessel-related proteins, VEGF-C, LYVE-1, and FLT-4. These observations suggest that pamidronate-treated RAW 264.7 cells do not participate in immediate inflammatory reactions and vascular capillary production, but that they still provide some support for lymphatic drainage.

Pamidronate was found to widely affect the expressions of proteins in different signaling pathways in RAW 264.7 cells. Its global protein expression changes were illustrated in Fig. 8, exhibiting dynamic impacts on epigenetic modification, protein translation, RAS signaling, NFkB signaling, cellular proliferation, protection, differentiation, survival, apoptosis, inflammation, angiogenesis, and osteoclastogenesis. Highly up- and down-regulated proteins for each cellular functions were summarized in Fig. 9. Pamidronate induced marked over- and under-expression of some elective proteins more than 20% compared to non-treated controls, which may play pathogenetic roles (biomarkers) for cellular differentiation, inflammation, apoptosis, angiogenesis, and osteoclastogenesis in RAW 254.7 cells.

Conclusions

Summarizing, pamidronate was found to alter the expressions of many important proteins in RAW 264.7 cells. It upregulated proliferation-related proteins associated with p53/Rb/E2F and Wnt/β-catenin signaling and inactivated epigenetic modification and protein translation. In addition, RAS (cellular growth) and NFkB (cellular stress)

signalings were markedly affected by pamidronate. Pamidronate-treated cells showed that upstream of RAS signaling was stimulated by up-regulation of some growth factors, while downstream of RAS signaling was attenuated by down-regulation of ERK-1 and p-ERK-1, resulted in reduction of cMyc/MAX/MAD network expression. They also showed suppression of NFkB signaling by downregulating p38 and p-p38 and upregulating mTOR. Consequently, pamidronate affects global protein expression in RAW 264.7 cells by downregulating the expressions of immediate inflammation, cellular differentiation, survival, angiogenesis, and osteoclastogenesis-related proteins, but by upregulating PARP-1- and FAS-mediated apoptosis, protection, and proliferation-related proteins. These findings suggest pamidronate has potent anti-inflammatory, anti-angiogenesis, and anti-osteoporotic effects together with cellular stresses dysregulating RAS signaling, NFkB signaling, apoptosis, and proliferation. The present study explored the global expressions of representative essential proteins (n=218) in pamidronate-treated RAW 264.7 cells, but some affected proteins were so dynamic and variable that they should be continuously monitored by IP-HPLC, if pamidronate treatment will be prolonged. Finally, we suggest further molecular biologic studies be undertaken on interactions between pamidronate and target proteins.

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Figure 1

In situ proliferation assayof RAW 264.7 cells

Figure 1. *In situ* proliferation assay of RAW 264.7 cells. Increases in cell numbers were determined by counting on Petri dishes (A-F), and proliferation indices (%) were calculated by expressing cell growths (final-initial cell counts) as percentages of initial cells counts. Pamidronate-treated (6.5 µM) RAW 264.7 cells had a slightly higher mean proliferation index (73.1 ± 2.32% at 24 h and 74.7 ± 2.8% at 48 h) than non-treated controls (69.9 ± 2.46%) (G).

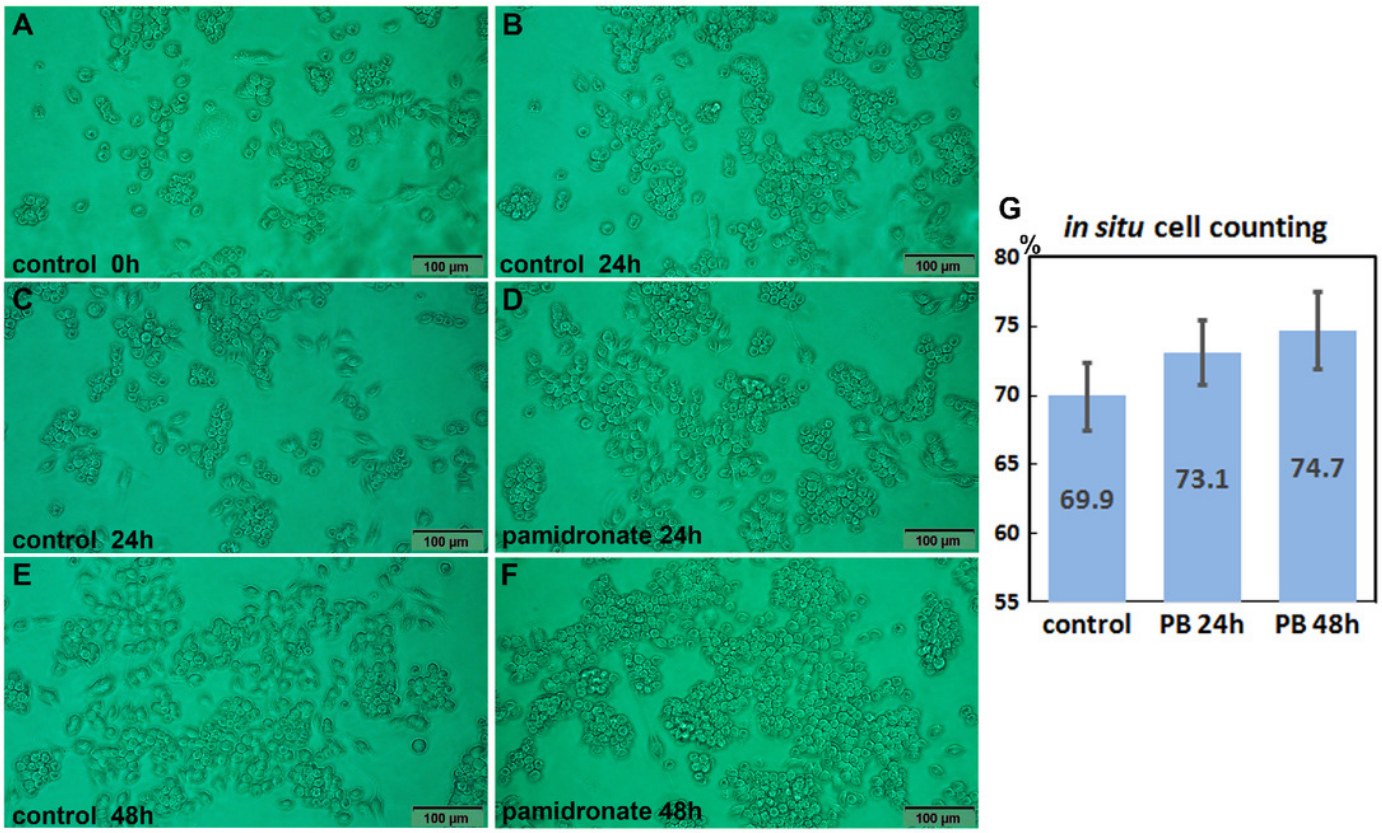

Figure 2

Expressions ofproliferation-related proteins, cMyc/MAX/MAD network proteins,p53/Rb/E2F signaling proteins, and Wnt/β-catenin signaling proteins

Figure 2. Expressions of proliferation-related proteins (A and B), cMyc/MAX/MAD network proteins (C or D), p53/Rb/E2F signaling proteins (E and F), and Wnt/β-catenin signaling proteins (F or H) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, E, and G show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, F, and H) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).

