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[Continued on next page]

(54) Title: METHODS OF INHIBITING RSK FOR TREATMENT OF BREAST CANCER

(57) Abstract: A method of treating triple negative breast cancer in a subject is provided. The method involves administering an effective dose of an inhibitor against the p90 ribosomal S6 kinase (RSK) family of kinases to the subject. The method may involve selective inhibition of RSK 1 or RSK2. The inhibitor may be a siRNA, BI-D1870 or SL0101.

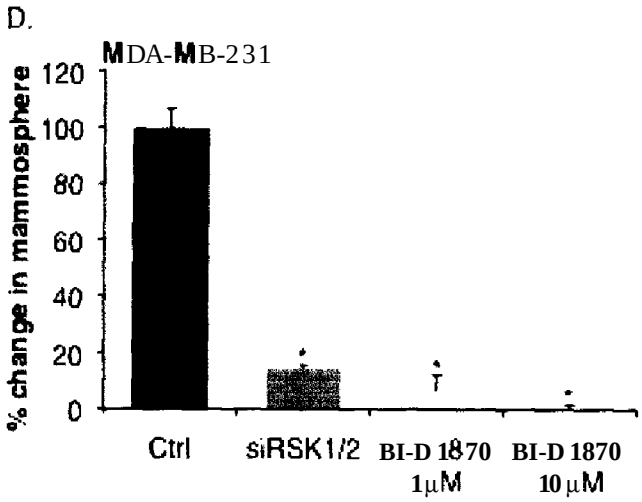

Figure 4

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METHODS OF INHIBITING RSK FOR TREATMENT OF BREAST CANCER

CROSS REFERENCE TO RELATED APPLICATION

[001] This application claims priority from United States Provisional Patent Application Serial Number US 61/655,220 filed on June 4, 2012, entitled METHODS AND USES OF RSK FOR THE DIAGNOSIS AND TREATMENT OF BREAST CANCER, which is expressly incorporated by reference herein to the extent permitted by law.

FIELD OF THE INVENTION

[002] The invention relates to treating breast cancer, and more particularly to treating breast cancer by inhibiting the p90 ribosomal S6 kinase (RSK) family of kinases.

BACKGROUND OF THE INVENTION

[003] The treatment of triple negative breast cancer (TNBC) suffers from a lack of targeted therapies. While these tumors often initially respond well to chemotherapy, they commonly become resistant in the long-term leading to relapse (Dent *et al.,* 2007; *Clinical Cancer Research,* 13:4429-4434; Liedtke *et al.,* 2008; *Journal of Clinical Oncology,* 26(8):1275-1281). Targeted therapies for treating TNBC are not available.

[004] Tumor initiating cells (TICs), which are $CD44^{+}/CD24^{-}$, are more frequent in TNBC than in other breast cancer subtypes, and have been linked to tumor recurrence (Honeth *et al.,* 2008; *Breast Cancer Research,* 10:R53; Charafe-Jauffret *et al.,* 2009; *Cancer Research,* 69:1302-1313; Nakshatri *et al.,* 2009; *Current Stem Cell Research and Therapies,* 4:50-60; and Park era/., 2010; *Clinical Cancer Research,* 16(3):876-887). This is, in part, due to that fact that they are intrinsically resistant to traditional chemo- and radiotherapy, but also the percentage of TICs increases following chemotherapy (Creighton *et al.,* 2009; *Proceedings of the National Academy of Sciences of the United*

States of America, 106:13820-13825; Li *et al.,* 2008; *Journal of the National Cancer Institute,* 100(9):672-679; Phillips *et al.,* 2006; *Journal of the National Cancer Institute,* 98:1777-1785; and Fillmore and Kuperwasser, 2008; *Breast Cancer Research,* 10:R25). TICs are characterized as having the ability to self-renew, grow as mammospheres, invade, to resist traditional therapies such as radiation and chemotherapy, and initiate tumor formation in mice (Stratford *et al.,* 2010; *Expert Reviews in Molecular Medicine,* 12:e22; Dontu and Wicha, 2005 *Journal of Mammary Gland Biology and Neoplasia,* 10:75-86; and Ponti *et al.*, 2005; *Cancer Research,* 65:5506-5511).

[005] The p90 ribosomal S6 kinase (RSK) family of kinases are activated by receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR), fibroblast growth factor and insulin-like growth factor receptors which are commonly activated in TNBC (Carriere *et al.,* 2008; *Frontiers in Biosciences,* 13:4258-4275; Kang ei *al.,* 2007; *Cancer Cell,* 12(3):201-214; Law *et al.,* 2008; *Cancer Research,* 68(24):10238-10246; and Stratford *et al.,* 2008; *Breast Cancer Research,* 10(6):R99). This allows RSK to phosphorylate downstream targets involved in tumor growth, invasion and epithelial-mesenchymal transition (Carriere *et al.,* 2008; Anjum and Blenis, 2008; *Nature Reviews Molecular Cell Biology,* 9:747-758). These include transcription factors such as Y-box binding protein-1 (YB-1) (Stratford *et al.,* 2008), creb and c-fos (Chen *et al.,* 1993; *Proceedings of the National Academy of Sciences of the United States of America,* 90:10952-10956) as well as the anti-apoptosis protein BAD (Shimamura *et al.,* 2000; *Current Biology* 2000, 10:127-135), the translation factor GSK3p (Sutherland *et al.,* 1993; *Biochemical Journal,* 296:15-19) and histone H3 (Lau and Cheung, 2011; *Proceedings of the National Academy of Sciences of the United States of America,* 108:2801-2806 and Sassone-Corsi *et al.,* 1999; *Science,* 285:886-891). More specifically, RSK phosphorylates YB-1 at S102 leading to nuclear translocation and transcriptional activation, as has been demonstrated in *in vitro* biochemical studies (Stratford *et al.,*

2008). RSK is also activated by commonly used chemotherapies such as paciitaxel leading to the phosphorylation of YB-1 ^{S102} and ultimately to the induction of CD44 (To ef a/., 201 0; *Cancer Research,* 70:2840-2851).

SUMMARY OF INVENTION

[006] In one aspect, there is provided a method of treating TNBC in a subject. The method involves administering an effective dose of an inhibitor against the p90 ribosomal S6 kinase (RSK) family of kinases to the subject. The inhibitor may selectively inhibit RSK1 or RSK2.The inhibitor may be a small molecule inhibitor or a small interfering RNA (siRNA). The small molecule inhibitor may be BI-D1 870 or SL01 0 1. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. The subject may be a human.

