Role of interleukin-24 in the tumor-suppressive effects of interferon-β on melanoma

Running title: Role of IL-24 in IFN-β treatment against melanoma

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Abstract

Background: Type 1 interferons (IFNs) including IFN-β, are widely used in adjuvant therapy for patients who undergo surgery for malignant melanoma to inhibit recurrence and in-transit metastasis. The precise mechanisms underlying the tumor-suppressive effects of IFN-β on melanoma are not yet completely understood.

Objective: The purpose was to reveal the mechanisms underlying the tumor-suppressive effects of IFN-β via interleukin (IL)-24.

Methods: Genome-wide oligonucleotide microarray, quantitative real-time reverse transcription-polymerase chain reaction (PCR), enzyme-linked immunosorbent assay and western blotting assay were performed using four melanoma cell lines (A375, RPMI-7951, SK-MEL-5 and SK-MEL-1) treated with natural-type IFN-β to assess the expression of IL-24. Proliferation assay was performed using these melanoma cells and IL-24-knock-down melanoma cells.

Results: Genome-wide microarray analysis detected candidate genes up-regulated in IFN-β-sensitive cells after treatment with IFN-β. We focused on *IL24* among the candidate genes encoding secretory proteins. Peak *IL24* mRNA expression completely correlated with the order of sensitivity of melanoma cells to IFN-β. IFN-β-treatment induced extracellular IL-24 protein in IFN-β-sensitive cells, but did not induce intracellular IL-24 protein. Knockdown of IL-24 changed melanoma cells into IFN-β-resistant cells. The expression ratio of IL-22R1, one of IL-24 receptors, correlated with the order of sensitivity of melanoma cells to IFN-β. Treatment with recombinant human IL-24 did not have any effects on all the melanoma cell lines.

Conclusion: Our data suggest that IFN-β suppresses the proliferation of melanoma cells through extracellular IL-24 protein derived from melanoma cells.

1. Introduction

Melanoma is a neoplasm that develops *de novo* or from pre-existing melanocytic lesions [1]. Melanoma accounts for only 4% of all skin cancers, but is responsible for 80% of all deaths from skin cancers [1]. The prognosis of patients with melanoma is dependent on the clinical stage of the disease. The prognosis of patients with early-stage melanoma is excellent, and more than 80% of these patients achieve 5-year survival [2]. Conversely, prognosis remains poor for patients with advanced-stage melanoma, such as stage IIIC, IIID and IV [2].

New therapeutic agents for melanoma, including immune checkpoint inhibitors and BRAF and MEK inhibitors, have recently come into wide use [2,3], due to their great effectiveness in some patients with advanced melanoma. However, a multicenter, open-label, randomized, controlled, phase 3 study showed that objective response rates of pembrolizumab and ipilimumab, classified as immune checkpoint inhibitors, were 42% and 17%, respectively [4]. A double-blinded, phase 3 study of combination therapy with dabrafenib and trametinib (classified as BRAF and MEK inhibitors, respectively) showed an objective response rate of 68% patients with BRAF mutation [5], whereas rates of BRAF mutation in Caucasian and Japanese melanoma patients were only 40-50% and 20.6%, respectively [6,7]. Actually, the 5-year survival rate for all melanoma patients at stage IIID or IV was less than 40% [2].

Type 1 interferons (IFNs), including IFN-β, have been widely used as therapies for viral hepatitis and melanoma [8], because type 1 IFNs show potent antiviral and tumor suppressive effects. In terms of melanoma therapy, some studies have demonstrated that type 1 IFNs improved the overall survival rates for stage II and resectable stage III melanoma [9,10]. Prospective, randomized, multicenter treatment trials have demonstrated that high-dose IFN-α-2b and pegylated IFN-α improved relapse-free

survival [9]. Another study demonstrated that adjuvant therapy with IFN-α-2b significantly improved both relapse-free and overall survival rates in patients with high-risk melanoma [10].

Natural-type IFN-β is currently used in Japan as a postoperative adjuvant therapy for patients with melanoma. Yamamoto et al. reported that the 5-year overall survival rate was significantly higher in the patient group on IFN-β maintenance therapy with local injection of natural-type IFN-β at a dose of 3×10^6 international units (IU) once or twice every 4 weeks, than in the control group [11]. Considering these facts, IFN-β contributes to the inhibition of melanoma recurrence and in-transit metastasis.

However, some patients have experienced recurrence of melanoma and in-transit metastasis during IFN-β-adjuvant therapy, suggesting that some melanoma cells may be resistant to IFN-β. A better understanding of the precise mechanisms by which IFN-β inhibits melanoma cells will potentially lead to improvements in IFN-β therapy for melanoma. In this context, we have previously reported CXCL10 as a likely IFN-β-induced melanoma-suppressive molecule [12]. The present study identified interleukin (IL)-24 as an IFN-β-induced melanoma-suppressive molecule.

2. Material and methods

2.1. Cells and culture

Human melanoma cell lines (SK-MEL-1, SK-MEL-5, A375 and RPMI-7951) and human primary epidermal melanocytes (HEMs) were purchased from the American Type Culture Collection (Manassas, VA) [13–15]. All cells were cultured in a humidified atmosphere (5% CO_2) at 37 °C according to the instructions from the manufacturer.

2.2. Treatment with IFN-β and recombinant human IL-24

Natural-type IFN-β protein produced by human fibroblasts, which contains sugar chains, was kindly provided by Toray Medical (Tokyo, Japan). Melanoma cells (RPMI-7951, A375, SK-MEL-5 and SK-MEL-1) and HEMs were treated with IFN-β at a final concentration of 1,000 IU/ml in complete culture medium. Melanoma cells were treated with recombinant human IL-24 protein (rhIL-24) (R&D Systems, Minneapolis, MN) at a final concentration of 0, 20 or 100 ng/ml in complete culture medium.

2.3. Oligonucleotide microarray analysis

Using an RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany), total RNA was extracted from melanoma cells after treatment with or without IFN-β at a concentration of 1,000 IU/ml for 48 h. For expression profiling, oligonucleotide microarray analysis was performed at Hokkaido System Science (Sapporo, Japan) using an Agilent Human Genomic microarray 8 × 60 K version 2.0 (Agilent Technologies, Santa Clara, CA). Raw data were normalized with a locally weighted scatterplot smoothing linear algorithm using Feature Extraction 10.7.3.1 software (Agilent Technologies). Principal Component Analysis (PCA), Gene Ontology (GO) enrichment analysis and hierarchy

cluster analysis including a heatmap were performed using GeneSpring (Agilent Technologies) at Hokkaido System Science (Sapporo, Japan). Data are available from the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) database (accession no. GSE 124759).

2.4. Quantitative real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted from cultured cells using the RNeasy Plus Mini Kit (QIAGEN). The cDNA was synthesized using the Super-Script III First-Strand Synthesis System (Life Technologies, Rockville, MD). Quantitative RT-PCR was performed with the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The mRNA quantity of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers and PCR conditions are shown in Supplementary Table 1.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of IL-24 in culture supernatant on days 0, 1, 2, 3 and 4 after treatment with IFN-β was determined with ELISA using the Human IL-24 Duoset ELISA kit (R&D Systems) according to the instructions from the manufacturer. Luminescence intensity was quantified with a DTX 800 Multimode Detector plate reader (Beckman Coulter, Fullerton, CA) at an optical density of 450 nm.

2.6. Cell apoptosis analysis

Cell apoptosis in RPMI-7951 and A375 cells were detected using an Annexin V apoptosis kit, following the procedure recommended by the manufacturer (BioVision, Milpitas, CA). A375 and RPMI-7951 cells were treated for 2 and 4 days with or without

IFN-β at a concentration of 1,000 IU/ml. Treated cells were collected by centrifugation and suspended in 500 μl of 1× binding buffer. These cells were stained with Annexin V-fluorescein isothiocyanate (FITC) conjugates and Propidium Iodide (PI) for 15 min and detected by flow cytometry (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.7. Plasmid transfection and generation of stable clones

To generate stable *IL24*-knock-down clones of A375 and RPMI-7951 cells, A375 cells were transfected with HuSH short hairpin RNA (shRNA) against *IL24* (5'-CCACAATAGAACAGTTGAAGTCAGGACTC-3') and RPMI-7951 cells were transfected with shRNA against *IL24* (5'-GCCAACAACTTTGTTCTCATCGT-GTCACA-3'). These genes were encoded with puromycin N-acetyl-transferase (OriGene, Rockville, MD). A non-effective 29-mer scrambled shRNA cassette was used as a control. Transfections were performed with TurboFectin 8.0 reagent (OriGene) according to the instructions from the manufacturer. Stable clones were selected and maintained under a final puromycin concentration of 1.0 μg/ml.

2.8. Western Blotting Analysis

Samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membrane was blocked with 5% skim milk in TBS (20 mM Tris and 137 mM NaCl [pH7.3]) containing 0.1% Tween 20 (TBS-Tween), then incubated with the primary antibodies: rabbit monoclonal anti-IL-24 (1:2000) (Abcam, Cambridge, MA) or mouse monoclonal anti-β-actin (1:2000) (Sigma-Aldrich, St. Louis, MO) in TBS-Tween. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam) for anti-IL-24 primary antibody or HRP-conjected rabbit

anti-mouse IgG (Sigma-Aldrich) for anti-β-actin primary antibody. The membrane was incubated for 1 minute with ECL (Bio-Rad), and positive bands were detected by chemiluminescence using a ChemiDoc Touch Imaging system (Bio-Rad).

2.9. Statistical analysis

Statistical analysis was performed using SPSS version 18 software (SPSS Japan, Tokyo, Japan). Significant differences in laboratory data were analyzed by the Wilcoxon rank-sum test and paired t-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Melanoma cell lines are classified into IFN-β-sensitive and -resistant groups

To determine the sensitivity of four melanoma cell lines to IFN-β, a cell proliferation assay was performed using melanoma cell lines (RPMI-7951, A375, SK-MEL-5 and SK-MEL-1). Proliferation of RPMI-7951 and A375 cells was markedly suppressed by treatment with IFN-β compared with non-treated cells, whereas proliferation of SK-MEL-1 and SK-MEL-5 cells was weakly suppressed by treatment with IFN-β (Supple. Fig. 1). This experiment confirmed similar results to the previous study [12]. We therefore classified RPMI-7951 and A375 as IFN-β-sensitive cell lines, while SK-MEL-5 and SK-MEL-1 were classified as IFN-β-resistant cell lines.