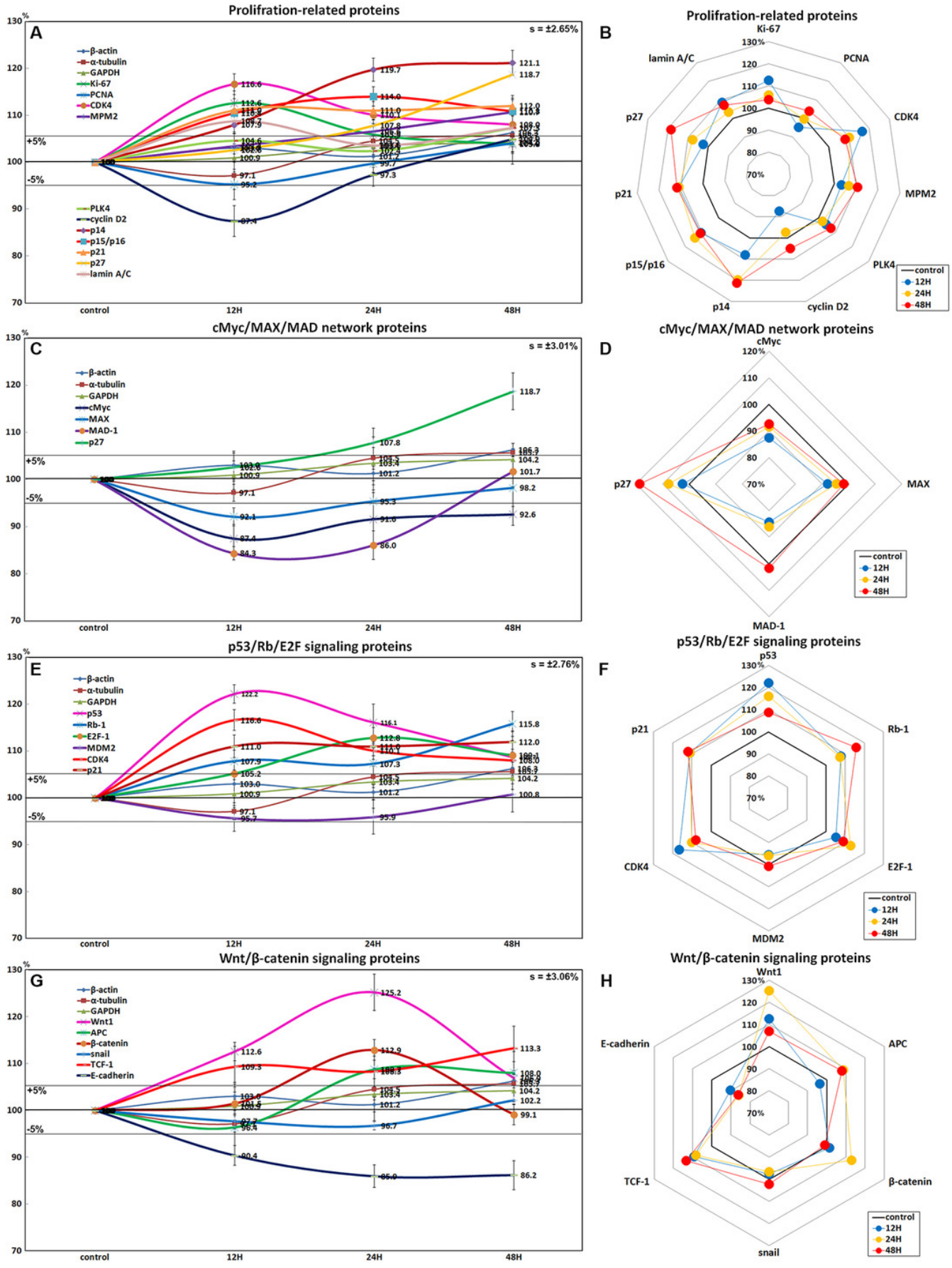
Prolifration-related proteins
A
s = ±2.65%
β-actin
α-tubulin
GAPDH
Ki-67
PCNA
CDK4
MPM2
PLK4
cyclin D2
p14
p15/p16
p21
p27
lamin A/C
+5%
-5%
control
12H
24H
48H
B
Prolifration-related proteins
Ki-67
PCNA
CDK4
MPM2
PLK4
cyclin D2
p14
p15/p16
p21
p27
lamin A/C
control
12H
24H
48H
cMyc/MAX/MAD network proteins
C
s = ±3.01%
β-actin
α-tubulin
GAPDH
cMyc
MAX
MAD-1
p27
D
cMyc/MAX/MAD network proteins
cMyc
MAX
MAD-1
p27
p53/Rb/E2F signaling proteins
E
s = ±2.76%
β-actin
α-tubulin
GAPDH
p53
Rb-1
E2F-1
MDM2
CDK4
p21
F
p53/Rb/E2F signaling proteins
p53
Rb-1
E2F-1
MDM2
CDK4
p21
Wnt/β-catenin signaling proteins
G
s = ±3.06%
β-actin
α-tubulin
GAPDH
Wnt1
APC
β-catenin
snail
TCF-1
E-cadherin
H
Wnt/β-catenin signaling proteins
Wnt1
APC
β-catenin
snail
TCF-1
E-cadherin

Figure 3

Expressions ofepigenetic modification-related proteins, protein translation-relatedproteins, growth factors, and RAS signaling proteins

Figure 3. Expressions of epigenetic modification-related proteins (A and B), protein translation-related proteins (C or D), growth factors (E and F), and RAS signaling proteins (G or H) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, E, and G show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, F, and H) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).

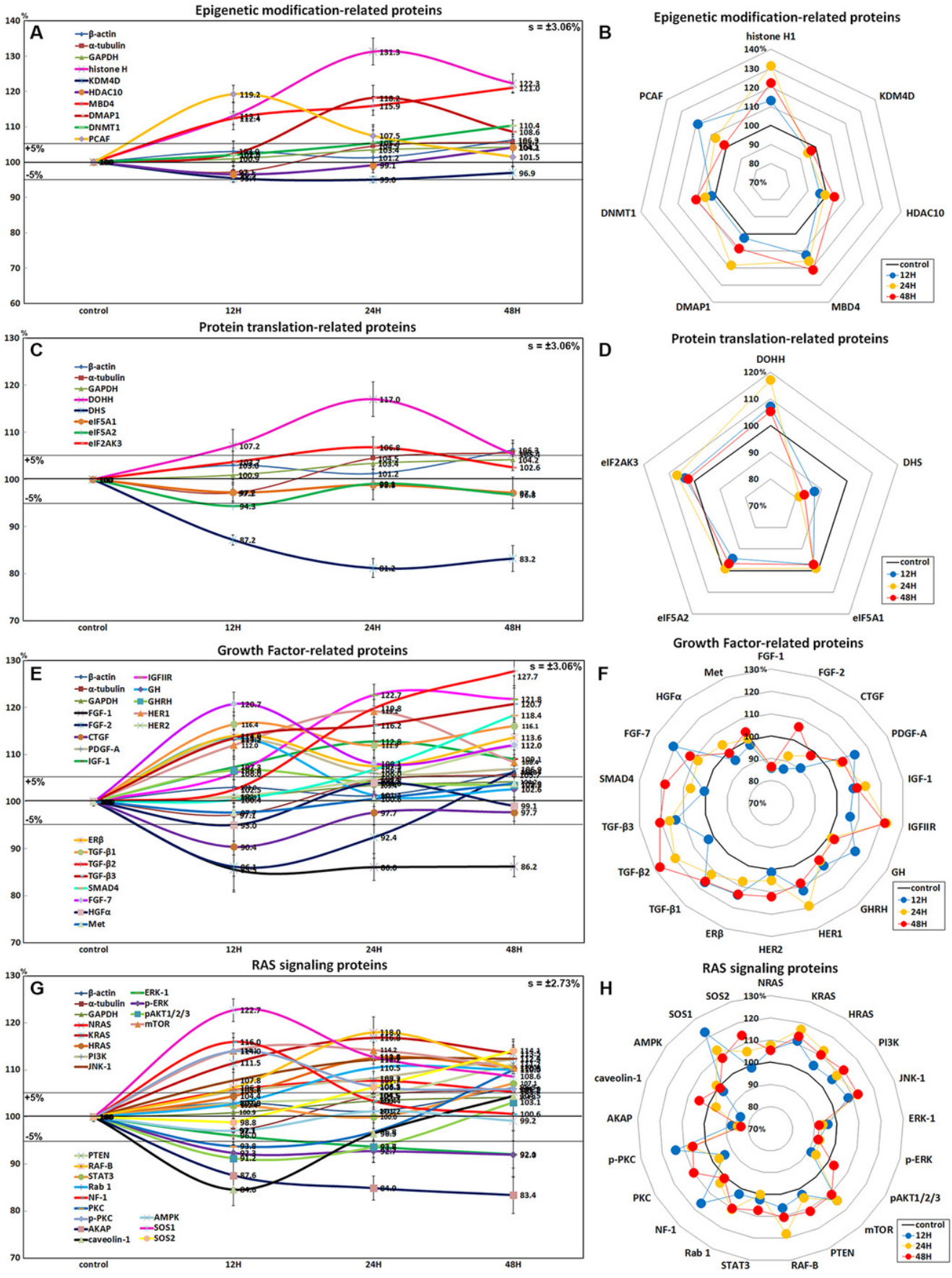
Epigenetic modification-related proteins
A
s = ±3.06%
β-actin
α-tubulin
GAPDH
histone H
KDM4D
HDAC10
MBD4
DMAP1
DNMT1
PCAF
+5%
-5%
control
12H
24H
48H
Epigenetic modification-related proteins
B
histone H1
KDM4D
HDAC10
MBD4
DMAP1
DNMT1
PCAF
control
12H
24H
48H
Protein translation-related proteins
C
s = ±3.06%
β-actin
α-tubulin
GAPDH
DOHH
DHS
eIF5A1
eIF5A2
eIF2AK3
Protein translation-related proteins
D
DOHH
DHS
eIF5A1
eIF5A2
eIF2AK3
Growth Factor-related proteins
E
s = ±3.06%
IGFIIR
GH
GHRH
HER1
HER2
FGF-1
FGF-2
CTGF
PDGF-A
IGF-1
ERβ
TGF-β1
TGF-β2
TGF-β3
SMAD4
FGF-7
HGFα
Met
Growth Factor-related proteins
F
FGF-1
FGF-2
CTGF
PDGF-A
IGF-1
IGFIIR
GH
GHRH
HER1
HER2
ERβ
TGF-β1
TGF-β2
TGF-β3
SMAD4
FGF-7
HGFα
Met
RAS signaling proteins
G
s = ±2.73%
ERK-1
p-ERK
pAKT1/2/3
mTOR
NRAS
KRAS
HRAS
PI3K
JNK-1
PTEN
RAF-B
STAT3
Rab 1
NF-1
PKC
p-PKC
AKAP
caveolin-1
AMPK
SOS1
SOS2
RAS signaling proteins
H
NRAS
KRAS
HRAS
PI3K
JNK-1
ERK-1
p-ERK
pAKT1/2/3
mTOR
PTEN
RAF-B
STAT3
Rab 1
NF-1
PKC
p-PKC
AKAP
caveolin-1
AMPK
SOS1
SOS2