[007] In another aspect, a method of inducing apoptosis in a tumor cell in a subject is provided. The method involves administering an effective dose of an inhibitor against the RSK family of kinases to the subject.

[008] In another aspect, a method of decreasing CD44 expression in a tumor cell in a subject is provided. The method involves administering an effective dose of an inhibitor against the RSK family of kinases to the subject. The tumor cell may be a TNBC tumor cell. The inhibitor may selectively inhibit RSK1 . The inhibitor may selectively inhibit RSK2. The inhibitor may be a small molecule inhibitor or a siRNA. The small molecule inhibitor may be BI-D1870 or SL0101. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Further and optionally, the tumor cell may express any one or more of CD49f and EpCam. The tumor cell may be a tumor-initiating cell. The subject may be a human.

[009] In another aspect, a method of diagnosing breast cancer in a subject is provided. The method involves identifying phosphorylated RSK in a breast tissue of the subject. In another aspect, a method of diagnosing breast cancer in a subject is provided. The method involves identifying mRNA expression of RSK2 in a breast tissue of the subject. With respect to the aforementioned methods, the breast cancer may be a TNBC. The subject may be a human.

[0010] In another aspect, a method for diagnosing a TNBC in a human subject is provided. The method involves: (a) determining phosphorylated RSK protein levels in a biological sample obtained from the subject; and (b) making a TNBC determination based on the phosphorylated RSK protein levels in the subject sample.

[0011] In another aspect, a method of monitoring RSK inhibition in a biological sample is provided. The method involves: (a) obtaining a first expression level reading for each of phosphorylated-YB-1, phosphorylated-GSK3p, and phosphorylated-histone H3 from the biological sample; (b) introducing a compound which may or may not inhibit RSK in the biological sample; and (c) obtaining a second expression level reading for each of P-YB-1, P-GSK3β, and P-histone H3, wherein when the second expression level is lower than the first expression level, there is RSK inhibition in the biological sample as a result of the compound introduced in step (b). The biological sample may be a TNBC.

[0012] In another aspect, a method of treating breast cancer in a subject is provided. The method involves administering a chemotherapeutic agent together with an inhibitor against the RSK family of kinases to the subject. The chemotherapeutic agent may be a taxane or an anthracycline. The inhibitor may selectively inhibit RSK1. The inhibitor may selectively inhibit RSK2. The inhibitor may be a small molecule inhibitor or a siRNA. The small molecule inhibitor may be BI-D1870 or SL0101. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ

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[0013] In another aspect, a method of diagnosing breast cancer risk in a subject is provided. The method involves identifying the presence or absence of phosphorylated Y-box binding protein-1 (YB-1) in a breast tissue of said subject, wherein the presence of phosphorylated YB-1 is indicative of a higher risk of breast cancer associated relapse and/or patient survival and wherein the absence of phosphorylated YB-1 is indicative of a lower risk to breast cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 demonstrates that YB-1-induced $CD44^{h¾h}$ cells remain sensitive to RSK inhibition by BI-D1870. In Panel A, stable incorporation of Flag-YB-1 into the MDA-MB-231 cell line was validated by Western blot and 500 cells were injected into the 4^{th} inguinal mammary fat pads of NOD/SCID mice. In Panel B, tumors from cells expressing Flag-YB-1 had increased growth rates compared to empty vector control tumors. In Panel C, cells isolated from tumors expressing Flag-YB-1 had higher expression of the TIC markers CD44 and CD49f, as well as P-YB-1^{S102} as assessed by Western blot. In Panel D, RSK inhibition via BI-D1870 (10μM) suppressed growth and P-YB-1^{S102} in the Flag-YB-1 population with comparable efficacy as in the control empty vector cells. Control Western blot demonstrates P-YB-1^{S102} suppression by BI-D1870 in both cell lines.

[0015] **Figure 2** demonstrates that inhibiting RSK suppresses growth of TNBC cell lines. In Panel A, SUM149 cells transfected with siRNA against RSK1 and/or RSK2 for 10 days

showed 50-100% growth suppression. Western blot demonstrates loss of protein after 72hrs. In Panel B, doses of BI-D1870 as low as 2μM results in 80% reduction of SUM149 cell growth. Western blot demonstrates decreased P-YB-1^{S102} across a range of BI-D1870 concentrations at 96hrs. In Panel C, the effect of BI-D1870 on SUM149 cell growth could be partially rescued through expression of an activated YB-1 mutant (D102). Transgene expression was validated by Western blot. In Panel D, cells which survived 72hrs BI-D1870 treatment were seeded at low density in the RSK inhibitor and allowed to grow for 10 days. Treated cells did not grow in this clonogenic assay. In Panel E, treatment of SUM149 cells with BI-D1870 resulted in the induction of apoptosis as measured by PI uptake.

[0016] Figure 3 demonstrates that RSK inhibition decreases CD44 expression. In Panel A, treating SUM149 cells with BI-D1870 decreases nuclear localization of P-YB-1^{S102}. Immunofluorescence shows P-YB-1^{S102} (grey) and hoechst. Scale bar is 20μm. When inhibited by BI-D1870, there is decreased nuclear staining of P-YB-1^{S102} *{i.e.,* loss of grey). In Panel B, inhibiting RSK with BI-D1870 decreases CD44 promoter activity. In Panel C, CD44 transcript levels decrease after treatment with RSK siRNA in the SUM149 and MDA-MB-231 cell lines. In Panel D, the CD44^{h¾h} fraction in cell populations was reduced upon RSK inhibition with BI-D1870 (10μM). In Panel E, paclitaxel, but not BI-D1870 induces CD44 expression. Further, the combination of BI-D1870 and paclitaxelprevented the induction of CD44.

[0017] Figure 4 demonstrates that targeting RSK suppresses growth of TICs. In Panel A, CD44^{+} cells express high levels of P-histone H3^{S10} (creating a grey nuclear halo). Scale bar is 15μm. In Panels B-C, treatment with BI-D1870 (1-10μM) reduces P-histone H3^{S10} (diminishes the grey halo around the nucleus) and CD44 protein levels respectively. In Panel D, MDA-MB-231 cells treated with BI-D1870 or RSK1/2 siRNA had a significantly

reduced ability to form mammospheres after 7 days. In Panel E, mammosphere number was reduced following treatment of established spheres with BI-D1870 (10μM). The RSK isoforms (RSK1 or RSK2) were individually silenced with siRNA and again loss of either isoform suppressed mammosphere growth (Panel F). The combination of RSK1 and RSK2 also inhibited growth in this assay by >90% (Panel F). To illustrate the potency of each siRNA the controls are shown where loss of RSK1 inhibited protein expression but had no effect on RSK2 (Panel G). The propensity of cell to form tumors is mice if often preclinically assessed using soft agar growth assays also referred to as anchorage-independent growth. Silencing RSK1 , RSK2 or both isoforms simultaneously suppressed the growth of the TNBC cell lines SUM149 and MDA-MB-231s in soft-agar assays (Panel H). The controls for this are shown where loss of RSK1 reduced RSKImRNA by >90% but had no effect on RSK2 and visa versa (Panel I).