3.2. IFN-β induces apoptosis in RPMI-7951, but not in A375

To determine whether apoptosis was induced by treatment with IFN-β in IFN-β-sensitive cells, cell apoptosis assay was performed for RPMI-7951 and A375 cells. We defined Annexin-V-FITC-positive and PI-negative cells as early apoptotic cells, and Annexin-V-FITC-positive and PI-positive cells as late apoptotic cells. The number of A375 cells staining with Annexin-V-FITC, which indicates both early and late apoptosis, was not increased on days 2 and 4 by treatment with IFN-β, while that of RPMI-7951 cells stained with Annexin-V-FITC was significantly increased by treatment with IFN-β (Supple. Fig. 2a). The rates of early and late apoptotic cells were 24.2% and 10.6% of RPMI-7951 cells treated with IFN-β for 2 days, respectively. On the other hand, rates of early and late apoptotic cells were 16.6% and 25.3% of RPMI-7951 cells treated with IFN-β for 4 days, respectively (Supple. Fig. 2b).

3.3. Genome-wide microarray indicates up-regulation of IL-24

To detect genes expressed in melanoma cells after treatment with IFN-β, analysis of a genome-wide microarray with 62,743 probes was performed using RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 cell lines. For reference, clustering analysis including heatmap (Supple. Fig. 3), gene ontology (GO) enrichment analysis (Supple. Fig. 4), and principal component analysis (PCA) (data not shown) were also performed. A total of 241 genes were expressed >5-fold in both RPMI-7951 and A375 cells treated with IFN-β compared with non-treated cells. Of the 241 upregulated genes in RPMI-7951 and A375 cells, 24 genes were expressed < 2-fold in SK-MEL-5 and SK-MEL-1 cells treated with IFN-β compared with non-treated cells (Table 1). Among the 24 genes, we focused on IL-24, because IL-24 is known as a secreted protein associated with the inhibition of melanoma [16].

3.4. IL-24 mRNA is significantly induced by IFN-β in IFN-β-sensitive melanoma cells

To elucidate the association between *IL24* mRNA expression level and sensitivity of each melanoma cell line to IFN-β, we assessed quantities of *IL24* mRNA in cells after treatment with IFN-β using RT-PCR. Peak levels of *IL24* mRNA expression in melanoma cells correlated completely with the order of sensitivity to IFN-β. Furthermore, marked differences were identified between *IL24* mRNA in IFN-β-sensitive cells and that in IFN-β-resistant cells (RPMI-7951> A375>> SK-MEL-5> SK-MEL-1) (Fig. 1).

3.5. *Treatment with IFN-β induces extracellular, but not intracellular IL-24 protein*

To assess the association between sensitivity of each melanoma cell line to IFN-β and expression of extra- and intracellular IL-24 protein, we performed ELISA and western blotting assay using four melanoma cell lines after treatment with IFN-β. Peak

extracellular IL-24 protein in melanoma cells correlated nearly exactly with the order of sensitivity to IFN-β. Extracellular IL-24 protein was significantly elevated in IFN-β-sensitive cells treated with IFN-β compared with non-treated cells. A marked difference was evident between extracellular IL-24 protein in IFN-β-sensitive cells and IFN-β-resistant cells (A375 >RPMI-7951 > >SK-MEL-5 > SK-MEL-1) at day 4 after treatment with IFN-β (Fig. 2a). Quantities of intracellular IL-24 protein in all the melanoma cells were decreased or unchanged after treatment with IFN-β (Fig. 2b). Expression levels of intracellular IL-24 protein did not correlate with the order of sensitivity of melanoma cells to IFN-β.

3.6. Treatment with recombinant human IL-24 protein has little suppressive effect on any of the melanoma cells

To assess whether quantities of extracellular rhIL-24 protein are related to proliferation of melanoma cells, we performed cell proliferation assay with melanoma cells on days 0, 2 and 4 after treatment with rhIL-24. Proliferation was not decreased in any melanoma cell lines after treatment with rhIL-24 (Supple. Fig. 5).

3.7. Knockdown of IL-24 decreases the effectiveness of IFN-β on melanoma cells

To assess whether the quantities of melanoma-derived extracellular IL-24 protein correlated with proliferation of melanoma cells, we performed knock-down of *IL24* in IFN-β-sensitive cells (PRMI-7951 and A375 cell lines) and performed proliferation assays using these knock-down cells. We confirmed the suppression of extracellular IL-24 protein in IL-24-knock-down melanoma cells after treatment with IFN-β. *IL-24*-knock-down RPMI-7951 and A375 cells were less sensitive to IFN-β than RPMI-7951 and A375 cells transduced with non-effective shRNA, respectively (Fig. 3).

3.8. IFN-β induces IL-22 receptor 1 and IL-20 receptor 2

To elucidate the association between sensitivity to IFN-β and expression of IL-24 receptor components, comprising IL-22R1, IL-20R1 and IL-20R2, on melanoma cells, we assessed the expression of IL-24 receptor components using RT-PCR. The expression ratio of *IL22R1* in RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 cells treated with IFN-β was 3.31-fold, 2.96-fold, 2.48-fold and 1.51-fold untreated cells, respectively. Expression levels of *IL22R1* correlated completely with the order of sensitivity of melanoma cells to IFN-β (Fig. 4a). Expression ratios of *IL20R2* in RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 cells treated with IFN-β were 1.02-fold, 1.29-fold, 0.77-fold and 0.94-fold compared with untreated cells, respectively. Expression levels of *IL20R2* correlated nearly exactly with sensitivity to IFN-β (Fig. 4b). *IL20R1* was not expressed in almost all the melanoma cell lines (data not shown).

3.9. IFN-β induced IL-24 in HEMs

To elucidate the expression of IL-24 mRNA and extracellular IL-24 protein in HEMs, we performed proliferation assay and assessed the quantities of IL-24 mRNA and extracellular IL-24 protein using RT-PCR and ELISA, respectively. Proliferation of HEMs was weakly suppressed after treatment with IFN-β, so we classified HEMs as IFN-β-resistant cells (Supple. Fig. 6a). Expressions of IL-24 mRNA and extracellular IL-24 protein in HEMs increased significantly after treatment with IFN-β compared with those in all the melanoma cell lines (Supple. Fig. 6b and 6c).

4. Discussion

The present study demonstrated that natural-type IFN-β exerts tumor suppressive effects on melanoma cells through IL-24 protein derived from melanoma cells.

Some melanomas do not respond to treatment with IFN-β [11]. Yamamoto et al. reported that recurrence or in-transit metastasis of melanoma occasionally occurs during treatment with IFN-β [11]. Furthermore, the present and previous studies demonstrated that *in vitro* treatment with IFN-β is ineffective against some melanoma cell lines [12]. Based on these findings, the degree of tumor-suppressive effects of IFN-β on melanoma varies with each melanoma. However, factors determining the sensitivity of melanoma to IFN-β have not been completely identified. Lebedeva et al. reported IL-24 as a tumor-suppressor for various cancers, including melanoma [17], and Jiang et al. reported that IFN-β induces IL-24 in the H0-1 melanoma cell line [18]. Accordingly, we hypothesized that the effectiveness of IFN-β on melanoma depends on the IL-24 expression level in the tumor microenvironment, leading to the investigation of whether IL-24 determines the sensitivity of melanoma to IFN-β.

The present study demonstrated that: i) *IL24* mRNA was increased in IFN-β-sensitive cells treated with IFN-β; ii) extracellular IL-24 protein was increased in IFN-β-sensitive cells treated with IFN-β; iii) intracellular IL-24 protein was unchanged in all the melanoma cell lines treated with IFN-β; iv) treatment with mouse-derived rhIL-24 protein did not suppress proliferation of melanoma cells; and v) knockdown of IL-24 reduced the tumor-suppressive effects of IFN-β. Based on these findings, we suggest that extracellular IL-24 protein induced by IFN-β from melanoma cells regulates the sensitivity of melanoma to IFN-β, resulting in suppression of the proliferation of the melanoma cells.

The present study demonstrated that: i) IFN-β markedly suppressed proliferation of

RPMI-7951 and A375 cells; ii) IFN-β induced apoptosis in RPMI-7951 cells; iii) IFN-β did not induce apoptosis in A375 cells; iv) knockdown of IL-24 diminished some of the tumor-suppressive effects of IFN-β on RPMI-7951 cells; and v) knockdown of IL-24 completely diminished the tumor-suppressive effects on A375 cells. From these results, IFN-β suppressed A375 cells mainly through IL-24 from tumor cells. In contrast, IFN-β suppressed RPMI-7951 cells through both apoptotic mechanism and IL-24 from tumor cells. We therefore suggested that extracellular IL-24 protein may play a major role in cell growth inhibition in melanoma cells in which apoptosis is not induced by IFN-β. On the other hand, extracellular IL-24 protein may have some involvement in cell growth inhibition in melanoma cells in which apoptosis is induced by IFN-β.

In this study, extracellular rhIL-24 protein derived from mouse cells did not inhibit the proliferation of melanoma cells, compatible with the data reported by Kreis et al. [19], although our data indicated that IFN-β suppressed the proliferation of melanoma cells via increases in extracellular IL-24. Furthermore, previous data from Sauane et al. showed that extracellular IL-24 protein derived from human cells transfected with Ad-*IL-24*, a replication-incompetent adenovirus vector designed to express *IL24* cDNA, suppressed the proliferation of melanoma cells [20]. The effectiveness of extracellular IL-24 protein thus remains controversial. To elucidate these paradoxical results about the origin of extracellular IL-24 protein, we hypothesized that IL-24 protein derived sorely from human cells may suppress the proliferation of melanoma cells. To confirm our hypothesis, we performed a proliferation assay for IL-24-knockdown human melanoma cells, resulting in knockdown of extracellular IL-24 protein and thus reducing its tumor-suppressive effects. Based on these results, we confirmed that extracellular IL-24 protein derived from human cells was effective against melanoma cells. The human specific IL-24 protein structure including human-specific

glycosylation and phosphorylation may contribute to tumor-suppression. In addition, these findings are compatible with the facts that the SK-MEL-5 and SK-MEL-1 cell lines, in which extracellular IL-24 protein is not expressed, are basically resistant to IFN-β.