Figure 4

Expressions of NFkBsignaling proteins, inflammatory proteins were upregulated,and inflammatory proteins downregulated

Figure 4. Expressions of NFkB signaling proteins (A and B), inflammatory proteins were upregulated (C or D), and inflammatory proteins downregulated (E and F) in pamidronate treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, and E show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, and F) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).

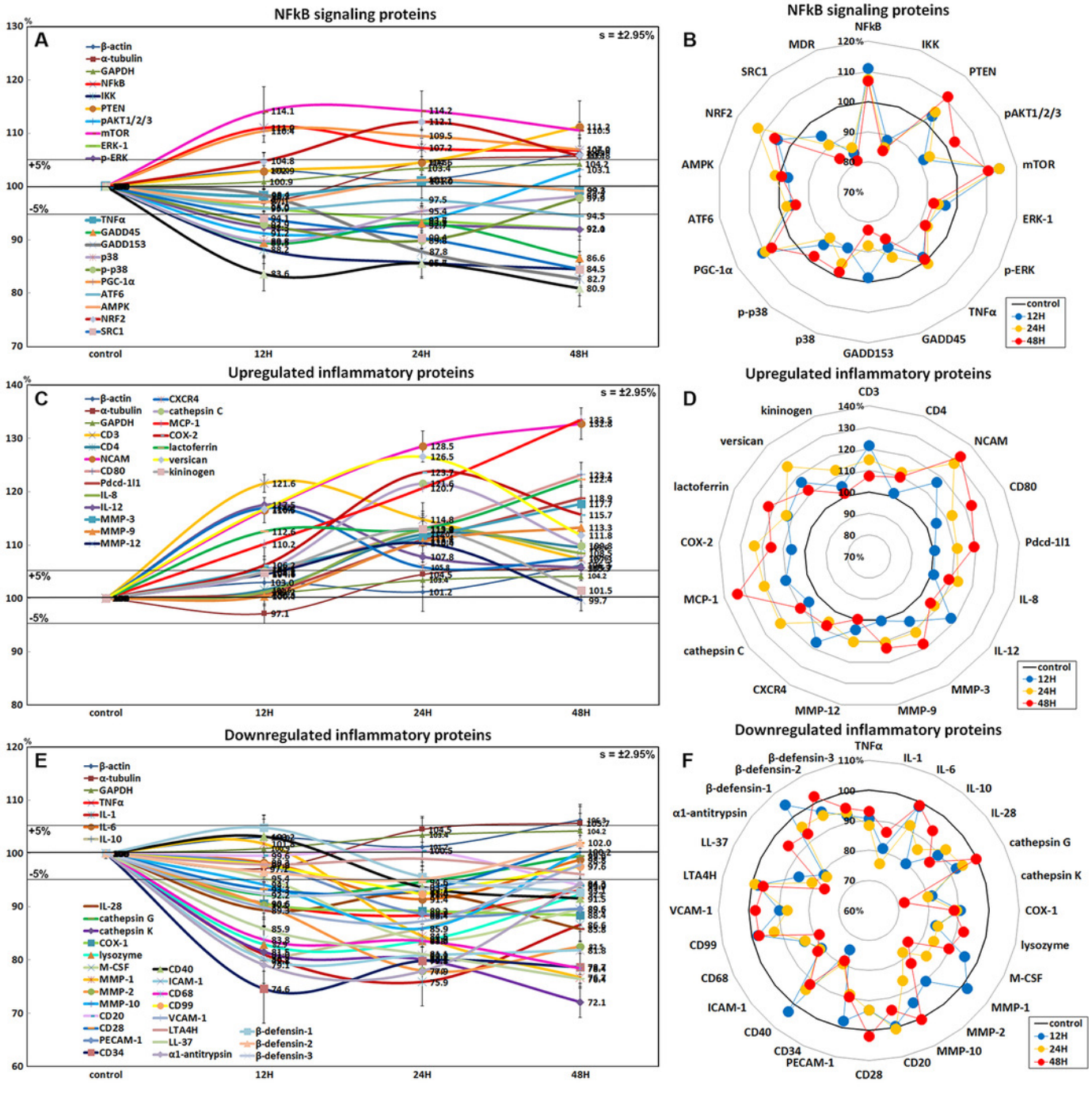
NFkB signaling proteins
A
s = ±2.95%
β-actin
α-tubulin
GAPDH
NFkB
IKK
PTEN
pAKT1/2/3
mTOR
ERK-1
p-ERK
TNFα
GADD45
GADD153
p38
p-p38
PGC-1α
ATF6
AMPK
NRF2
SRC1
+5%
-5%
control
12H
24H
48H
NFkB signaling proteins
B
NFkB
MDR
IKK
PTEN
pAKT1/2/3
mTOR
ERK-1
p-ERK
TNFα
GADD45
GADD153
p38
p-p38
PGC-1α
ATF6
AMPK
NRF2
SRC1
control
12H
24H
48H
Upregulated inflammatory proteins
C
s = ±2.95%
β-actin
α-tubulin
GAPDH
CD3
CD4
NCAM
CD80
Pdcd-1l1
IL-8
IL-12
MMP-3
MMP-9
MMP-12
CXCR4
cathepsin C
MCP-1
COX-2
lactoferrin
versican
kininogen
+5%
-5%
control
12H
24H
48H
Upregulated inflammatory proteins
D
CD3
CD4
NCAM
CD80
Pdcd-1l1
IL-8
IL-12
MMP-3
MMP-9
MMP-12
CXCR4
cathepsin C
MCP-1
COX-2
lactoferrin
versican
kininogen
control
12H
24H
48H
Downregulated inflammatory proteins
E
s = ±2.95%
β-actin
α-tubulin
GAPDH
TNFα
IL-1
IL-6
IL-10
IL-28
cathepsin G
cathepsin K
COX-1
lysozyme
M-CSF
MMP-1
MMP-2
MMP-10
CD20
CD28
PECAM-1
CD34
CD40
ICAM-1
CD68
CD99
VCAM-1
LTA4H
LL-37
α1-antitrypsin
β-defensin-1
β-defensin-2
β-defensin-3
+5%
-5%
control
12H
24H
48H
Downregulated inflammatory proteins
F
TNFα
IL-1
IL-6
IL-10
IL-28
cathepsin G
cathepsin K
COX-1
lysozyme
M-CSF
MMP-1
MMP-2
MMP-10
CD20
CD28
PECAM-1
CD34
CD40
ICAM-1
CD68
CD99
VCAM-1
LTA4H
LL-37
α1-antitrypsin
β-defensin-1
β-defensin-2
β-defensin-3
control
12H
24H
48H

Figure 5

Expressions ofp53-mediated apoptosis-related proteins, FAS-mediated apoptosis-relatedproteins, and cell survival-related proteins

Figure 5. Expressions of p53-mediated apoptosis-related proteins (A and B), FAS-mediated apoptosis-related proteins (C or D), and cell survival-related proteins (E and F) in RAW 264.7 cells treated with pamidronate for different times as determined by IP-HPLC. Line graphs, A, C, and E show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, and F) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).