[0018] Figure 5 demonstrates that inhibiting RSK suppresses growth in $CD44^+$ cells. In Panel A, $CD44^+/CD24^-$ sorted cells have a much higher level of activated RSK and YB-1 than CD447CD24* cells. In Panel B, quantification of the level of P-$RSK^{S221/7}$ in $CD44^+$ compared to $CD44^-$ cells along with representative images of P-$RSK^{S221/7}$ staining. In Panel C, treatment of $CD44^+/CD24^-$ sorted cells with a single dose of BI-D1870 (1μM-10μM) results in a -90% decrease in growth after 72hrs. In Panel D, treatment of $CD447CD24^-$ sorted cells with BI-D1870 induces apoptosis in TICs as indicated by PI uptake. In Panel E, knockdown of RSK2 in $CD447CD24^-$ sorted SUM149 cells resulted in a -70% decrease in growth after 96hrs. Western blot demonstrates loss of protein. In Panel F, suppression of RSK2 with siRNA led to an increase in apoptosis as demonstrated by elevated PI uptake.

[0019] Figure 6 demonstrates that inhibiting RSK2 delays tumor initiation. In Panel A, MDA-MB-231 cells are ubiquitously $CD447CD24^-$ as demonstrated by flow cytometry and

these cells form tumors in mice within two weeks. In Panel B, a reduction in RSK2 transcript and protein (inset) was achieved at 48hrs in MDA-MB-231 cells. In Panel C, Transient RSK2 knockdown inhibited tumor initiation of MDA-MB-231 cells ($1x10^6$ cells/injection) in NOD/SCID mice (p=0.058).

[0020] Figure 7 demonstrates that activated RSK is expressed in TNBC patient samples. In Panel A, RSK2 mRNA was associated with poor survival in 244 breast cancer cases who had not received chemotherapy (log-rank $p=8.3x10^{-6}$; Cox proportional hazards $p=1x10^{-5}$) (left). When examining RSK2 expression in a cohort of 771 breast cancer cases representing all subtypes it was highest in the basal-like subtype (centre) (N=771;p<0.005) and in those of high grade (right) (N=771; p<0.05). In Panel B, P-$_{RSK\ s22i/7\ wgs}$ detected j$_n$ 13/18 aggressive breast tumor samples. P-YB-1^{S102} and CD44 expression also correlated (see Table 4). In Panel C, expression of activated RSK and YB-1 was not detected in normal breast tissue. Scale bars represent 100pm.

[0021] Figure 8 demonstrates that RSK1 and RSK2 kinase activity is inhibited with BI-D1870 when using a YB-1 peptide including the S102 residue as the substrate (see: Panel A). Similar results were observed using a peptide to S6 kinase, a known RSK substrate (data not shown). In Panel B, BI-D1870 (10μM) prevents activation of two downstream RSK substrates P-YB-1^{S102} and GSK3p^{S9}.

[0022] Figure 9, Panel A demonstrates that BI-D1870 inhibits growth of SUM149 and MDA-MB-231 cell lines after 72hrs. P-YB-1^{S102} is decreased at this time point (inset). Panel B demonstrates that SL0101 inhibits growth of SUM149 cells after 72hrs. Panel C demonstrates that treatment of SUM149 cells with BI-D1870 induces apoptosis as shown by induction of P-H2AX^{S139} and PARP cleavage, as shown in Panel D. Panel E demonstrates that apoptosis was also assessed by Annexin-V staining after treatment

with BI-D1870 in the SUM149 cells at 48hrs and (in Panel F) in the MDA-MB-231 cells at 72hrs using flow cytometry.

[0023] Figure 10 demonstrates that treatment of SUM149 cells with BI-D1870 (10μM) resulted in a decrease in CD44 transcript levels.

[0024] Figure 11, Panel A demonstrates that CD44* cells are more actively under going cell division and express a higher proportion of mitotic figures and P-histone $H3^{S10}$ staining when compared to non-$CD44^+$ cells. Panel B demonstrates that sorted $CD44^+/CD24^-$ SUM149 cells had increased mammosphere-forming ability compared to CD447CD24* cells. Panel C demonstrates mammosphere formation in SUM149 cells was inhibited by both BI-D1870 and siRNA against RSK1/2. Panel D demonstrates that PI uptake was increased in $CD44^+$ compared to $CD44^-$ SUM149 cells.

[0025] Figure 12 demonstrates colony formation was not inhibited in hematopoietic progenitor cells treated with increasing doses of BI-D1870 at concentrations below 4-6 μM however in cancer cells and TIC's this RSK inhibitor killed cells. IC50s for erythroid and myeloid progenitors were 6 and 4.6μM respectively.

[0026] Figure 13, Panel A demonstrates BI-D1870 dose response on immortalized normal breast epithelial 184htert cells. There was no effect on growth at doses that killed cancer cells. Panel B demonstrates that SL0101 inhibited growth of SUM149 cells by 90% but had no effect on 184htert cells. Panel C demonstrates suppression of RSK using siRNA had no effect on 184htert cell growth.

[0027] Figure 14, Panel A demonstrates that RSK2 knockdown, but not RSK1, resulted in a decrease in CD44 expression in the MDA-MB-231 cells at 96hrs by immunoblotting.

Panels B-C demonstrate MDA-MB-231 cells were transfected with RSK2 siRNA for 7, 10 and 14 days. Knockdown was measured by Western blot analysis or qRT-PCR respectively. Panel D demonstrates that RSK2 mRNA was measured by qRT-PCR in tumors taken from the mice upon termination of the experiment to confirm RSK2 re-expression.

[0028] Figure 15 demonstrates real-time quantitatve PCR data demonstrating RSK2 mRNA levels normalized to T47D breast cancer cells. RSK2 levels tended to be higher in TNBC cell lines as compared to non-TNBC cell lines.

[0029] Figure 16 demonstrates that P-YB-1 ^{S102} is associated with poor overall survival (death due specifically to breast cancer) (Panel B) and relapse (Panel C) in a cohort of 1057 patients with invasive breast cancer based on quantitative reverse phase arrays, where 0=low P-YB-1; ^intermediate P-YB-1; and 2=high P-YB-1.