IL-24 is known to inhibit the proliferation of cancer cells without affecting normal cells [21]. Our study demonstrated that: i) HEM was resistant to IFN-β; and ii) IL-24 mRNA and extracellular IL-24 protein were significantly expressed in HEMs compared with all the melanoma cells (Supple. Fig. 3). These data suggest that extracellular IL-24 protein does not suppress proliferation of HEMs. On the other hand, we suggested that extracellular IL-24 protein suppresses proliferation of melanoma cells. These findings imply that malignant transformation from normal melanocyte to melanoma cell potentially requires a decrease in the expression of extracellular IL-24 protein.

IL-24 protein binds to two heterodimeric receptor complexes, IL-22R1/IL-20R2 and IL-20R1/IL-20R2 [22]. Our study demonstrated that: i) the expression ratio of IL-22R1 was increased after treatment with IFN-β in all the melanoma cell lines; ii) the expression ratio of IL-22R1 correlated completely with the order of the sensitivity to IFN-β; and iii) the expression ratio of IL-20R2 correlated nearly exactly with the order of sensitivity to IFN-β. These data suggest that the expressions of IL-22R1 and IL-20R2 are closely associated with the sensitivity of melanoma cells to IFN-β.

The final concentration of IFN-β is a critical factor for the *in vitro* condition in this study. Yamamoto et al. demonstrated that IFN-β accumulated at 310-4,012 IU/g in regional nodes 4 h after local administration of 6×10^6 IU of IFN-β into the primary site of melanoma [23]. Given the results in the clinical study, setting the dose range of IFN-β concentrations as 300-4,000 IU/ml may be reasonable. Chawla-Sarkar et al. showed that 1,000 IU/ml of IFN-β inhibits A375 cells by 50% (IC_{50}) at day 4 after

treatment with IFN-β under *in vitro* conditions [24]. Based on those findings, we determined the final concentration of IFN-β as 1,000 IU/ml under *in vitro* conditions.

One limitation in this study was that no *in vivo* experiments were performed. In the tumor microenvironment, tumor cells are surrounded by extracellular matrix and various cells including fibroblasts, inflammatory cells and vascular endothelial cells [25]. Previous *in vivo* studies have reported that IL-24 is induced from tumor-surrounding cells, including fibroblasts and endothelial cells [26], and plays important roles in the inhibition of invasion, migration and angiogenesis in the tumor microenvironment [27]. *In vivo* experiments may contribute to a further understanding of the tumor-suppressive effects of IFN-β through IL-24 in the tumor microenvironment.

In conclusion, our data suggest that IFN-β suppresses melanoma through extracellular IL-24 protein derived from melanoma cells.

AUTHOR CONTRIBUTION

YW and YN designed the study; YW, YN and MI performed the research; YW and YN analyzed the data; YW wrote the manuscript; AA and HN supervised the study.

All authors have read and approved the final manuscript.

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Figure Legends

Fig. 1.

Expression of *IL24* mRNA in IFN-β-treated melanoma cells

The mRNA expression of *IL24* was measured with RT-PCR in RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 treated with or without IFN-β for 0, 1, 2, 3, 4 and 5 days. Horizontal, depth and vertical axes indicate treatment time (days), type of cells and expression level of *IL24* mRNA divided by that of *GAPDH* mRNA, respectively. The data are representative of at least three independent experiments.

Fig. 2.

Expression of extra- and intracellular IL-24 protein in melanoma cell lines after treatment with IFN-β

a) Extracellular IL-24 protein was measured by ELISA in RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 treated with or without IFN-β. Horizontal, depth and vertical axes indicate treatment time (days), type of cells and concentration of extracellular IL-24 protein in the culture supernatant (ng/ml/cell), respectively. The data are representative of at least three independent experiments.

b) Intracellular IL-24 protein detected by western blotting analysis in RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 treated with or without IFN-β for 72 h. The left figure shows the membrane immunoblotted with anti-IL-24 antibody or anti-β-actin antibody at 1:2000 or 1:5000 dilutions, respectively. The right bar graph shows expression of IL-24 protein after normalization to β-actin protein. The data are representative of at least three independent experiments. Error bars indicate standard error of the mean.

Paired t tests were used to assess statistical significance (n=3, n.s: $p > 0.05$).

Fig. 3.

Proliferation assay of A375 and RPMI-7951 cells with knock-down of IL-24

IL24 was knocked down in RPMI-7951 (a, b, c) cells and A375 cells (d, e, f). The data are representative of at least three independent experiments.

a, d) The left bar graphs indicate expression of *IL24* mRNA and IL-24 protein in RPMI-7951 (a) and A375 (d) cells transduced with *IL24* shRNA (black bar) and non-effective scramble shRNA (gray bar) at 72 h after treatment with IFN-β. Expression of *IL24* mRNA and extracellular IL-24 protein was decreased in *IL24*-knock-down cells.

b, e) The center graphs indicate proliferation of RPMI-7951 (b) and A375 cells (e), which were transduced *IL24* shRNA (lower graph) and non-effective RNA (upper graph), at 0, 2 and 4 days after incubation with (solid line) or without (dotted line) IFN-β.

c, f) The right bar graphs represent the number of cells indicated by the center graph at 4 days after treatment with IFN-β. Vertical axes indicate the number of cells (non-effective shRNA transduced cells and *IL24*-knockdown cells) after treatment with (black bar) or without (gray bar) IFN-β. The difference between the change rate of IL-24-knockdown cells and that of mRNA-scramble cells are analyzed using the Wilcoxon rank-sum test (* $p < 0.05$).

Fig. 4.

Expression of *IL22R1* and *IL20R2* mRNA in melanoma cells after treatment with IFN-β

Bar graphs show expression ratio of *IL22R1* mRNA (a) and *IL20R2* mRNA (b). Expression of *IL22R1* and *IL20R2* measured by RT-PCR in RPMI-7951, A375, SK-MEL-5 and SK-MEL-1 treated with (black bar) or without (gray bar) IFN-β for 72 h. Horizontal and vertical axes indicate type of cells and the expression ratio of *IL22R1* and *IL20R2* receptors, respectively. Black bars of each graph indicate the expression ratio of *IL22R1* and *IL20R2* mRNA in IFN-β-treated cells relative to that in untreated cells (gray bar) (set as 1.0 for each untreated cell). The data are representative of at least three independent experiments.

Supple. Fig. 1.

Proliferation assay of melanoma cells after treatment with IFN-β

The proliferation assay of melanoma cells (RPMI-7951, A375, SK-MEL-5 and SK-MEL-1) after treatment with (solid line) or without (dotted line) IFN-β for 0, 2 and 4 days. Vertical and horizontal axes show numbers of cells and treatment time (days), respectively. Error bars represent standard deviation of duplicate data. The data are representative of at least three independent experiments.

Supple. Fig. 2

Cell apoptosis assay of RPMI-7951 and A375 after treatment with IFN-β

Cell apoptosis assay of RPMI-7951 cells and A375 cells at day 2 and 4 after treatment with IFN-β was performed using Annexin-V-FITC and PI.

a, b) RPMI-7951 cells (a) and A375 cells (b) treated with or without IFN-β are labeled

with Annexin-V-FITC and PI. Flow cytometry scatter plots show the presence of cells that are positive for PI alone in the upper left quadrant, for Annexin-V-FITC alone in the lower right quadrant, for PI and Annexin-V-FITC in the upper right quadrant, and negative for PI and Annexin-V-FITC in the lower left quadrant.

c, d) The graphs show the percentage of early apoptotic cells (c) and late apoptotic cells (d) in RPMI-7951 and A375 cells. Vertical and horizontal axes show the percentage of early or late apoptotic cells, and the type of cells (RPMI-7951 and A375) at day 2 or 4 after treatment with or without 1,000 IU/ml IFN-β, respectively.

Supple. Fig. 3

Heatmap and cluster analysis of differentially expressed genes (DEGs) in melanoma cell lines treated and untreated with IFN-β

In microarray analysis, 318 DEGs satisfied the two following conditions: i) genes expressed more than 2-fold in IFN-β-sensitive cells treated with IFN-β compared with cells untreated with IFN-β; and ii) genes expressed less than 1.5-fold in IFN-β-resistant cells treated with IFN-β compared with cells untreated with IFN-β.

The heatmap shows DEGs in each melanoma cell line treated and untreated with IFN-β. In the heatmap, red color represents high expression and blue color represents low expression. The left panel shows the enlarged panel of a part of the heatmap focusing on IL-24 to recognize the position of IL-24 and genes similar to IL-24 expression in the heatmap.

Supple. Fig. 4

Gene ontology (GO) enrichment analysis in melanoma cells treated with IFN-β

Based on 318 DEGs selected according to cluster analysis, GO enrichment analysis was performed.

The graph shows the 30 most relevant GO terms associated with 318 genes. The vertical axis shows GO terms in ascending order of p-values from the top. The horizontal axis shows the numbers of genes classified by individual GO terms. GO terms are classified into three ontologies: biological processes (green bars); molecular functions (red bars); and cellular components (none). The bars surrounded by black lines show GO terms relevant to IL-24 genes. Significant genes identified from *P-values* < 0.01 (calculated using Fisher's exact test) as the cutoff criterion for GO functional enrichment analysis.

Supple. Fig. 5.

Proliferation assay of melanoma cells after treatment with recombinant human IL-24 protein (rhIL-24)

Proliferation assay of melanoma cells (RPMI-7951, A375, SK-MEL-5 and SK-MEL-1) after treatment with rhIL-24 protein (0, 20 and 100 ng/ml). Vertical and horizontal axes indicate number of cells and treatment time (day 0, 2 and 4), respectively. White, gray and black bars indicate concentrations of rhIL-24 protein at 0, 20 and 100 ng/ml, respectively. Error bars represent the standard deviation of duplicate data. Data are representative of at least three independent experiments.

Supple. Fig. 6.

Proliferation assay of human epidermal melanocytes (HEMs) and expression of *IL24* mRNA and extracellular IL-24 protein in HEMs

a) Proliferation assay of HEMs after treatment with (solid line) or without (dotted line)

IFN-β for 0, 2 and 4 days. Vertical and horizontal axes show number of cells and treatment time (days), respectively.

b, c) Expression of *IL24* mRNA and extracellular IL-24 protein in HEMs and melanoma cells after treatment with (black bar) or without (gray bar) IFN-β for 72 h was performed using RT-PCR and ELISA, respectively. Horizontal axes show type of cells (HEM, RPMI-7951, A375, SK-MEL-5 and SK-MEL-1) and vertical axes indicate quantities of *IL24* mRNA divided by that of *GAPDH* mRNA (b) and the concentration of extracellular IL-24 protein (c).