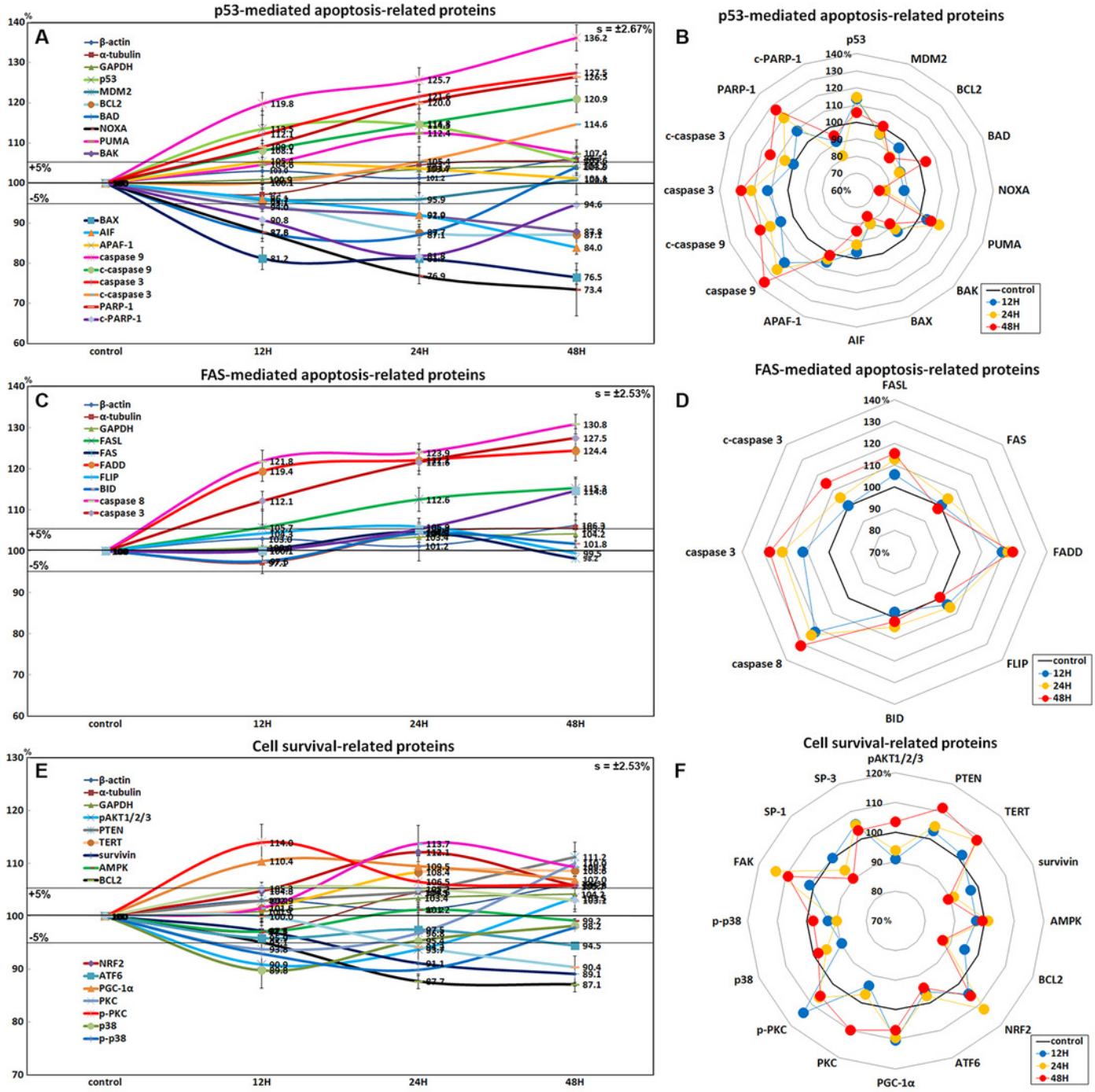
p53-mediated apoptosis-related proteins
A
s = ±2.67%
β-actin
α-tubulin
GAPDH
p53
MDM2
BCL2
BAD
NOXA
PUMA
BAK
BAX
AIF
APAF-1
caspase 9
c-caspase 9
caspase 3
c-caspase 3
PARP-1
c-PARP-1
+5%
-5%
control
12H
24H
48H
p53-mediated apoptosis-related proteins
B
p53
MDM2
BCL2
BAD
NOXA
PUMA
BAK
BAX
AIF
APAF-1
caspase 9
caspase 3
c-caspase 9
c-caspase 3
PARP-1
c-PARP-1
control
12H
24H
48H
FAS-mediated apoptosis-related proteins
C
s = ±2.53%
β-actin
α-tubulin
GAPDH
FASL
FAS
FADD
FLIP
BID
caspase 8
caspase 3
+5%
-5%
control
12H
24H
48H
FAS-mediated apoptosis-related proteins
D
FASL
FAS
FADD
FLIP
BID
caspase 8
caspase 3
c-caspase 3
control
12H
24H
48H
Cell survival-related proteins
E
s = ±2.53%
β-actin
α-tubulin
GAPDH
pAKT1/2/3
PTEN
TERT
survivin
AMPK
BCL2
NRF2
ATF6
PGC-1α
PKC
p-PKC
p38
p-p38
+5%
-5%
control
12H
24H
48H
Cell survival-related proteins
F
pAKT1/2/3
PTEN
TERT
survivin
AMPK
BCL2
NRF2
ATF6
PGC-1α
PKC
p-PKC
p38
p-p38
FAK
SP-1
SP-3
control
12H
24H
48H

Figure 6

Expressions of cellprotection-related proteins, differentiation-related proteins,and oncogenesis-related proteins

Figure 6. Expressions of cell protection-related proteins (A and B), differentiation-related proteins (C or D), and oncogenesis-related proteins (E and F) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A, C, and E show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B, D, and F) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).