[0030] Figure 17 demonstrates the combined effect of chemotherapeutic agents and BI-D1870 on cell viability in MDA-MB-231 cells. The chemotherapeutic agents used are epirubicin (Epi) and paclitaxel (paclit).

[0031] Figure 18 demonstrates the **combined** effect of chemotherapeutic agents (methotrexate, epirubicine, 5-fluorouracil or paclitaxel) and BI-D1870 on cell viability in MDA-MB-231 cells.

DETAILED DESCRIPTION

[0032] In one aspect, there is provided a method of treating a triple negative breast cancer "TNBC" in a subject. The method involves administering an effective dose of an inhibitor against the p90 ribosomal S6 kinase (RSK) family of kinases to the subject.

[0033] As used herein, the term "triple negative breast cancer" is also referred to herein as TNBC. The term "triple negative breast cancer" includes tumors of the basal-like subtype.

[0034] The inhibitor may selectively inhibit RSK1 or RSK2.The inhibitor may be a small molecule inhibitor or a small interfering RNA (siRNA). The small molecule inhibitor may be BI-D1870 or SL0101. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. The subject may be a human.

[0035] In another aspect, a method of inducing apoptosis in a tumor cell in a subject is provided. The method involves administering an effective dose of an inhibitor against the RSK family of kinases to the subject.

[0036] In another aspect, a method of decreasing CD44 expression in a tumor cell in a subject is provided. The method involves administering an effective dose of an inhibitor

against the RSK family of kinases to the subject. The tumor cell may be a TNBC tumor cell. The inhibitor may selectively inhibit RSK1 . The inhibitor may selectively inhibit RSK2.

[0037] The inhibitor may be a small molecule inhibitor or a siRNA. The small molecule inhibitor may be BI-D1 870 or SL01 0 1. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Further and optionally, the tumor cell may express any one or more of CD49f and EpCam. The tumor cell may be a tumor-initiating cell. As used herein, the term "tumor-initiating cell" also refers to a breast cancer stem cell and a tumor repopulating cell. The subject may be a human.

[0038] In another aspect, a method of diagnosing breast cancer in a subject is provided. The method involves identifying phosphorylated RSK in a breast tissue of the subject. In another aspect, a method of diagnosing breast cancer in a subject is provided. The method involves identifying mRNA expression of RSK2 in a breast tissue of the subject. With respect to the aforementioned methods, the breast cancer may be a TNBC. The subject may be a human.

[0039] In another aspect, a method for diagnosing a TNBC in a human subject is provided. The method involves: (a) determining phosphorylated RSK protein levels in a biological sample obtained from the subject; and (b) making a TNBC determination based on the phosphorylated RSK protein levels in the subject sample.

[0040] In another aspect, a method of monitoring RSK inhibition in a biological sample is provided. The method involves: (a) obtaining a first expression level reading for each of phosphorylated-YB-1 , phosphorylated-GSK3p, and phosphorylated-histone H3 from the biological sample; (b) introducing a compound which may or may not inhibit RSK in the biological sample; and (c) obtaining a second expression level reading for each of P-YB-1, P- GSK3β, and P-histone H3, wherein when the second expression level is lower than the first expression level, there is RSK inhibition in the biological sample as a result of the compound introduced in step (b). The biological sample may be a TNBC.

[0041] In another aspect, a method of treating breast cancer in a subject is provided. The method involves administering a chemotherapeutic agent together with an inhibitor against the RSK family of kinases to the subject. The chemotherapeutic agent may be a taxane or an anthracycline. Further, a non-limiting list of chemotherapeutic agents includes: doxorubicin, epirubicine, etoposide, 5-fluorouracil, cisplatin, methotrexate, and cyclophosphamide. The inhibitor may selectively inhibit RSK1 . The inhibitor may selectively inhibit RSK2. The inhibitor may be a small molecule inhibitor or a siRNA. The small molecule inhibitor may be BI-D1 870 or SL01 0 1. Optionally, the siRNA may include a sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6. Optionally, the siRNA may include a

sequence that has at least 80% sequence identity to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. Optionally, the sequence identity may be 90% or 95% to the aforementioned sequences. Further, the siRNA may include a sequence according to the following siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16. The subject may be a human.

[0042] In another aspect, a method of diagnosing breast cancer risk in a subject is provided. The method involves identifying the presence or absence of phosphorylated Y-box binding protein-1 (YB-1) in a breast tissue of said subject, wherein the presence of phosphorylated YB-1 is indicative of a higher risk of breast cancer associated relapse and/or patient survival and wherein the absence of phosphorylated YB-1 is indicative of a lower risk to breast cancer.

[0043] As used herein, the term "risk" includes a risk of a subject dying from breast cancer and alternately includes a higher risk of suffering from recurrent breast cancer. The term "risk" also includes a risk for increased tumor cell growth, and a risk for increased tumorigenic potential and an increased propensity to relapse. The term also includes the pathological development of breast cancer.

Materials and Methods

[0044] Cell Lines and reagents. SUM149 (Asterand, Detroit, Ml) and MDA-MB-231, MDA-MB-468, HCC1143, HCC1937 and MDA-MB-453 (ATCC; Manassas, VA) cells were used as models of TNBC and cultured as previously described (Stratford *et al.,* 2008). For high-throughput cell-based screening, breast tumor cell lines were purchased from either Asterand or ATCC and maintained according to the distributor's instructions. 184htert,

immortalized normal breast epithelial cells, were cultured as previously described (To *et al.,* 2007; *Molecular Pharmacology,* 72(3):641-652). The RSK specific inhibitor SL0101 (Toronto Research Chemicals Inc., North York, ON) was dissolved in methanol (Smith *et al.,* 2005), and BI-D1870 from Stemgent (Cambridge, MA) was dissolved in DMSO. RSK1 and RSK2 siRNA were obtained from Qiagen (Mississauga, ON). RSK1 and RSK2 siRNA sequences are detailed as follows:

RSK1
1. Hs_RPS6KA1_9 (RSK1a)

Target sequence: 5'-CCCAACATCATCACTCTGAAA-3' (SEQ ID NO: 1)
Sense strand: 5'-CAACAUCAUCACUCUGAAATT-3' (SEQ ID NO: 2)
Antisense strand: 5'-UUUCAGAGUGAUGAUGUUGGG-3' (SEQ ID NO: 3)