Data are representative of at least three independent experiments.

Fig. 1

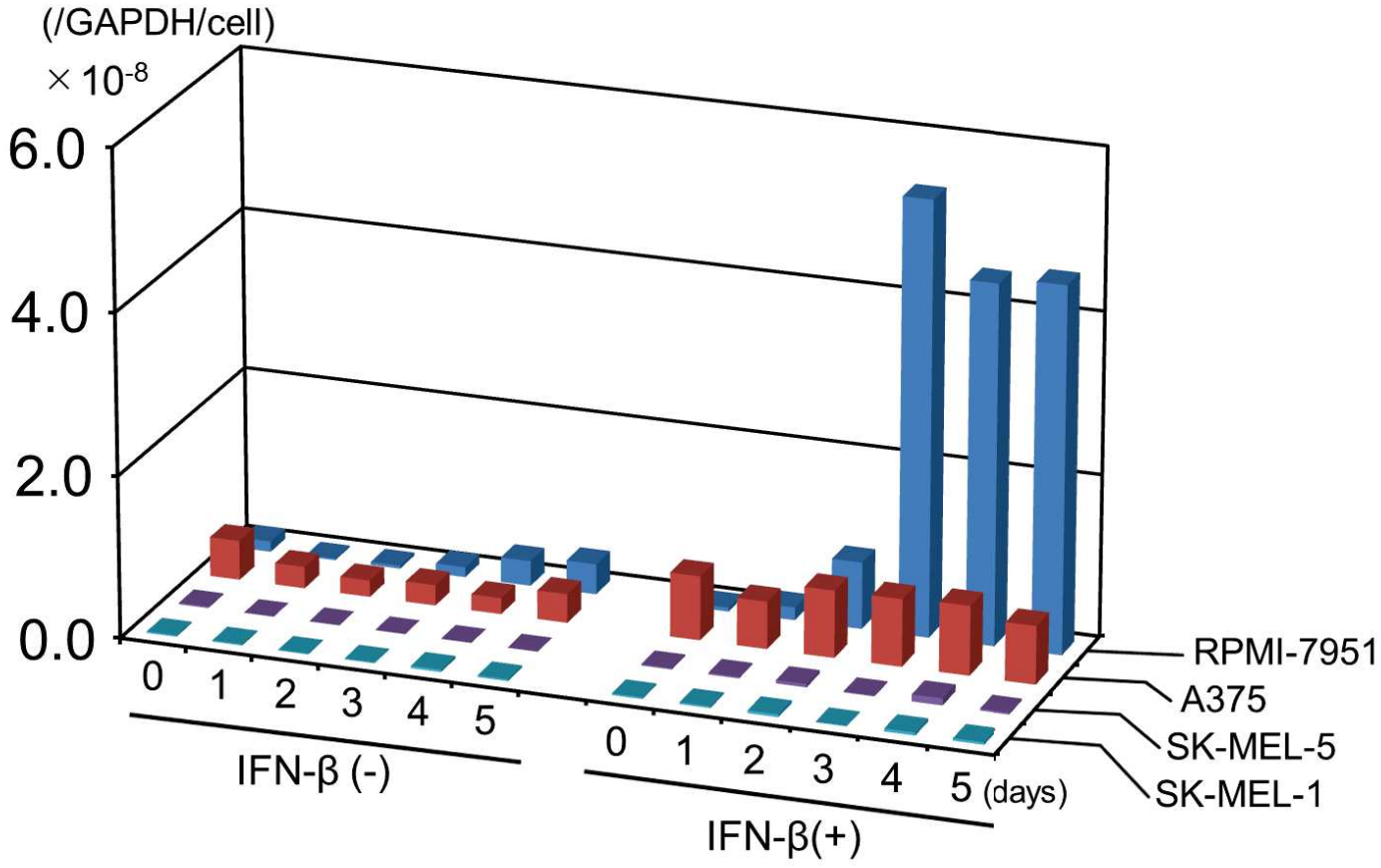
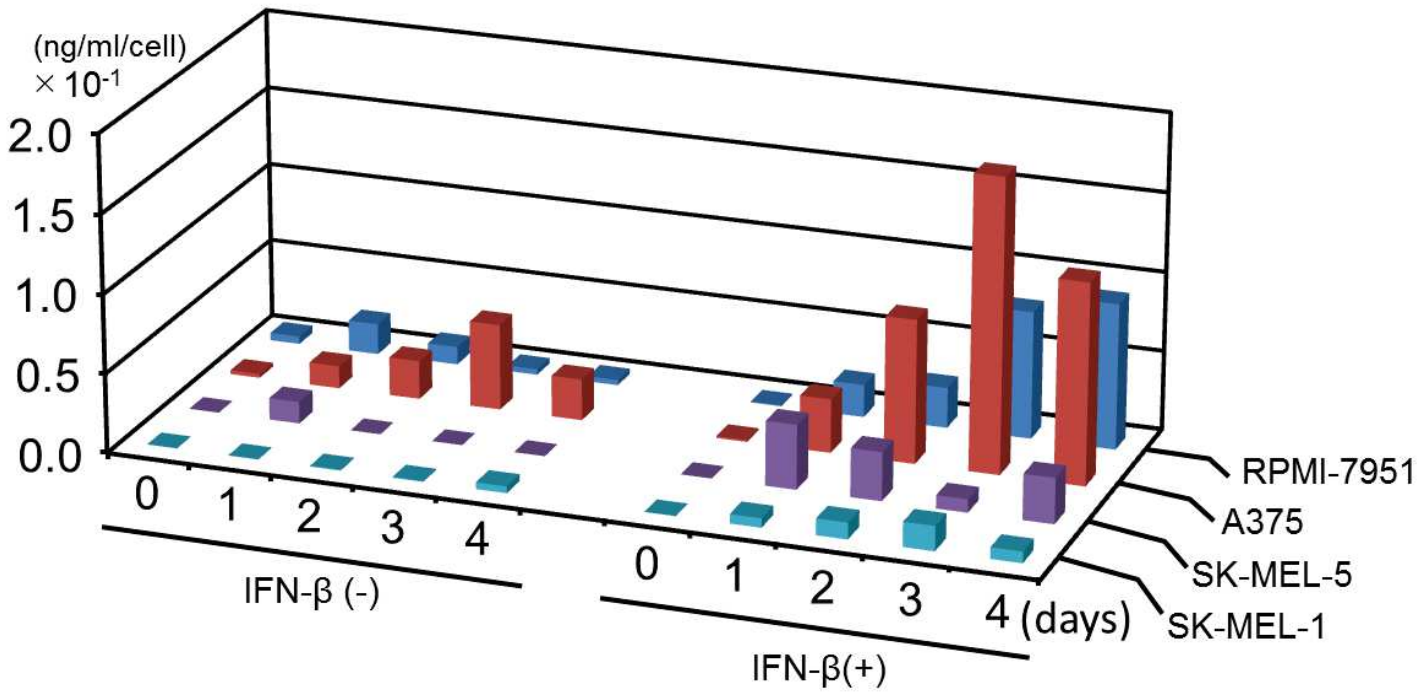
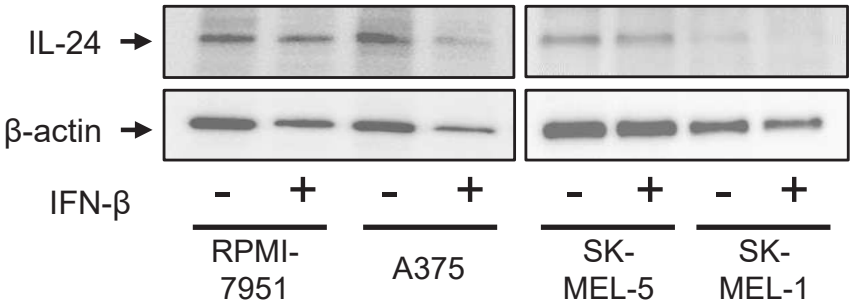

(/GAPDH/cell)
× 10-8
6.0
4.0
2.0
0.0
0 1 2 3 4 5
IFN-β (-)
0 1 2 3 4 5 (days)
IFN-β(+)
RPMI-7951
A375
SK-MEL-5
SK-MEL-1

Fig. 2

a


(ng/ml/cell)
× 10-1
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4
IFN-β (-)
0 1 2 3 4 (days)
IFN-β(+)
RPMI-7951
A375
SK-MEL-5
SK-MEL-1

b


IL-24
β-actin
IFN-β
- + - + - + - +
RPMI-7951
A375
SK-MEL-5
SK-MEL-1

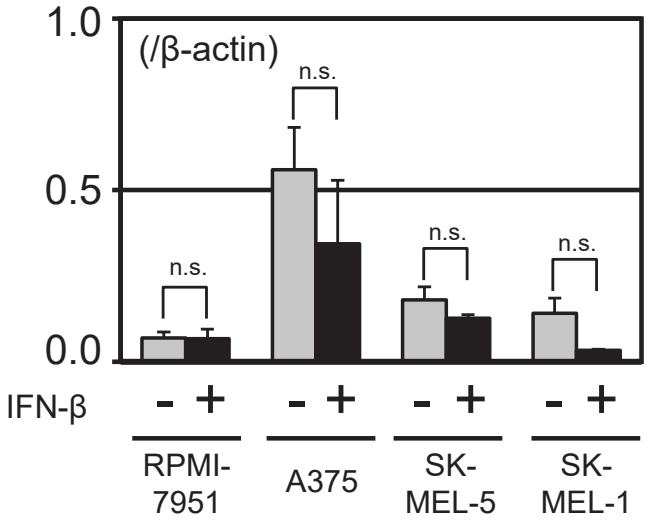

1.0
0.5
0.0
(/β-actin)
n.s.
n.s.
n.s.
n.s.
IFN-β
- + - + - + - +
RPMI-7951
A375
SK-MEL-5
SK-MEL-1