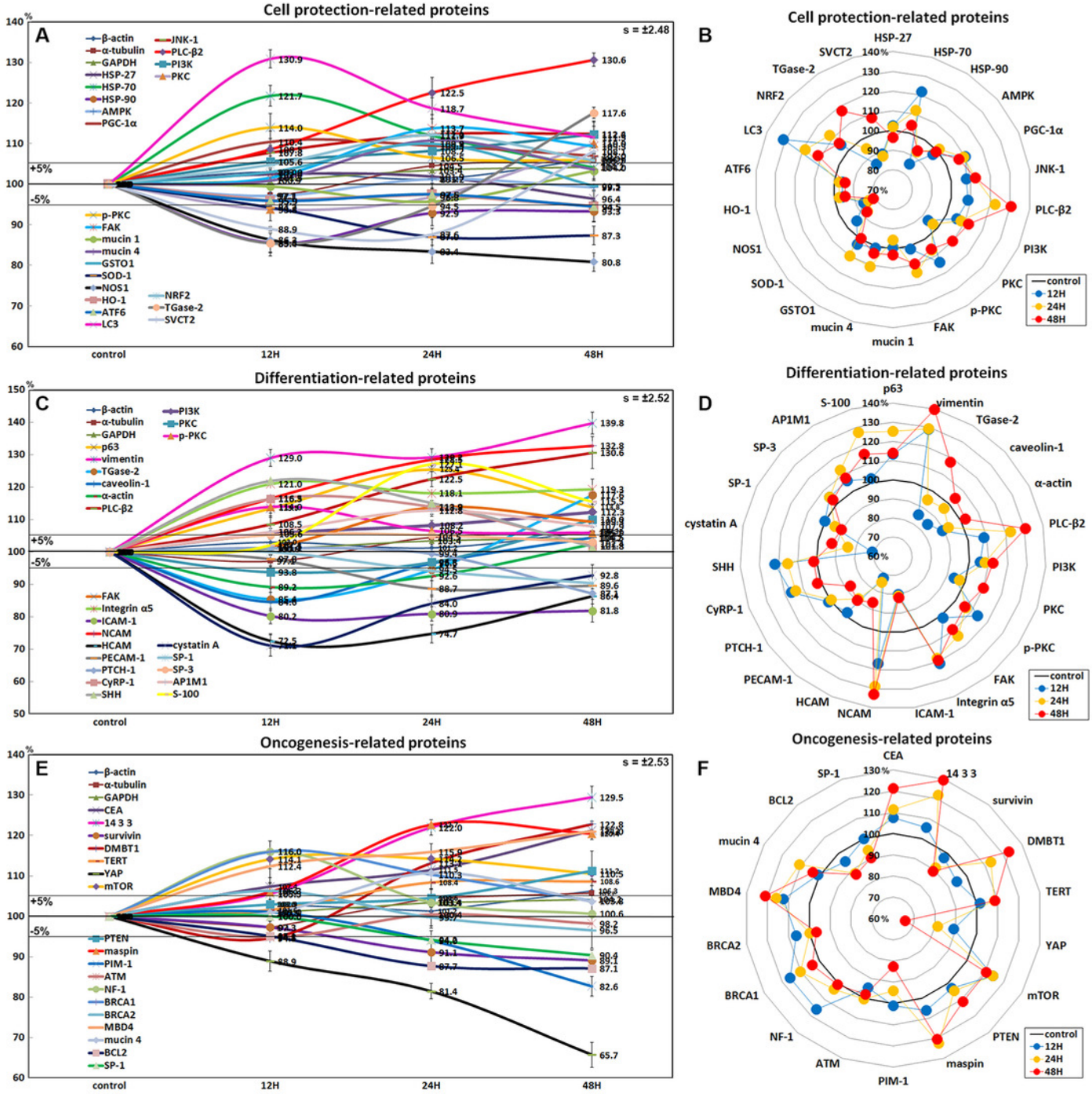
Cell protection-related proteins
A
s = ±2.48
β-actin
α-tubulin
GAPDH
HSP-27
HSP-70
HSP-90
AMPK
PGC-1α
JNK-1
PLC-β2
PI3K
PKC
p-PKC
FAK
mucin 1
mucin 4
GSTO1
SOD-1
NOS1
HO-1
ATF6
LC3
NRF2
TGase-2
SVCT2
+5%
-5%
control
12H
24H
48H
Cell protection-related proteins
B
HSP-27
HSP-70
HSP-90
AMPK
PGC-1α
JNK-1
PLC-β2
PI3K
PKC
p-PKC
FAK
mucin 1
mucin 4
GSTO1
SOD-1
NOS1
HO-1
ATF6
LC3
NRF2
TGase-2
SVCT2
control
12H
24H
48H
Differentiation-related proteins
C
s = ±2.52
β-actin
α-tubulin
GAPDH
p63
vimentin
TGase-2
caveolin-1
α-actin
PLC-β2
PI3K
PKC
p-PKC
FAK
Integrin α5
ICAM-1
NCAM
HCAM
PECAM-1
PTCH-1
CyRP-1
SHH
cystatin A
SP-1
SP-3
AP1M1
S-100
+5%
-5%
control
12H
24H
48H
Differentiation-related proteins
D
p63
vimentin
TGase-2
caveolin-1
α-actin
PLC-β2
PI3K
PKC
p-PKC
FAK
Integrin α5
ICAM-1
NCAM
HCAM
PECAM-1
PTCH-1
CyRP-1
SHH
cystatin A
SP-1
SP-3
AP1M1
S-100
control
12H
24H
48H
Oncogenesis-related proteins
E
s = ±2.53
β-actin
α-tubulin
GAPDH
CEA
14 3 3
survivin
DMBT1
TERT
YAP
mTOR
PTEN
maspin
PIM-1
ATM
NF-1
BRCA1
BRCA2
MBD4
mucin 4
BCL2
SP-1
+5%
-5%
control
12H
24H
48H
Oncogenesis-related proteins
F
CEA
14 3 3
survivin
DMBT1
TERT
YAP
mTOR
PTEN
maspin
PIM-1
ATM
NF-1
BRCA1
BRCA2
MBD4
mucin 4
BCL2
SP-1
control
12H
24H
48H

Figure 7

Expressions ofangiogenesis-related proteins and of osteogenesis-related proteins

Figure 7. Expressions of angiogenesis-related proteins (A and B) and of osteogenesis-related proteins (C or D) in pamidronate-treated RAW 264.7 cells as determined by IP-HPLC. Line graphs, A and C show protein expressional changes on the same scale (%) versus culture time (12, 24, or 48 hours), whereas the star plots (B and D) showed the differential expression levels of proteins after 12, 24, or 48 hours of treatment on appropriate scales (%). Standard error (s).