2. Hs_RPS6KA1_10 (RSK1b)

Target sequence: 5'-TGCCACGTACTCCGCACTCAA-3' (SEQ ID NO: 4)
Sense strand: 5-CCACGUACUCCGCACUCAATT-3' (SEQ ID NO: 5)
Antisense strand: 5'-UUGAGUGCGGAGUACGUGGCA-3' (SEQ ID NO: 6)

RSK2
1. HsRPS6KA3_5 (RSK2a)

Target sequence: 5'-AGCGCTGAGAATGGACAGCAA-3' (SEQ ID NO: 7)
Sense strand: 5'-CGCUGAGAAUGGACAGCAATT-3' (SEQ ID NO: 8)
Antisense strand: 5'-UUGCUGUCCAUUCUCAGCGCT-3' (SEQ ID NO: 9)

2. HsRPS6KA3_6 (RSK2c)

Target sequence: 5'-TCCAAACATTATCACTCTAAA-3' (SEQ ID NO: 10)
Sense strand: 5'-CAAACAUUAUCACUCUAAATT-3' (SEQ ID NO: 11)
Antisense strand: 5'-UUUAGAGUGAUAAUGUUUGGA-3' (SEQ ID NO: 12)

RSK2
1. Stealth RNA (siRNA) (RSK2b)
Sense strand: 5'-CCUCAGAUGAUGAAAGCCAAGCUAU-3' (SEQ ID NO: 13)
Antisense strand: 5'-AUAGCUUGGCUUUCAUCAUCUGAGG-3' (SEQ ID NO: 14)

2. Stealth RNA (siRNA) (RSK2d)
Sense strand: 5'-GGGAGGAGAUUUGUUUACACGCUUA-3' (SEQ ID NO: 15)
Antisense strand: 5'-UAAGCGUGUAAACAAAUCUCCUCCC-3 (SEQ ID NO: 16)

[0045] Unless otherwise noted, the siRNA experiments contained herein utilized RSK1 sequences SEQ ID NO:2 and SEQ ID NO:3 and RSK2 sequences SEQ ID NO:8 and

SEQ ID NO:9. The other disclosed siRNA sequences also worked for their intended function. It is specifically contemplated that variants of these sequences (e.g., 80% sequence similarity, 90% sequence similarity, and 95% sequence similarity) will also work so long as the intended functionality remains viable *(e.g.,* variants of the aforementioned sequences will be able to inhibit their respective RSK targets).

[0046] siRNA transfections. Cells were transfected with siRNA (20nM) (Qiagen; Madison, WI) with RNAiMAX (Invitrogen; Burlington, ON) using the fast forward protocol. All experiments were performed once the cells had been transfected for 72hrs unless otherwise stated.

[0047] Semi-quantitative real-time PCR. RNA was extracted from cells and xenograft tissue (RNeasy mini kit; Qiagen) and converted into cDNA (superscript III; Invitrogen). Quantitative realtime PCR (qRT-PCR) was performed to detect CD44 (catalogue number 4331182; Hs00153304_m1), RSK1 (catalogue number 4331 182; Hs01546665_m1), RSK2 (catalogue number 4331182; Hs00177936_m1), PPIA (cat no. 4333763F) and 18s ribosomal subunit using Taqman gene expression assays (Applied Biosystems/Life Technologies; Carlsbad, CA, USA).

[0048] Western blot analysis. Immunoblotting was **performed** as **previously** described (Wu era/., 2006; *Cancer Research,* 66:4872-4879). Antibodies were used as listed in the Supplemental Methods section herein.

[0049] Mammosphere assay. Cells were seeded (SUM149: 20,000 cells/well; MDA-MB-231: 50,000 cells/well) in ultra-low adherent 6 well plates (StemCell Technologies, Inc. Vancouver, BC) and grown in MammoCult® (StemCell Technologies) supplemented with

hydrocortisone and heparin. Spheres were counted after 7 days. For post-treatment of established spheres, BI-D1870 was added in fresh media to wells after 72hrs.

[0050] Anchorage-independent growth assay. RSK isoforms were silenced for 72 hrs and then the cells were placed in soft agar (SUM149 cells 5000 cells/well and MDA-MB-231 cells 5000 cells/well). Colonies were counted 21 days later.

[0051] Growth and Apoptosis Assays. Cells were seeded (3,000 - 5,000 cells/well) in 96 well plates, treated with inhibitors or siRNA and allowed to grow for a further 72hrs - 10 days (siRNA refreshed every 3 days). Cells were stained as previously described (Law ***et ai,*** 2008). Apoptosis was measured by PI uptake, P-H2AX^{S139} staining, PARP and Caspase-3 cleavage. For further details see the Supplemental Methods section herein.

[0052] Clonogenic Assay. SUM149 cells were seeded in a 6 well plate ($4x10^5$ cells/well) and treated with DMSO or BI-D1870 (10μM) for 72hrs. Surviving cells were counted and re-seeded in 6 well plates (1000 cells/well). After 7 days clonal colonies were visualized with crystal violet and manually counted.

[0053] CD44 Promoter Assay. SUM149 cells were transfected with a CD44 promoter construct, as previously described (To ***et ai.,*** 2010). Cells were treated with BI-D1870 (10μM) 6hrs prior to harvest.

[0054] Immunofluorescence. Staining for nuclear localization of P-YB-1^{S102} and P-histone H3^{S10} was performed using SUM149 (5,000 cells/well) seeded in a 96 well plate and treated with BI-D1870 at 24hrs then allowed to grow for a further 48hrs. For details of staining procedure see the Supplemental Methods section herein.

[0055] YB-1 $^{D_{10}2}$ rescue. SUM149 cells were seeded in 6 well plates ($4x10^5$ cells/well) and transfected 24hrs later with 5µg 3xflag:EV or 3xflag:YB-1S102D (D102) using FuGene HD (Roche, Laval, QC). After 24hrs cells were reseeded in 96 well plates (5,000 cells/well). Remaining cells were collected for protein analysis. Plated cells were treated with BI-D1870 for 72hrs and then stained for Hoechst as described in the Supplemental Methods section herein.

[0056] FACS analysis. A single cell suspension of SUM149 cells was obtained as previously described (To ef a/., 2010). Cells were stained with CD44-PE conjugated (BD Pharmingen), CD24-FITC conjugated (StemCell Technologies), and 7-aminoactinomycin D (7-AAD) viability dye (BD Pharmingen) and sorted for the top 10% $CD44^+/CD24^-$ population.

[0057] *In vivo* tumor growth xenograft model. All experimentation involving mice were conducted in accordance with the standard protocol approved by the University Committee on the Use and Care of Animals at the University of British Columbia.