Fig. 3

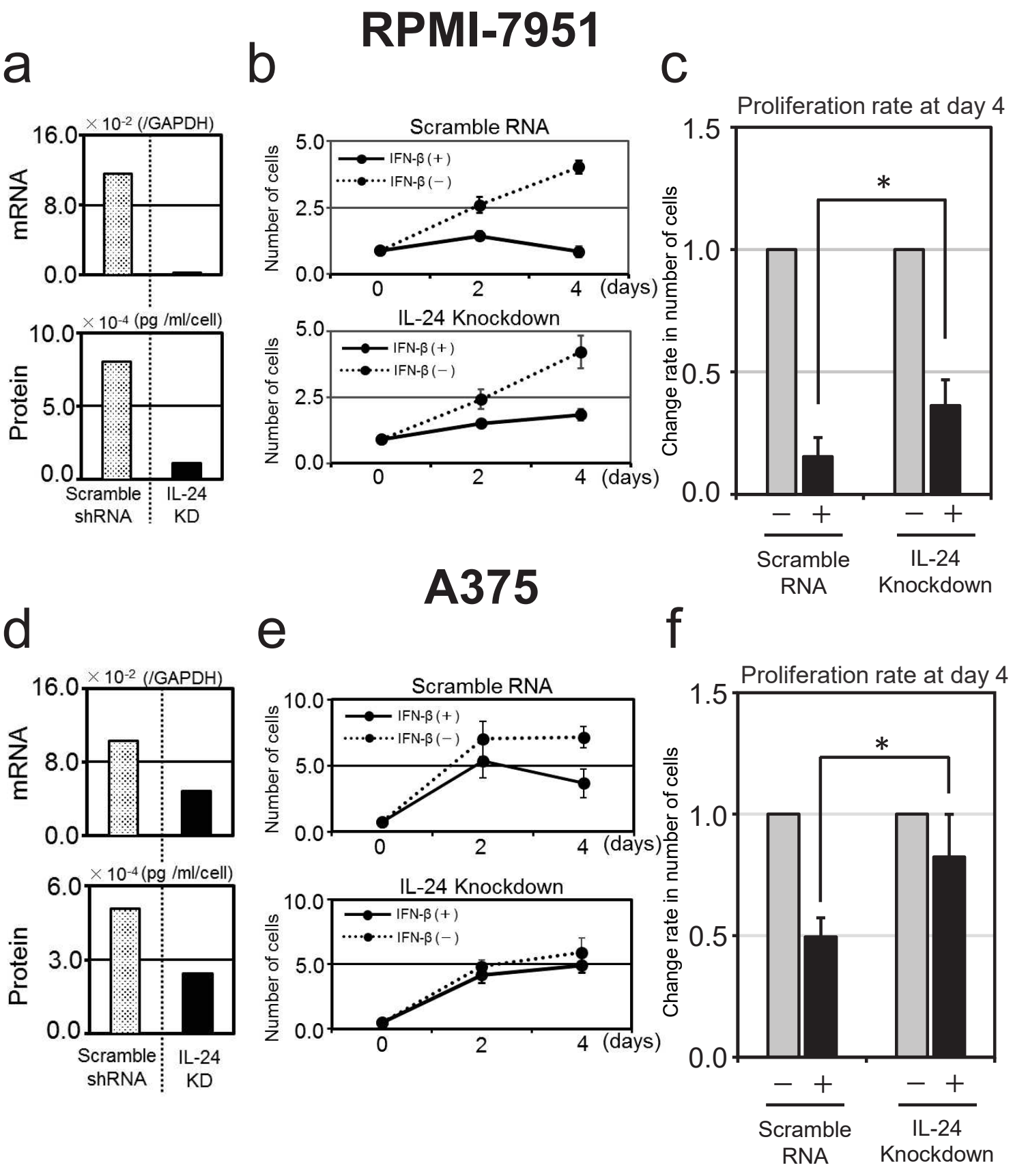

RPMI-7951
a
b
c
× 10-2 (/GAPDH)
mRNA
16.0
8.0
0.0
× 10-4 (pg /ml/cell)
Protein
10.0
5.0
0.0
Scramble shRNA
IL-24 KD
Scramble RNA
IL-24 Knockdown
Number of cells
IFN-β (+)
IFN-β (−)
5.0
2.5
0.0
0
2
4
(days)
Proliferation rate at day 4
Change rate in number of cells
1.5
1.0
0.5
0.0
*
− +
Scramble RNA
IL-24 Knockdown
A375
d
e
f
6.0
3.0
10.0

Fig. 4

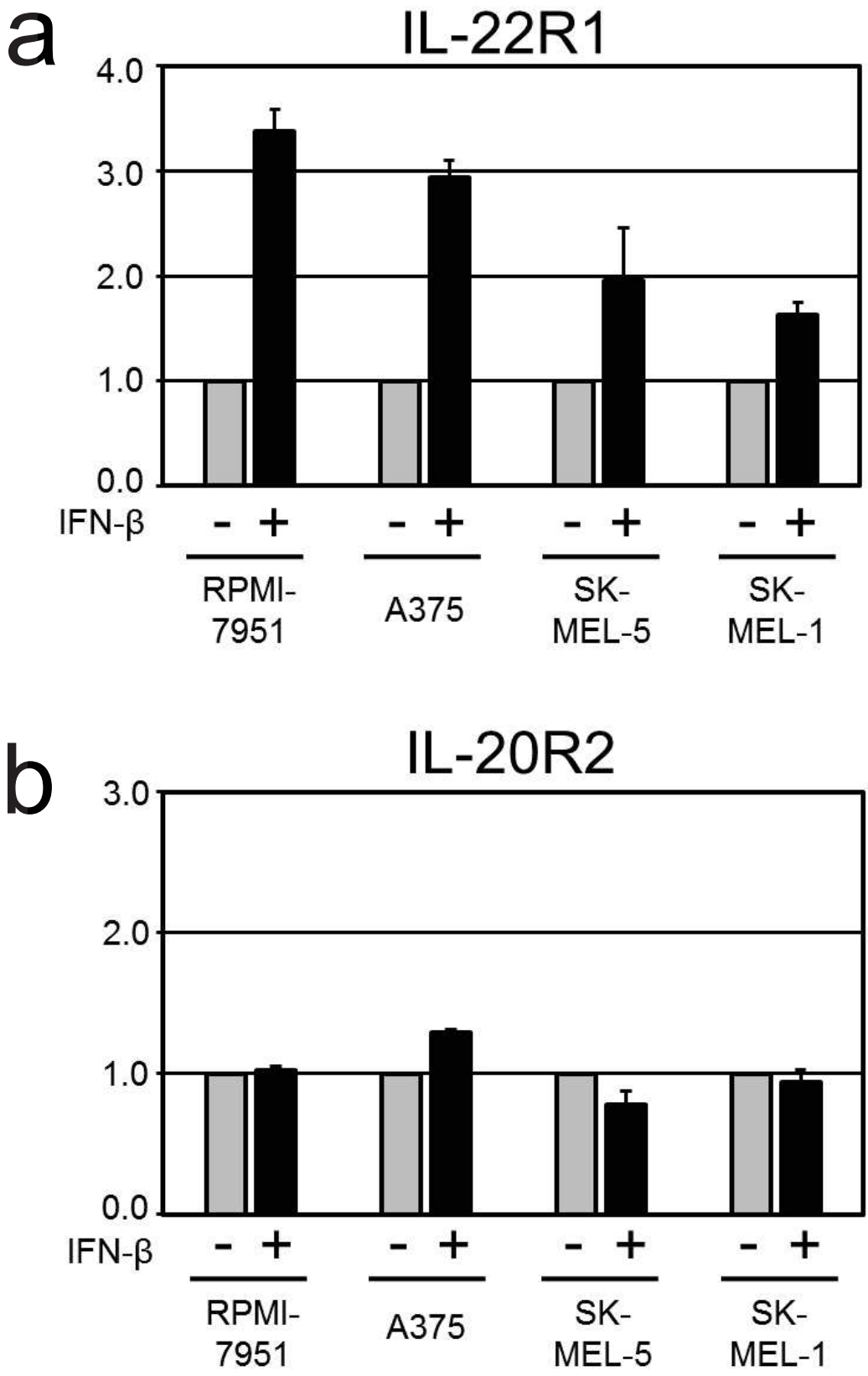
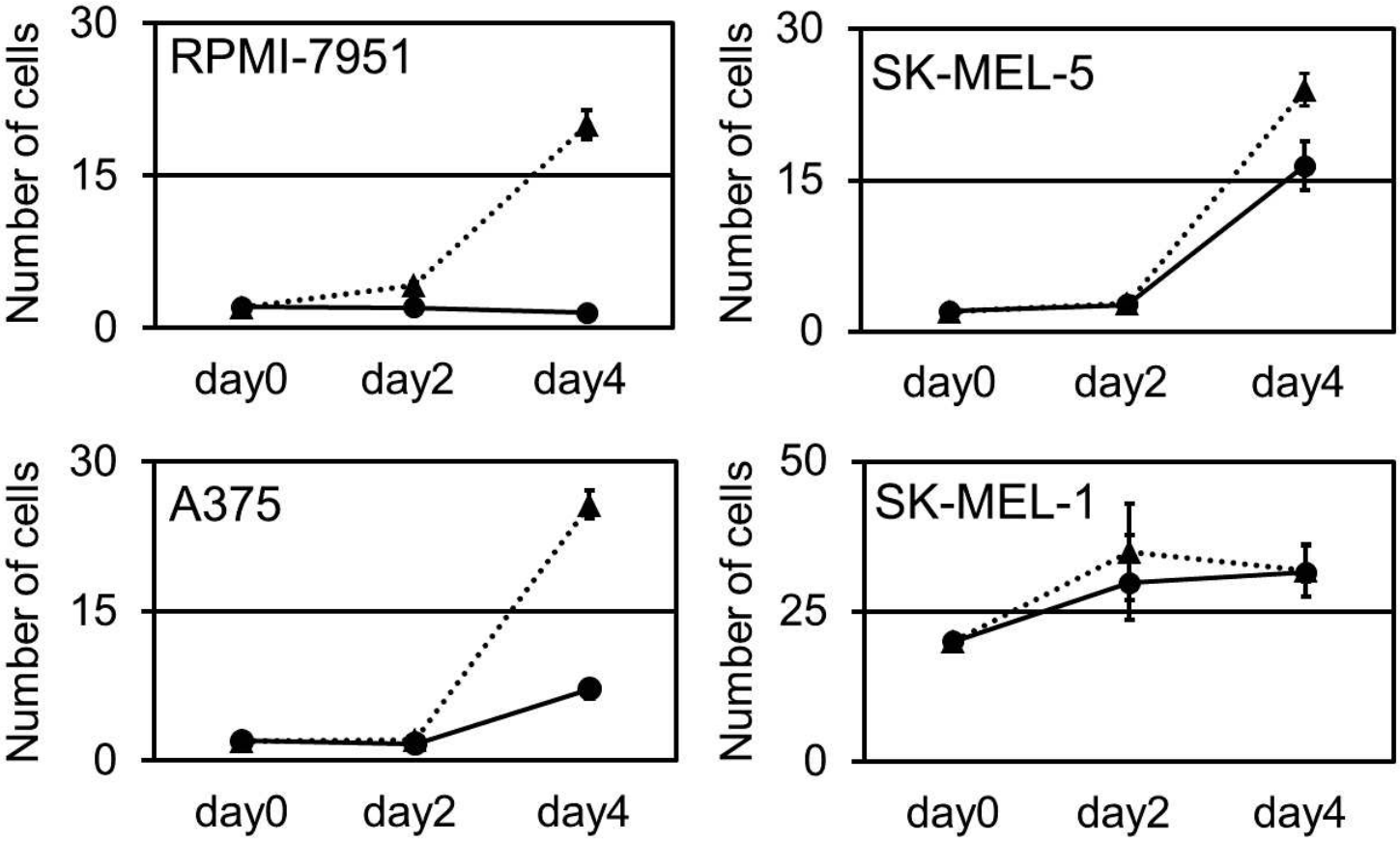