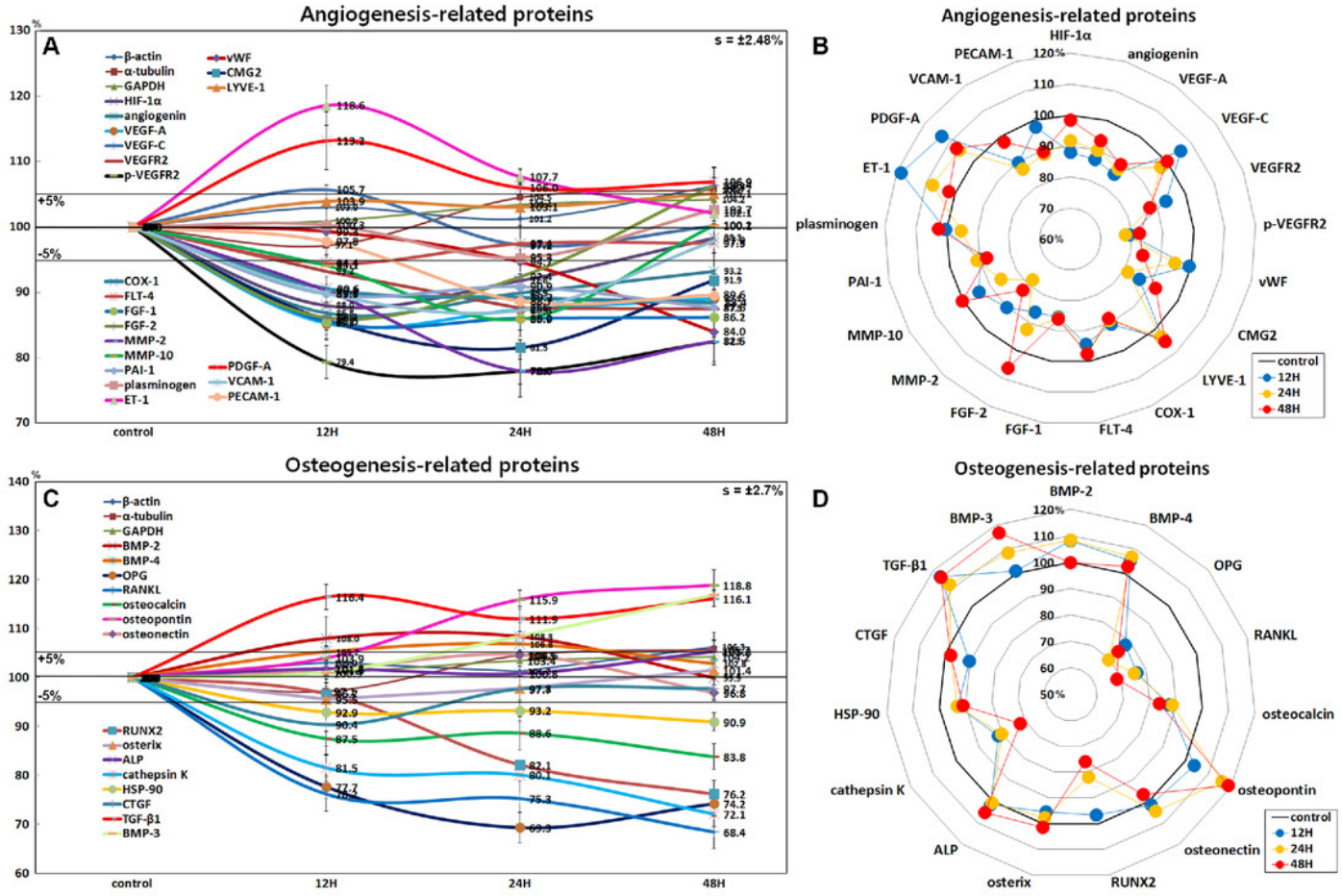

Figure 8

Star plot of global protein expression inpamidronate-treated RAW 264.7 cells

Figure 8. Star plot of global protein expression in pamidronate-treated RAW 264.7 cells. Representative proteins (n=73) of each signaling pathway are plotted in a circular manner. The expressions of proliferation, some growth factors, cellular apoptosis, protection, and differentiation-related proteins were upregulated, while the expressions of protein translation-, cell survival-, angiogenesis-, and osteogenesis-related proteins were downregulated. RAS signaling and NFkB signaling were suppressed by the up-regulations of the downstream effector proteins, ERK-1 (p-ERK-1) and p38 (p-p38), respectively. The expressions of inflammatory proteins and oncogenesis-related proteins in RAW 264.7 cells were variably altered, but epigenetic methylation was increased by pamidronate treatment. Blue, yellow, and red spots indicate after 12, 24, and 48 hours of pamidronate treatment, respectively.

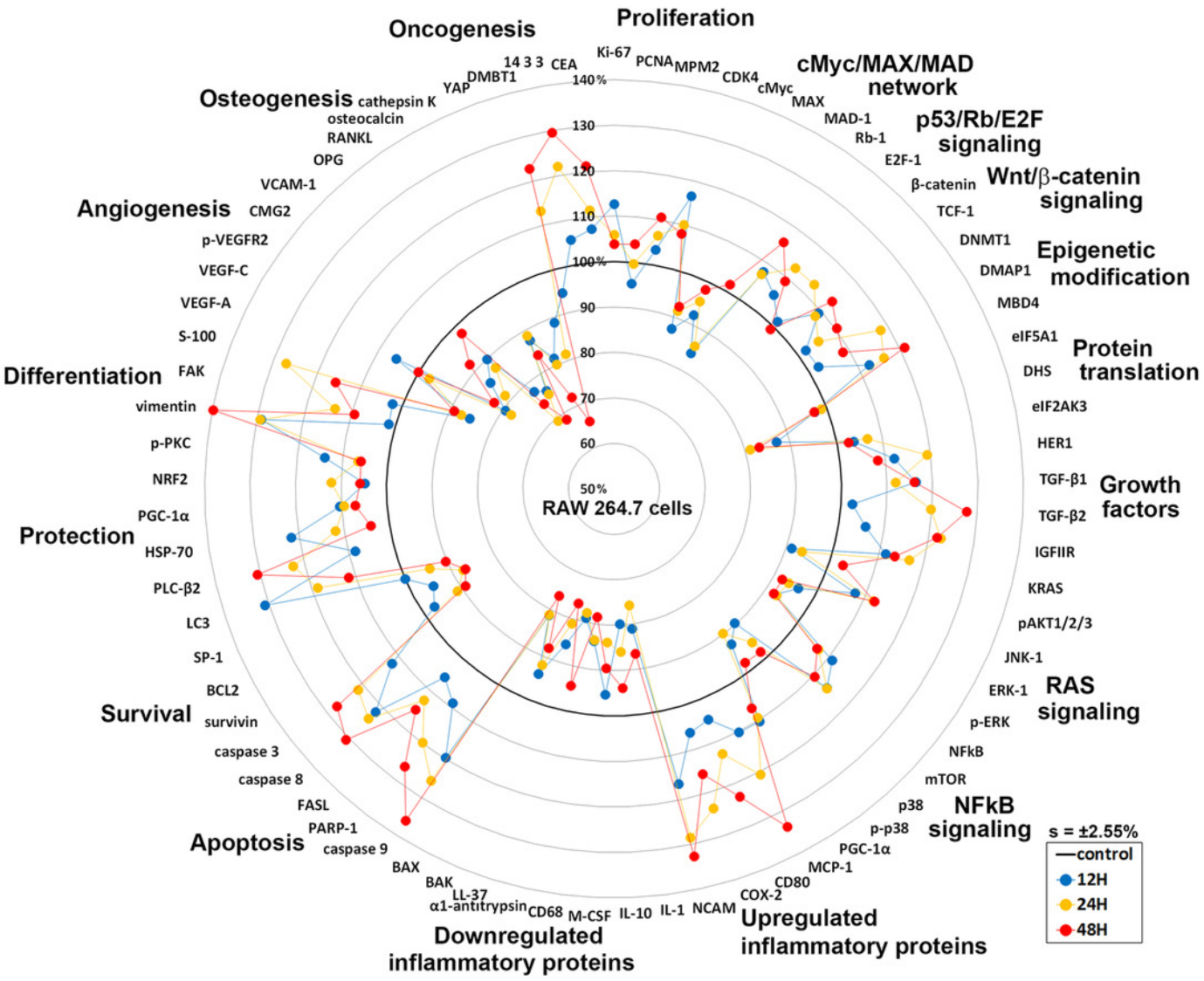
Proliferation
Ki-67
PCNA
MPM2
CDK4
cMyc/MAX/MAD network
cMyc
MAX
MAD-1
p53/Rb/E2F signaling
Rb-1
E2F-1
β-catenin
Wnt/β-catenin signaling
TCF-1
DNMT1
DMAP1
Epigenetic modification
MBD4
eIF5A1
DHS
Protein translation
eIF2AK3
HER1
TGF-β1
TGF-β2
Growth factors
IGFIIR
KRAS
pAKT1/2/3
JNK-1
ERK-1
RAS signaling
p-ERK
NFkB
mTOR
p38
p-p38
NFkB signaling
PGC-1α
MCP-1
CD80
COX-2
NCAM
IL-1
IL-10
M-CSF
CD68
α1-antitrypsin
LL-37
BAK
BAX
caspase 9
PARP-1
FASL
caspase 8
caspase 3
survivin
Apoptosis
BCL2
SP-1
LC3
PLC-β2
HSP-70
Protection
PGC-1α
NRF2
p-PKC
vimentin
Differentiation
FAK
S-100
VEGF-A
VEGF-C
p-VEGFR2
CMG2
Angiogenesis
VCAM-1
OPG
RANKL
osteocalcin
cathepsin K
Osteogenesis
YAP
DMBT1
14 3 3
CEA
140%
130
120
110
100%
90
80
70
60
50%
RAW 264.7 cells
Downregulated inflammatory proteins
Upregulated inflammatory proteins
s = ±2.55%
control
12H
24H
48H

Figure 9

Highly up- and down-regulated proteins by pamidronatein RAW 264.7 cells

Figure 9. Highly up- and down-regulated proteins by pamidronate in RAW 264.7 cells. The cells were reactive to pamidronate by marked upregulation of some proteins for cellular proliferation, protection, differentiation, RAS signaling, NFkB signaling, and oncogenic proteins, but gradually degenerated by marked downregulation of M2 macrophage differentiation-related inflammatory proteins and survival-related proteins and by marked upregulation of apoptosis-related proteins. The major protein expressions for angiogenesis and osteoclastogenesis were dramatically suppressed (A-C). Blue, yellow, and red spots indicate after 12, 24, and 48 hours of pamidronate treatment, respectively.