[0058] *YB-1 overexpressing cells.* Injections were performed using stable cell lines containing either Flag-YB-1 or control empty vector in the MDA-MB-231 created as previously described (To *et ai,* 2010). Cells (500 cells/injection) were resuspended in PBS containing 25% matrigel (BD biosciences) and injected into the 4th inguinal mammary gland of 6-8 week old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles River). Tumors were measured using a digital caliper and volume was calculated using $V = W^2 \times L/2$.

[0059] *Effect of RSK2 on tumor initiation.* To test the effects of transient RSK2 knockdown on tumor initiation MDA-MB-231 cells were transfected with either RSK2

siRNA or control siRNA for 48hrs. Knockdown was validated using both qRT-PCR and Western blot analysis. Orthotopic mammary fat pad injections were performed in a similar manner as described above. In order to achieve tumor initiation at a time that would capture the effects of RSK2 knockdown mice were injected with 1 x 10^6 cells/injection. A tumor was considered to be anything measuring r > 2.5 mm.

[0060] Dissociation of tumors. Mice were humanely euthanized when tumors reached 500 mm^3. Tumors were harvested, dissociated mechanically and digested in DMEM containing collagenase/hyaluronanidase (StemCell Technologies) at 37°C for 4hrs. Red blood cell lysis was performed by incubating the suspension in ammonium chloride. To ensure a single cell suspension the cells were passed through a 40pm nylon mesh. This was then used to perform flow cytometry, Western blot analysis and drug treatment experiments.

[0061] Flow analysis. Cultured or xenograft cells were resuspended in FACS buffer (PBS containing 2% FBS and 5mM EDTA then stained with CD44-PE conjugated (BD Pharmingen), CD24-FITC conjugated (StemCell Technologies), and 7-aminoactinomycin D (7-AAD) viability dye (BD Pharmingen). Cells were washed once and resuspended at ~10 x 10^6 cells/mL in FACS buffer and collected using a FACS Calibur. Analysis was performed using Flowjo software.

[0062] RSK2 survival and subtype analyses. RSK2 mRNA levels were assessed in RMA-normalised Affymetrix HG-U133A or HG-U133PLUS2 microarray data from 771 clinically annotated breast tumors drawn from five breast cancer cohorts as previously described (Lasham *et al.,* 2012; *Journal of the National Cancer Institute,* 104:1-14). Using the only probe set for RSK2 (RPS6KA3- 203843_at), expression levels were plotted against histological grade and subtype for all 771 patients, followed by one-way

analysis of variance and Tukey's Honestly Significant Difference Test to determine the statistical significance, using the R statistical environment. In addition Kaplan-Meier survival analysis with both log-rank significance tests (Harrington and Fleming, 1982; *Biometrika,* 69:553-566) (comparing above -vs- below RSK2 median expression) and significance tests using Cox proportional hazards models (http://cran.r-project.org/web/packages/survival/) were performed on those 244 patients who had not received any kind of adjuvant treatment. Patients with events ≥ 12 years were excluded, since over 82% of these patients came from a single cohort (Desmedt *et al.,* 2007; *Clinical Cancer Research,* 13:3207-3214).

[0063] Immunohistochemistry. A breast cancer TMA of 18 patients with high-grade infiltrating ductal tumors was obtained and stained. The sections were stained with P-YB-1^{S102} (1:100), P-$RSK^{S221/7}$ (1:200), or CD44 (1:100) antibodies. Statistical analyses were performed using JMP version 8.0.2 (SAS Institute Inc). Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients. Differences were considered statistically significant for p values <0.05. Scale bars on images represent 100pm.

Supplemental methods

[0064] Western blot antibodies. RSK1; 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), RSK2; 1:500 (Santa Cruz Biotechnology), YB-1; 1:2000 (Cell Signaling Technology, Boston, MA), YB-1; 1:1000 (Epitomics, Burlingame, CA), CD44; 1:1000 (Abeam, Cambridge, MA), Flag; 1:2000 (Sigma, Oakville, ON), P-$RSK^{S221/7}$; 1:1000 (Invitrogen), P-YB-1^{S102}; 1:1000 (Cell Signaling Technology), P-Histone $H2AX^{S139}$; 1:1000 (Abeam), GSK3P; 1:1000 (Cell Signaling Technology) α/β-Tubulin; 1:1000 (Cell Signaling Technology), Vinculin; 1:1000 (Upstate, MA) and Pan-actin; 1:1000 (Cell Signaling Technology).

[0065] Apoptosis assays. Sorted and unsorted SUM149 cells treated with BI-D1870 were assessed for indicators of apoptosis. In all instances two of the following assays were performed; PARP cleavage, caspase-3 cleavage, propidium iodide uptake or phosphorylation of histone $H2AX^{S139}$. PARP and caspase-3 cleavage and P-histone $H2AX^{S139}$ were measured by immunoblotting. Propidium iodide (^g/ml) was added to the medium of unfixed cells and incubated at 37°C for 1h. Signal was quantified on the Cellomics, ArrayScan VTI.

[0066] Immunofluorescence. Primary antibodies used were P-histone $H3^{S10}$ (Cell Signaling, 1:200), P-YB-1^{S102} (Cell Signaling, 1:100), CD44-PE conjugated and Hoechst dye (Vg/ml). Secondary antibody used was Alexafluor 488 anti-rabbit. Cells were mounted with Prolong Gold antifade media with DAPI (Invitrogen) and signal was quantified on the Cellomics, ArrayScan VTI, as previously described (Law *et al.,* 2008).

[0067] RSK kinase assays. RSK kinase assays were conducted with and without BI-D1870 as previously described using 50mM ATP (Stratford *et* al., 2008).

[0068] Annexin V Staining. SUM149 and MDA-MB-231 cells were treated with BI-D1870 and collected at 48hrs and 72hrs respectively. Cells were trypsinised and a single cell suspension was obtained. PE Annexin V Apoptosis Detection Kit (BD Pharmingen) was used to detect apoptosis as per the manufacturer's protocol. Cells were stained for Annexin V (1:20) on ice for 20 minutes then analyzed by flow cytometry.

[0069] Hematopoietic Stem Cell Assay. Hematopoietic stem cell differentiation and growth was assessed following treatment with BI-D1870 (0.015μM-15μM) as performed by Stem Cell Technologies.