a
IL-22R1
4.0
3.0
2.0
1.0
0.0
IFN-β
- + - + - + - +
RPMI-7951
A375
SK-MEL-5
SK-MEL-1
b
IL-20R2
3.0
2.0
1.0
0.0
IFN-β
- + - + - + - +
RPMI-7951
A375
SK-MEL-5
SK-MEL-1

Table. 1

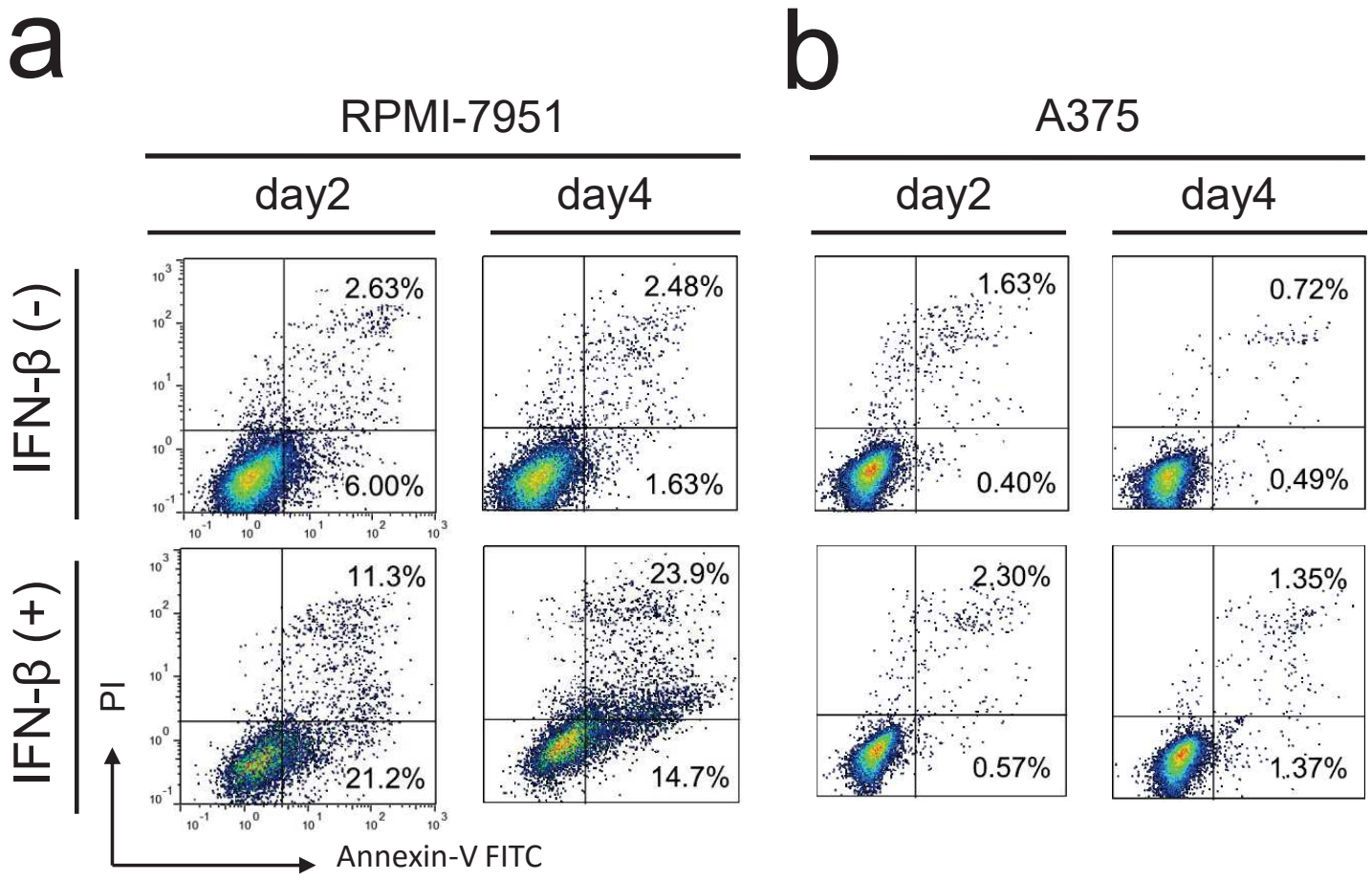
List of genes detected > 5-fold in IFN-β-sensitive cells (RPMI-7951, A375)
< 2-fold in IFN-β-resistant cells (SK-MEL-5, SK-MEL-1)

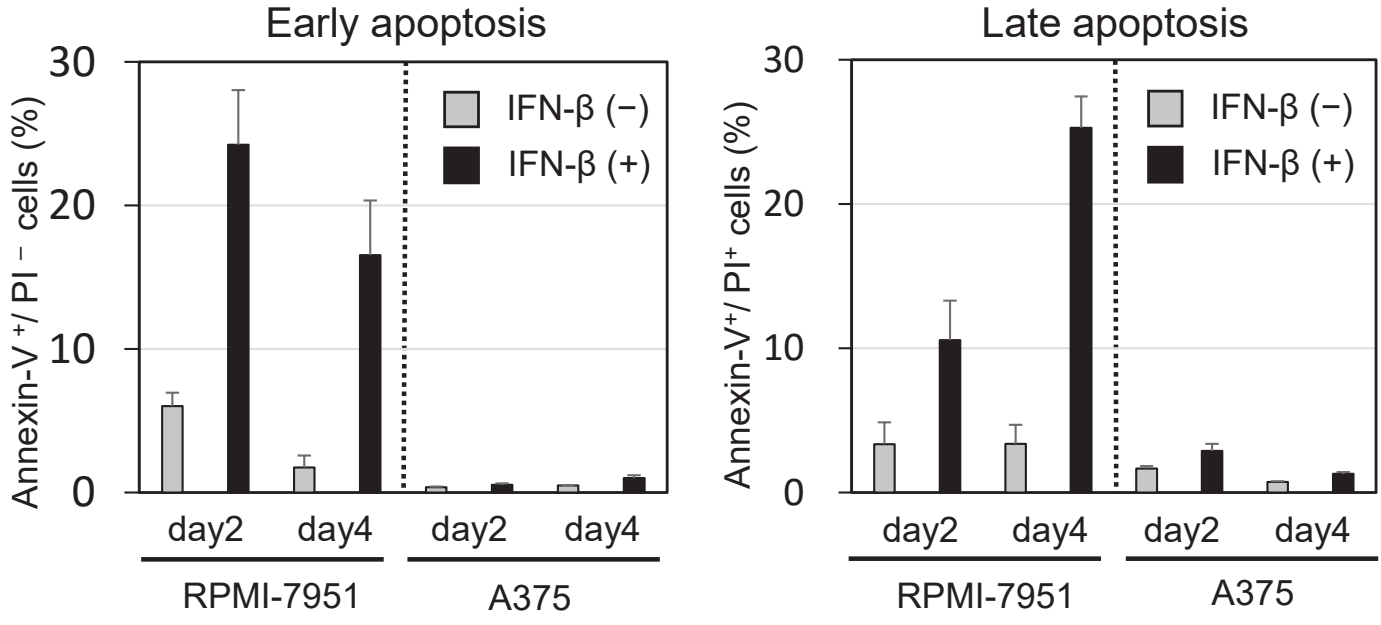
Gene symbol	Description	Fold-change			
		RPMI-7951	A375	SK-MEL-5	SK-MEL-1
XLOC_000027	BROAD institute lincRNA	32.25	10.09	0.68	0.69
GDF15	growth differentiation factor 15	26.02	7.18	1.30	1.50
LGALS9	lectin, galactoside-binding, soluble, 9	16.72	6.50	1.19	1.10
LOC100131733	uncharacterized LOC100131733	12.19	24.78	1.01	1.20
CYP2J2	cytochrome P450, family 2, subfamily J, polypeptide 2	11.86	6.15	1.26	1.66
IL-24	Interleukin-24	9.32	6.59	1.40	0.80
CXCL10	chemokine (C-X-C motif) ligand 10	9.13	7.26	0.66	0.58
LPAR6	lysophosphatidic acid receptor 6	8.94	9.66	0.79	0.81
LMO2	LIM domain only 2	8.02	5.12	1.35	1.64
HIST1H2AC	histone cluster 1 H2A, family member c	7.82	33.55	1.00	1.51
XLOC_l2_007063	BROAD institute lincRNA	7.40	7.30	1.89	1.47
IDO1	indoleamine 2,3-dioxygenase 1	7.34	8.02	1.93	0.94
C15orf48	chromosome 15 open reading frame 48	5.41	8.55	1.16	1.30
SPP1	secreted phosphoprotein 1	5.34	9.13	1.27	1.17
XLOC_l2_000297	guanylate binding protein 1, partial	5.17	12.45	0.29	1.04
XLOC_l2_015121	BROAD institute lincRNA	5.14	4.96	0.90	0.65
GMPR	guanosine monophosphate reductase	5.08	8.18	1.11	1.27