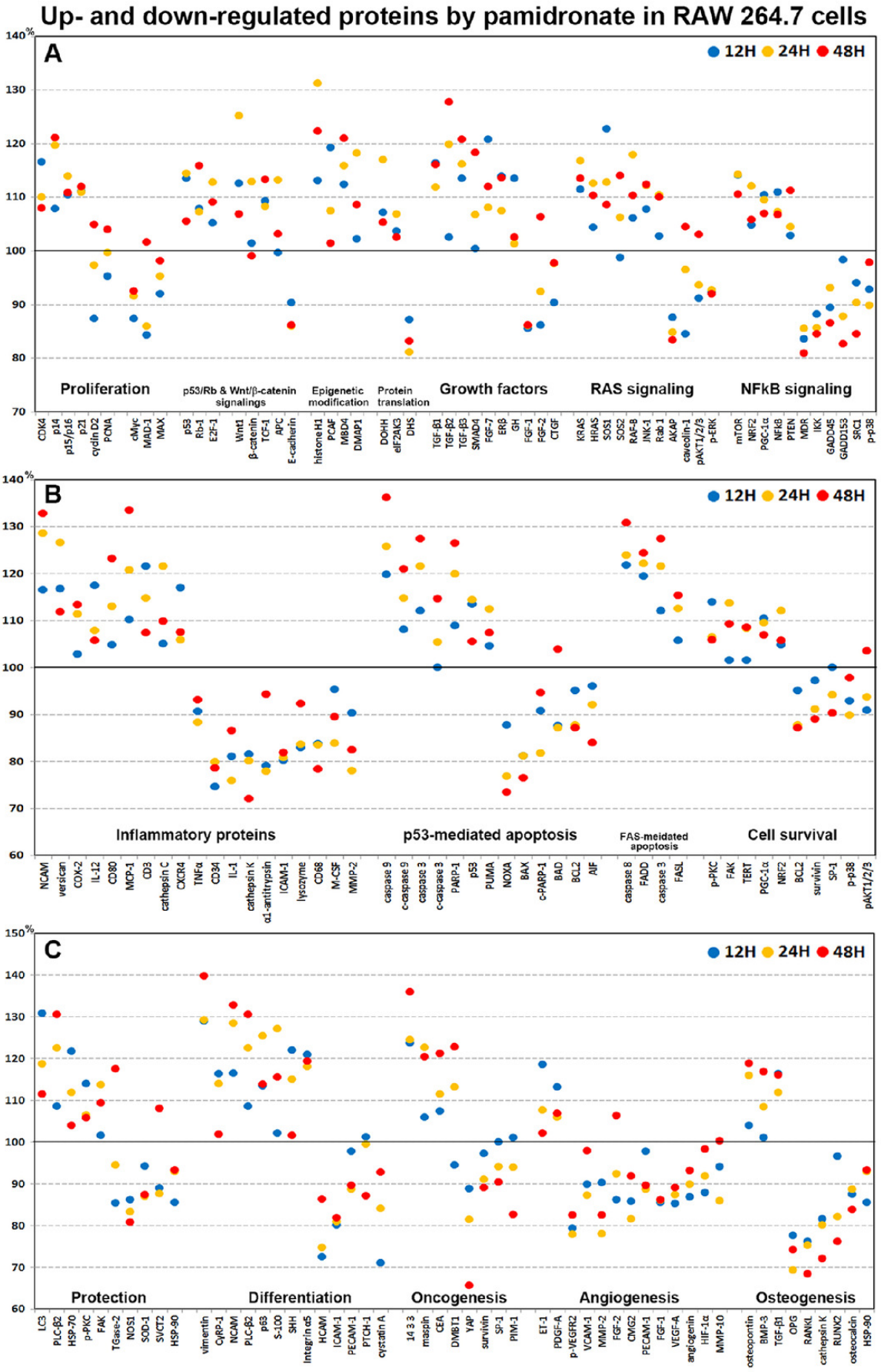
Up- and down-regulated proteins by pamidronate in RAW 264.7 cells
A
12H 24H 48H
Proliferation
p53/Rb & Wnt/β-catenin signalings
Epigenetic modification
Protein translation
Growth factors
RAS signaling
NFkB signaling
B
Inflammatory proteins
p53-mediated apoptosis
FAS-meidated apoptosis
Cell survival
C
Protection
Differentiation
Oncogenesis
Angiogenesis
Osteogenesis

Table 1(on next page)

Antibodies used in the study

Antibodies used in the study.

Table 1. Antibodies used in the study.

Proteins	No.	Antibodies
Proliferation-related	11	Ki-67*, PCNA*, CDK4*, MPM2*, PLK4*, cyclin D2, p14*, p16*, p21*, p27*, lamin A/C
cMyc/MAX/MAD network	3 (1)	cMyc*, MAX*, MAD-1*, (p27)
p53/Rb/E2F signaling	4 (2)	p53, Rb-1#, E2F-1*, MDM2, (p21, CDK4)
Wnt/β-catenin signaling	6	Wnt1*, β-catenin*, APC*, snail*, TCF-1*, E-cadherin
Epigenetic modification	7	histone H1*, DMAP1*, KDM4D$, HDAC-10$, MBD4*, DNMT1*, PCAF*
Protein translation	5	DOHH!, DHS!, eIF5A-1!, eIF5A-2!, eIF2AK3*
Growth factor	18	FGF-1*, FGF-2*, FGF-7*, CTGF, HGFα*, TGF-β1#, TGF-β2*, TGF-β3*, SMAD4*, PDGF-A*, IGF-1*, IGFIIR*, GH*, GHRH*, HER1*, HER2*, ERβ*, Met*
RAS signaling	22	NRAS$, KRAS$, HRAS, STAT3*, PI3K, pAKT1/2/3, RAF-B*, JAK2$, JNK-1*, ERK-1*, p-ERK-1$, Rab 1*, mTOR, PTEN, NF-1, AKAP, caveolin-1, AMPK@, SOS-1*, SOS-2*, PKC*, p-PKC@
NFkB signaling	12 (6)	NFkB*, IKK*, TNFα, GADD45*, GADD153*, MDR, p38*, p-p38*, PGC-1α, ATF6, NRF-2*, SRC-1*, (pAKT1/2/3, PTEN, ERK1*, p-ERK*, AMPK, mTOR@,)
Upregulated inflammatory proteins	17	CD3, CD4, NCAM (CD56), CD80 (B7-1), Pdcd-1/1 (CD279), IL-8, IL-12, MMP-3$, -9$, -12$, CXCR4*, cathepsin C, MCP-1, COX2*, lactoferrin, versican, kininogen
Downregulated inflammatory proteins	27 (1)	IL-1*, IL-6*, IL-10*, IL-28*, cathepsin G*, cathepsin K*, COX1, lysozyme*, M-CSF, MMP-1, -2, -10, CD20, CD28, PECAM-1 (CD31), CD34, CD40, ICAM-1 (CD54), CD68, CD99, VCAM-1 (CD106), LTA4H&, LL-37, α1- antitrypsin &, β-defensin-1, β-defensin-2, β-defensin-3, (TNFα@)
p53-mediated apoptosis	15 (2)	PUMA*, NOXA*, BCL2*, BAX*, BAD*, BAK*, BID*, AIF*, APAF-1*, caspase 9*, c-caspase 9*, caspase 3*, c-caspase 3*, PARP-1*, c-PARP-1*, (MDM2*, p53*)
FAS-mediated apoptosis	5 (3)	FASL*, FAS*, FADD*, FLIP*, caspase 8*, (BID*, caspase 3*, c-caspase 3*)
Cell survival-related	5 (11)	TERT*, survivin@, SP-1@, SP-3@, FAK, (pAKT1/2/3, PTEN, AMPK, BCL2, NRF2, ATF6, PGC-1α, PKC, p-PKC, p38, p-p38)
Protection-related	12 (13)	HSP-27*, HSP-70*, HSP-90*, TGase 2$, LC3, mucin 1, mucin 4, HO-1*, SOD-1*, GSTO1*, SVCT2&, NOS-1$, (PLC-β2, PI3K, PKC*, p-PKC*, FAK*, caveolin-1*, PGC-1α*, AMPK, JNK-1, PLC-β2, PI3K, ATF6, NRF2)
Differentiation-related	11 (11)	p63$, vimentin, α-actin, PTCH-1, CyRP, SHH, cystatin A, S-100. integrin α5, HCAM (CD44), (caveolin-1, SP-1, SP-3, PLC-β2, PI3K, PKC, p-PKC, FAK, AP1M1, ICAM-1 (CD54), NCAM (CD56), PECAM (CD31))
Oncogenesis-related	10 (10)	BRCA1&, BRCA2&, NF-1*, ATM*, CEA$, 14-3-3*, maspin*, DMBT1*, YAP, PIM1, (MBD4, BCL2, SP-1, PTEN&, mucin 1, mucin 4, survivin@, TERT*, pAKT1/2/3*, mTOR)
Angiogenesis-related	14 (9)	HIF-1α&, VEGF-A*, VEGF-C*, angiogenin$, LYVE-1*, CMG2$, vWF$, FLT-4$, ET-1*, PAI-1*, VEGFR2*, p-VEGFR2, plasminogen*, leptin*, (CD31, MMP-2, MMP-10, FGF-1, FGF-2, PDGF-A, PECAM-1 (CD31), VCAM (CD106), COX1)
Osteogenesis-related	11 (4)	OPG*, RANKL*, BMP-2*, BMP-3*, BMP-4*, ALP*, osteocalcin*, osteopontin*, osteonectin*, RUNX2*, osterix*, (HSP-90, cathepsin K, CTGF, TGF-β1)
Control housekeeping proteins	3	α-tubulin*, β-actin*, GAPDH*
Total	218 (73)	