Results

[0070] Example 1. ***RSK inhibitor BI-D1870 is effective at suppressing growth of a YB-1-induced CD44* population.*** Previously, YB-1 was identified as an oncogenic transcription factor with the ability to regulate a TIC phenotype including TIC markers CD44 and CD49f as well as mammosphere formation and drug resistance (To *et al.*, 2010). Using YB-1 as the driver to induce a CD44-high population, stable cell lines expressing a Flag-YB-1 transgene in MDA-MB-231 cells were created (Fig. 1A). When injected into the mammary fat pad of NOD/SCID mice (500 cells/mammary fat pad), cells overexpressing YB-1 demonstrated a significantly increased growth rate when compared to empty vector control cells (Fig. 1B). The tumors were isolated, cell lines established and characterized for TIC markers. We noted that the tumors arising from Flag-YB-1 cells had increased CD44 expression as indicated by immunoblotting (Fig. 1C). A second YB-1 target gene, CD49f was also confirmed to be induced in these explanted cell cultures (Fig. 1C). P-YB-1^{S102} was elevated in the Flag-YB-1 cells although it is still present in the EV cells (Fig. 1C). The induction of CD44 was further confirmed in a second pair of explanted tumors from MDA-MB-231 cells expressing the empty vector as compared to Flag-YB-1 (data not shown). Interestingly, despite having an increased CD44^{+} population, BI-D1870, a small molecule RSK ATP competitive inhibitor, was capable of inhibiting growth in the Flag-YB-1 cell population ex *vivo* suggesting that Flag-YB-1 relies on RSK for enhanced growth (Fig. 1D). In both *ex vivo* cell lines the target P-YB-1^{S102} was eliminated following treatment with BI-D1870 (Fig. 1D). To further elucidate the role of RSK1 and RSK2 separately on YB-1 activation, an *in vitro* kinase assay was performed using a YB-1 peptide as the substrate (Stratford *et al.*, 2008 and Law *et al.,* 2010; *PLoS one,* 5:e12661). Both RSK1 and RSK2 directly phosphorylate YB-1 at Ser102, which can be inhibited with BI-D1870 (Fig. 8A). Additionally, BI-D1870 was shown to block activation of a second downstream RSK substrate GSK33 (Fig. 8B). Thus, YB-1 appears to drive

tumor initiation *in vivo* and the tumors that arise have higher level of TICs. While CD44 is high in the emergent tumors they are sensitive to the RSK inhibitor.

[0071] More specifically, as shown in Figure 1, YB-1-induced $CD44^{high}$ cells remain sensitive to RSK inhibition by BI-D1870. As shown in Figure 1, Panel A, stable incorporation of Flag-YB-1 into the MDA-MB-231 cell line was validated by Western blot and 500 cells were injected into the 4^{th} inguinal mammary fat pads of NOD/SCID mice. As shown in Figure 1, Panel B, tumors from cells expressing Flag-YB-1 had significantly increased growth rates compared to empty vector control tumors. Further, as shown in Figure 1, Panel C, cells isolated from tumors expressing Flag-YB-1 had higher expression of the TIC markers CD44 and CD49f, as well as P-YB-1^{S102} as assessed by Western blot. Further, as shown in Figure 1, Panel D, RSK inhibition via BI-D1870 (10μM) suppressed growth and P-YB-1^{S102} in the Flag-YB-1 population with comparable efficacy as in the control empty vector cells. Control Western blot demonstrates P-YB-1^{S102} suppression by BI-D1870 in both cell lines.

[0072] Further, and as specifically shown in Figure 8 (Panel A) herein, RSK 1 and RSK2 kinase activity is inhibited with BI-D1870 when using a YB-1 peptide including the S102 residue as the substrate. Similar results were observed using a peptide to S6 kinase, a known RSK substrate. Further as shown in Figure 8, Panel B, BI-D1870 (10μM) prevents activation of two downstream RSK substrates P-YB-1^{S102} and GSK3p^{S9}.

[0073] Example 2. *RSK2 siRNA suppresses growth of TNBC cells.* Thereafter, an investigation was made as to whether there was a specific RSK isoform that supports the growth of TNBCs. The TNBC cell line SUM149 was treated with RSK siRNAs which led to a >90% loss in their target protein expression after 72hrs (Fig. 2A inset). This corresponded with a similar decrease in P-YB-1^{S102} (Fig. 2A inset). Targeting RSK2 with

siRNA every 72hrs for a total period of 10 days inhibited the growth of the TNBC cell line SUM149 by 90% (Fig. 2A). A consistent growth inhibition was observed at 72hrs using two distinct siRNAs (detailed herein) against both RSK 1 and 2 (see: Table 1 below).

Table 1. Growth inhibition of SUM149 cells by two different oligonucleotides against RSK1 and RSK2 after 72 hrs.

	Growth Inhibition	
	siRNA1	siRNA2
RSK1	51.33	79.08
RSK2	66.30	42.20

[0074] RSK2 inhibition was far more effective at suppressing tumor cell growth than RSK1 as loss of the former suppressed growth by almost 100% and the latter by 50% (Fig. 2A).

[0075] More specifically, as shown in Figure 2 herein, inhibiting RSK suppresses growth of TNBC cell lines. As shown in Figure 2, Panel A, SUM149 cells transfected with siRNA against RSK1 and/or RSK2 for 10 days showed 50-100% growth suppression. Western blot demonstrates loss of protein after 72hrs.

[0076] Example 3. ***BI-D1870 blocks the growth of TNBC cells.*** Thereafter, an investigation was made as to whether it was possible to achieve similar growth suppression by treating TNBC cell lines with small molecule RSK inhibitors. Inhibiting pan RSK kinase activity with BI-D1870 (2μM or above) reduced the growth of SUM149 cells by >90% after 10 days, with repeated dosing every 3 days (Fig. 2B). Suppression of P-YB-1^{S102} was confirmed by immunoblotting at 96hrs (Fig. 2B inset). Further, BI-D1870 (0.1-10μM) or SL0101 (25-100μM), a second RSK inhibitor, suppressed P-YB-1^{S102} and tumor cell growth by up to >90% after only 72hrs (Fig. 9A and 9B respectively). To

establish that P-YB-1^{S102} was a mediator of the effect observed following treatment with BI-D1870 we transfected SUM149 cells with activated YB-1 (D102) or empty vector (EV) and after 24hrs exposed these cells to BI-D1870 (5 and 10μM) for 72hrs. Cell growth was then measured and as expected in the EV transfected cells BI-D1870 killed >80% of the cells (Fig. 2C). This phenotype was partially rescued in the D102 transfected cells (50% growth inhibition) (Fig. 2C). Transgene expression was validated by immunoblotting (Fig. 2C inset).