Supple. Fig. 1

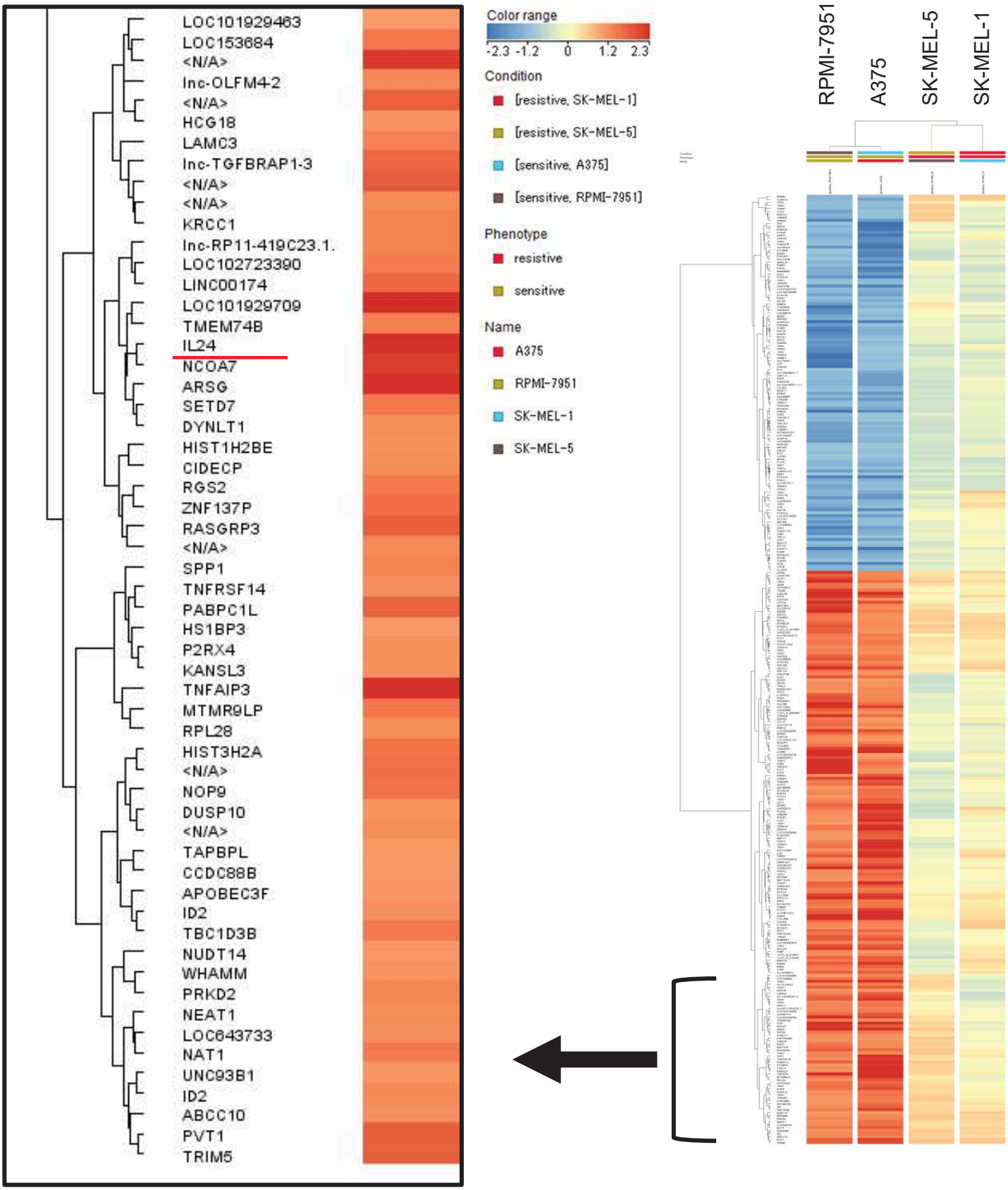

RPMI-7951
SK-MEL-5
A375
SK-MEL-1
Number of cells
30
15
0
50
25
day0
day2
day4

Supple. Fig. 2

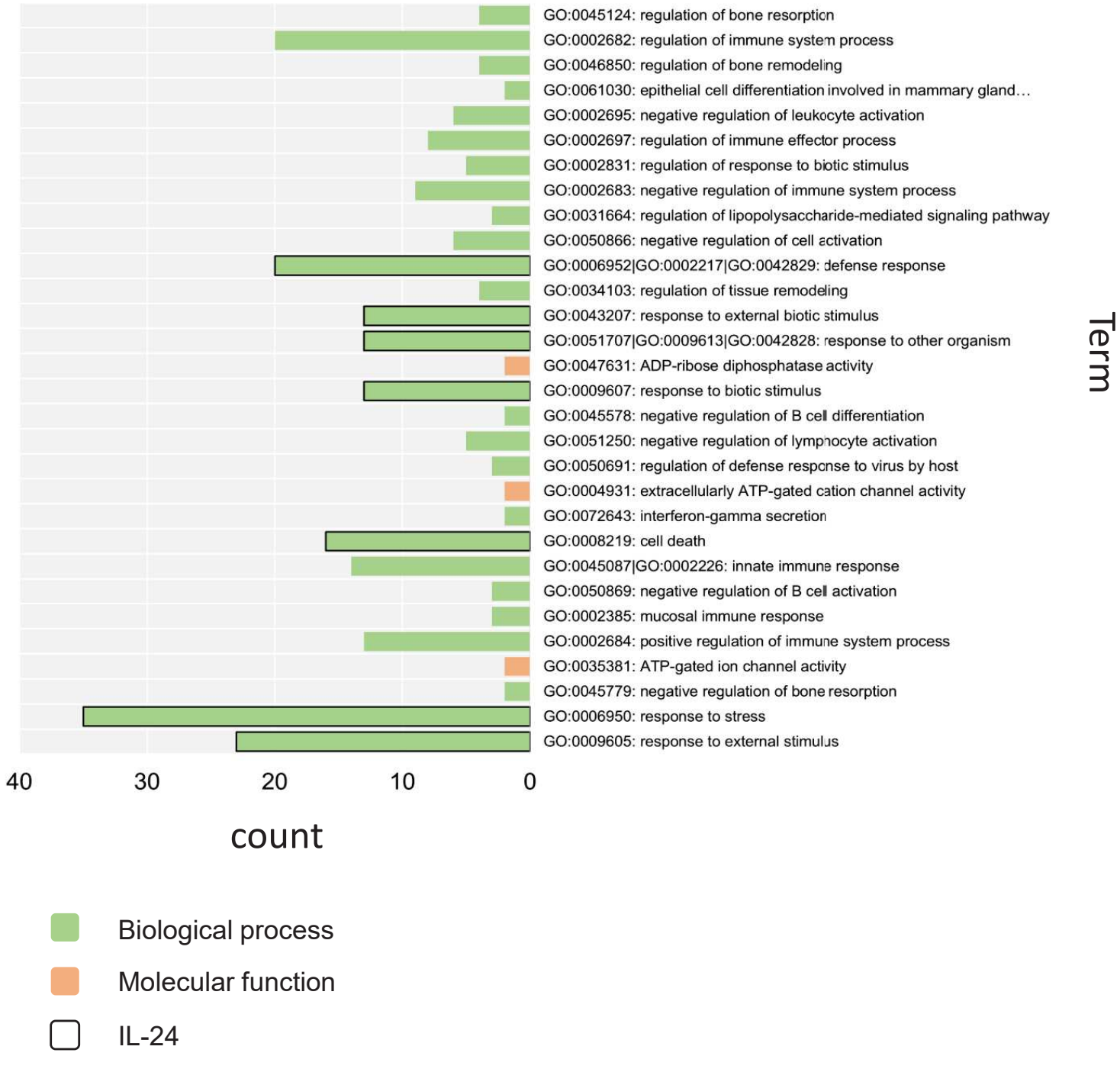

a
b
RPMI-7951
A375
day2
day4
day2
day4
IFN-β (-)
IFN-β (+)
2.63%
6.00%
2.48%
1.63%
1.63%
0.40%
0.72%
0.49%
11.3%
21.2%
23.9%
14.7%
2.30%
0.57%
1.35%
1.37%
PI
Annexin-V FITC


c
d
Early apoptosis
Late apoptosis
Annexin-V⁺/ PI⁻ cells (%)
Annexin-V⁺/ PI⁺ cells (%)
30
20
10
0
IFN-β (−)
IFN-β (+)
day2
day4
day2
day4
RPMI-7951
A375