* Santa Cruz Biotechnology, USA; # DAKO, Denmark; $ Neomarkers, CA, USA; @ ZYMED, CA, USA; &Abcam, Cambridge, UK; ! kindly donated from M. H. Park in NIH, USA (Park and Wolff 2018), the number of antibodies overlapped; ( ).

Abbreviations: AIF; apoptosis inducing factor, AKAP; A-kinase anchoring proteins, ALP; alkaline phosphatase, AMPK; AMP-activated protein kinase, pAKT; v-akt murine thymoma viral oncogene homolog, p-Akt1/2/3 phosphorylated (p-Akt, Thr 308), APAF-1; apoptotic protease-activating factor 1, APC; adenomatous polyposis coli, ATF6; activating transcription factor 6, ATM; ataxia telangiectasia caused by mutations, BAD; BCL2 associated death promoter, BAK; BCL2 antagonist/killer, BAX; BCL2 associated X, BCL-2; B-cell leukemia/lymphoma-2, BID; BH3 interacting-domain death agonist, BMP-2; bone morphogenesis protein

2, BRCA1; breast cancer type 1 susceptibility protein, c-caspase 3; cleaved-caspase 3, CD3; cluster of differentiation 3, CDK4; cyclin dependent kinase 4, CEA; carcinoembryonic antigen, CMG2: capillary morphogenesis protein 2, COX-1; cyclooxygenase-2, CTGF connective tissue growth factor, CXCR4; C-X-C chemokine receptor type 4, CyRP-1; cystein rich protein, DHS; deoxyhypusine synthase, DMAP1; DNA methyltransferase 1 associated protein, DMBT1; deleted in malignant brain tumors 1, DNMT1; DNA 5-cytosine methyltransferase 1, DOHH; deoxyhypusine hydroxylase, E2F-1; transcription factor, eIF2AK3 (PERK); eukaryotic translation initiation factor 2 (protein kinase R (PKR)-like endoplasmic reticulum kinase), elF5A-1; eukaryotic translation initiation factor 5A-1, ERK-1; extracellular signal-regulated protein kinase 1, ERβ; estrogen receptor beta, ET-1: endothelin-1, FADD; FAS associated via death domain, FAK; focal adhesion kinase, FAS; CD95/Apo1, FASL; FAS ligand, FGF-1; fibroblast growth factor-1, FLIP; FLICE-like inhibitory protein, FLT-4; Fms-related tyrosine kinase 4, GADD45; growth arrest and DNA-damage-inducible 45, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, GH; growth hormone, GHRH; growth hormone-releasing hormone, GSTO1; glutathione S-transferase ω 1, HCAM (CD44); homing cell adhesion molecule, HDAC-10; histone deacetylase 10,, HER1; human epidermal growth factor receptor 1, HGF-α; hepatocyte growth factor α, HIF-1α: hypoxia inducible factor-1α, HO-1; heme oxygenase 1, HRAS; GTPase HRas, HSP-70; heat shock protein-70, ICAM (CD54); intercellular adhesion molecule 1, IGF-1; insulin-like growth factor 1, IGFIIR; insulin-like growth factor 2 receptor, IKK; ikappaB kinase, IL-1; interleukin-1, JNK-1; Jun N-terminal protein kinase, KDM4D; Lysine-specific demethylase 4D, KRAS; V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, LC3; microtubule-associated protein 1A/1B-light chain 3, LTA4H; leukotriene A4 hydrolase, LYVE-1: lymphatic vessel endothelial hyaluronan receptor 1, MAD-1; mitotic arrest deficient 1, MAX; myc-associated factor X, MBD4; methyl-CpG-binding domain protein 4, MCP-1; monocyte chemotactic protein 1, M-CSF; macrophage colony-stimulating factor, MDM2; mouse double minute 2 homolog, MDR; multiple drug resistance, MMP-1; matrix metalloprotease-1, MPM2; mitotic protein monoclonal 2, mTOR; mammalian target of rapamycin, cMyc; V-myc myelocytomatosis viral oncogene homolog, NFkB; nuclear factor kappa-light-chain-enhancer of activated B cells, NCAM (CD56); neural cell adhesion molecule 1, NF-1; neurofibromin 1, NFkB; nuclear factor kappa-light-chain-enhancer of activated B cells, NOS-1; nitric oxide synthase 1, NOXA; Phorbol-12-myristate-13-acetate-induced protein 1, NRAS; neuroblastoma RAS Viral Oncogene homolog, NRF2; nuclear factor (erythroid-derived)-like 2, OPG; osteoprotegerin, PAI-1; plasminogen activator inhibitor-1, PARP-1; poly-ADP ribose polymerase 1, c-PARP-1; cleaved-PARP-1, PCNA; proliferating cell nuclear antigen, Pdcd-1/1 (CD279); programmed cell death protein 1, PDGF-A: platelet-derived growth factor-A, PECAM-1 (CD31); platelet endothelial cell adhesion molecule-1, PGC-1α; peroxisome proliferator-activated receptor gamma coactivator 1-α, PI3K; phosphatidylinositol-3-kinase, PIM-1; Proto-oncogene serine/threonine-protein kinase 1, PKC; protein kinase C, PLC-β2; 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterse β-2, PLK4; polo like kinase 4 or serine/threonine-protein kinase, PTEN; phosphatase and tensin homolog, PUMA; p53 upregulated modulator of apoptosis, Rab 1; Rab GTPases, RAF-B; v-Raf murine sarcoma viral oncogene homolog B, RANKL; receptor activator of nuclear factor kappa-B ligand, Rb-1; retinoblastoma-1, RUNX2; Runt-related transcription factor-2, SHH; sonic hedgehog, SMAD4; mothers against decapentaplegic, drosophila homolog 4, SOD-1; superoxide dismutase-1, SOS-1; son of sevenless homolog 1, SP-1; specificity protein 1, SRC1; steroid receptor coactivator-1, STAT3; signal transducer and activator of transcription-3, SVCT2; sodium-dependent vitamin C transporter 2, TERT; human telomerase reverse transcriptase, TGase-2; transglutaminase 2, TGF-β1; transforming growth factor-β1, TNFα; tumor necrosis factor-α, VCAM; vascular cell adhesion molecule-1, VEGF-A vascular endothelial growth factor A, VEGFR2: vascular endothelial growth factor receptor 2, p-VEGFR2: vascular endothelial growth factor receptor 2 (Y951), vWF: von Willebrand factor, Wnt1; proto-oncogene protein Wnt-1, YAP; Yes-associated protein.