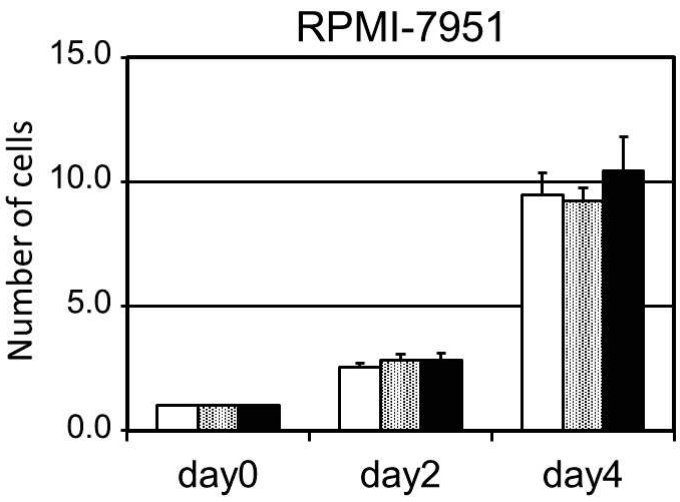
Supple. Fig. 3

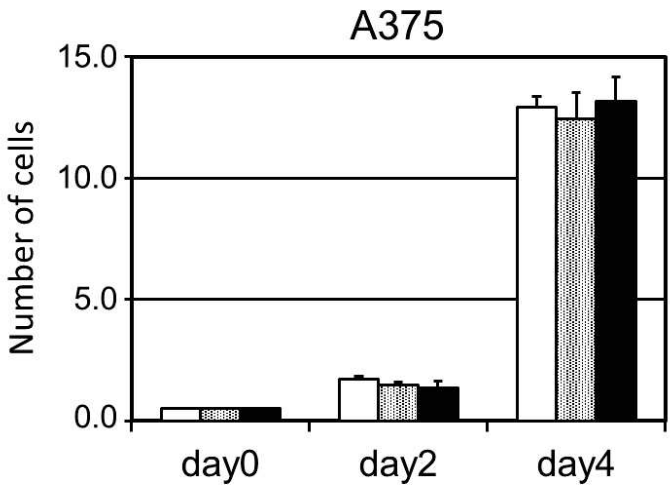

LOC101929463
LOC153684
<N/A>
lnc-OLFM4-2
<N/A>
HCG18
LAMC3
lnc-TGFBRAP1-3
<N/A>
<N/A>
KRCC1
lnc-RP11-419C23.1.
LOC102723390
LINC00174
LOC101929709
TMEM74B
IL24
NCOA7
ARSG
SETD7
DYNLT1
HIST1H2BE
CIDECP
RGS2
ZNF137P
RASGRP3
<N/A>
SPP1
TNFRSF14
PABPC1L
HS1BP3
P2RX4
KANSL3
TNFAIP3
MTMR9LP
RPL28
HIST3H2A
<N/A>
NOP9
DUSP10
<N/A>
TAPBPL
CCDC88B
APOBEC3F
ID2
TBC1D3B
NUDT14
WHAMM
PRKD2
NEAT1
LOC643733
NAT1
UNC93B1
ID2
ABCC10
PVT1
TRIM5
Color range
-2.3 -1.2 0 1.2 2.3
Condition
[resistive, SK-MEL-1]
[resistive, SK-MEL-5]
[sensitive, A375]
[sensitive, RPMI-7951]
Phenotype
resistive
sensitive
Name
A375
RPMI-7951
SK-MEL-1
SK-MEL-5
RPMI-7951
A375
SK-MEL-5
SK-MEL-1

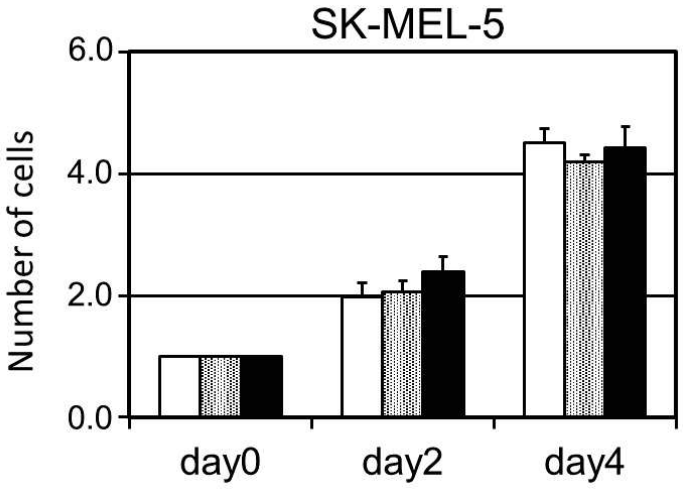
Supple. Fig. 4

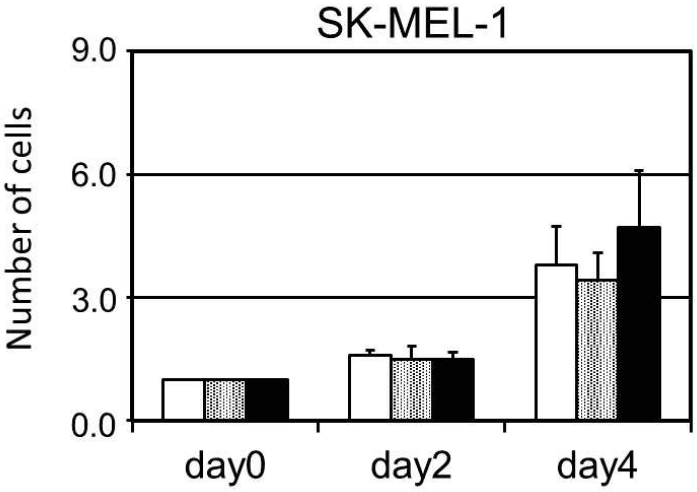

GO:0045124: regulation of bone resorption
GO:0002682: regulation of immune system process
GO:0046850: regulation of bone remodeling
GO:0061030: epithelial cell differentiation involved in mammary gland…
GO:0002695: negative regulation of leukocyte activation
GO:0002697: regulation of immune effector process
GO:0002831: regulation of response to biotic stimulus
GO:0002683: negative regulation of immune system process
GO:0031664: regulation of lipopolysaccharide-mediated signaling pathway
GO:0050866: negative regulation of cell activation
GO:0006952|GO:0002217|GO:0042829: defense response
GO:0034103: regulation of tissue remodeling
GO:0043207: response to external biotic stimulus
GO:0051707|GO:0009613|GO:0042828: response to other organism
GO:0047631: ADP-ribose diphosphatase activity
GO:0009607: response to biotic stimulus
GO:0045578: negative regulation of B cell differentiation
GO:0051250: negative regulation of lymphocyte activation
GO:0050691: regulation of defense response to virus by host
GO:0004931: extracellularly ATP-gated cation channel activity
GO:0072643: interferon-gamma secretion
GO:0008219: cell death
GO:0045087|GO:0002226: innate immune response
GO:0050869: negative regulation of B cell activation
GO:0002385: mucosal immune response
GO:0002684: positive regulation of immune system process
GO:0035381: ATP-gated ion channel activity
GO:0045779: negative regulation of bone resorption
GO:0006950: response to stress
GO:0009605: response to external stimulus
Term
40
30
20
10
0
count
Biological process
Molecular function
IL-24

Supple. Fig. 5


RPMI-7951
Number of cells
15.0
10.0
5.0
0.0
day0
day2
day4

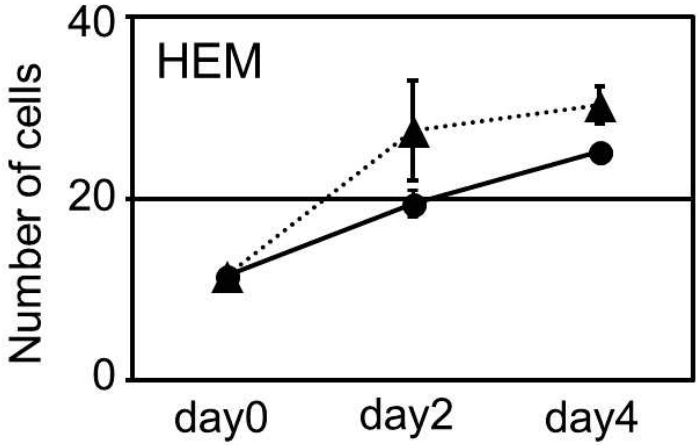

A375
Number of cells
15.0
10.0
5.0
0.0
day0
day2
day4


SK-MEL-5
Number of cells
6.0
4.0
2.0
0.0
day0
day2
day4

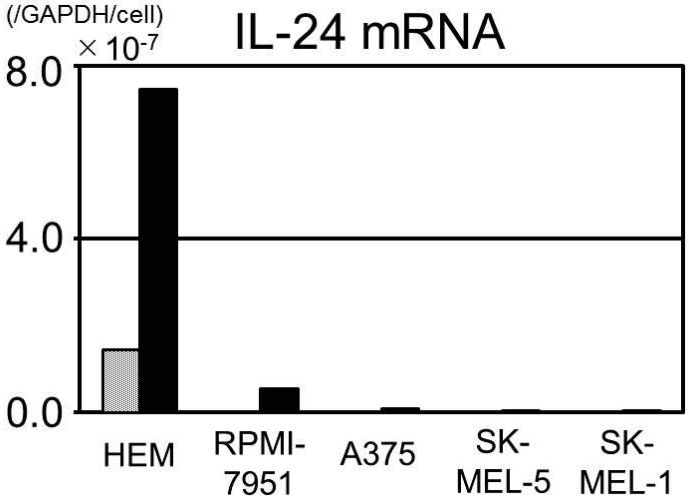

SK-MEL-1
Number of cells
9.0
6.0
3.0
0.0
day0
day2
day4

Supple. Fig. 6

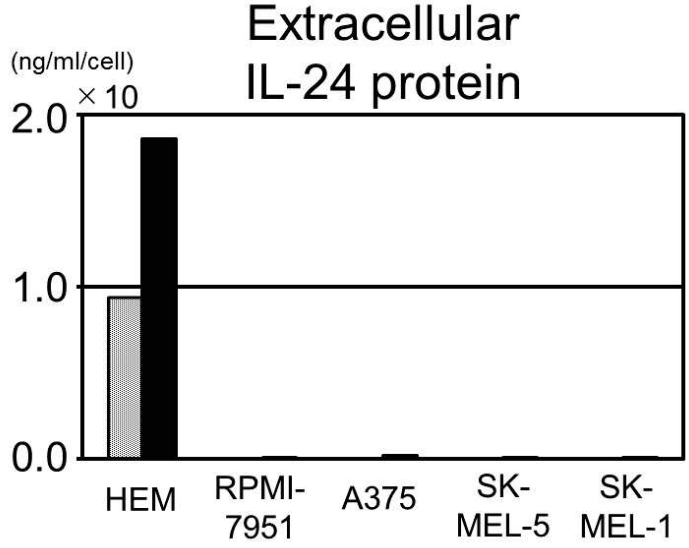
a



b



c



Supplementary table 1

Gene	Primer sequences (5' to 3')		AT(°C)*
	Forward	Reverse	
IL-24	*GGGAAGTGGACATTCTTCTGAC*	*CAGCATAGGAAGGGAGACTG*	65
IL-20R1	*CCATCTACCGATATATCCACGT*	*ATTTGACAAAAGATTCTTTG*	62
IL-20R2	*TCGCGCCTGGAGAAACAGT*	*AGCTGCTGGGGATCCAGATG*	65
IL-22R1	*CGTCGCAGTACTCTGCTACCTG*	*CGTCGCGCGAGTCCTGACTT*	65
GAPDH	*AGGTGAAGGTCGGAGTCAACG*	*AGGGGTCATTGATGGCAACA*	65

* Annealing temperature