[0077] Further, as shown specifically in Figure 2, Panel B, doses of BI-D1870 as low as 2μM results in 80% reduction of SUM149 cell growth. Western blot demonstrates decreased P-YB1^{S102} across a range of BI-D1870 concentrations at 96hrs. Further, as shown in Figure 2, Panel C, the effect of BI-D1870 on SUM149 cell growth could be partially rescued through expression of an activated YB-1 mutant (D102). Transgene expression was validated by Western blot. Further, as shown specifically in Figure 9, Panel A, BI-D1870 inhibits growth of SUM149 and MDA-MB-231 cell lines after 72hrs. P-YB-1^{S102} is decreased at this time point (inset). Further, as shown in Figure 9, Panel B, SL0101 inhibits growth of SUM149 cells after 72hrs.

[0078] Next, an investigation was made to determine whether the few cells which remain following treatment with BI-D1870 are in fact resistant to the drug. Cells which remained after 72hrs BI-D1870 treatment were plated at low density along with control treated cells. While the control treated cells formed colonies in this clonogenic assay, there was 100% growth supression of the BI-D1870 treated cells, indicating that these cells have not developed resistance to the drug (Fig. 2D). Having demonstrated a growth suppressive effect following RSK inhibition we then assessed induction of apoptosis. Treating the SUM149 cells with both BI-D1870 and RSK siRNA resulted in the induction of apoptosis. This is demonstrated by PI uptake (Fig. 2E), P-H2AX^{S139} (Fig. 9C) and PARP cleavage

(Fig. 9D). Additionally, both SUM149 and MDA-MB-231 cells stained positively for the apoptotic marker Annexin-V when treated with BI-D1870 (1, 5 or 10μM) for48-72hrs (Fig. 9E-F). Thus, TNBCs are dependent upon RSK signaling to sustain tumor cell growth and blocking it triggers cell death.

[0079] Further, as shown in Figure 2, Panel D, cells which survived 72hr BI-D1870 treatment were seeded at low density in the RSK inhibitor and allowed to grow for 10 days. Treated cells did not grow in this clonogenic assay. Further, as shown in Figure 2, Panel E, treatment of SUM149 cells with BI-D1870 resulted in the induction of apoptosis as measured by PI uptake. Further, as shown specifically in Figure 9, Panel C, treatment of SUM149 cells with BI-D1870 induces apoptosis as shown by induction of P-H2AX^{S139} and PARP cleavage (see: Figure 9, Panel C therein). As shown in Figure 9, Panel D, apoptosis was also assessed by Annexin-V staining after treatment with BI-D1870 in the SUM149 cells at48hrs and F) in the MDA-MB-231 cells at 72hrs using flow cytometry.

[0080] **Example** 4. ***Inhibiting RSK decreases CD44 expression.*** Keeping in mind that the frequency of TICs is higher in TNBC than in other breast cancer subtypes and that RSK inhibition decreases the growth of TNBC cell lines, it was suspected that it would also have an effect on TICs. As previously mentioned, TICs are induced by the RSK substrate YB-1 through binding to the CD44 promoter in a phosphorylation dependent manner (To *et al.,* 2010 and Stratford *et al.,* 2008). Site-directed mutants that prevent YB-1^{S102} phosphorylation stop nuclear trafficking and over-ride YB-1's ability to induce TICs (To *et al.,* 2010). BI-D1870 was therefore used as a chemical probe to mirror this effect. BI-D1870 inhibited the nuclear translocation of P-YB-1^{S102} in SUM149 cells (Fig. 3A) and CD44 promoter activity (Fig. 3B). Moreover, decreases in CD44 transcript levels were observed following treatment with RSK1/2 siRNA or BI-D1870 (Fig. 3C and Fig. 10) with a concomitant reduction in the number of cells expressing high levels of CD44 (Fig. 3D).

Interestingly, as with the effect on growth, suppression of RSK2 with siRNA resulted in a much larger decrease in CD44 transcript levels than that of RSK1 (Fig. 3C). A fundamental problem with many chemotherapeutic agents is that they induce CD44 expression (see, for *e.g.,* Creighton *et al.,* 2009) and this is thought to be involved in drug resistance and recurrence. Herein we show that treatment of SUM149 cells with paclitaxel induced CD44 expression but this was not the case with BI-D1870 (Fig. 3E). More importantly, combining paclitaxel treatment with BI-D1870 reduced the induction of CD44 by the former (Fig. 3E).

[0081] Further, as shown specifically in Figure 3, RSK inhibition decreases CD44 expression. Further, as shown in Figure 3, Panel A, treating SUM149 cells with BI-D1870 decreases nuclear localization of P-YB-1^{S102}. Immunofluorescence shows P-YB-1^{S102} (green) and hoechst (blue) and the scale bar is 20μm. Further, as shown in Figure 3, Panel B, inhibiting RSK with BI-D1870 decreases CD44 promoter activity. Further, as shown in Figure 3. Panel C, CD44 transcript levels decrease after treatment with RSK siRNA in the SUM149 and MDA-MB-231 cell lines. Further, as shown in Figure 3, Panel D, the CD44^{high} fraction in cell populations was reduced upon RSK inhibition with BI-D1870 (10μM). Further, as shown in Figure 3, Panel E, paclitaxel, but not BI-D1870 induces CD44 expression. Further, the combination of BI-D1870 and paclitaxel prevented the induction of CD44. **Further,** as shown in Figure 10 herein, treatment of SUM149 cells with BI-D1870 (10μM) resulted in a decrease in CD44 transcript levels.

[0082] Example 5. ***Targeting RSK inhibits TIC growth.*** While conducting high content screening, it was noticed that CD44$^+$ cells were more proliferative than the CD44$^-$ cells, having a greater number of mitotic figures based on Hoechst staining (Fig. 11A). To further validate this, a second marker of mitosis, P-histone H3^{S10}, an early M-phase marker, was stained. Consistently, it was found that CD44$^+$ cells had higher P-histone

$H3^{S10}$ expression and were more actively undergoing mitosis (Fig. 4A). The replicative capacity of $CD44^+$ cells was blunted by exposing the cells to increasing amounts of BI-D1870 (Fig. 4B). There were also fewer $CD44^+$ cells in total (Fig. 4C). As, P-histone $H3^{S10}$ is downstream of RSK signaling, the suppression in growth in the $CD44^+$ population may be in part due to a perturbed mitotic process (Lau and Cheung, 2011 and Sassone-Corsi *et al*., 1999). Thus RSK inhibition repressed TICs ability to replicate. Taking this further the impact of RSK inhibition on mammosphere growth was examined. $CD44^+$ cells have a higher capacity to form mammospheres as compared to $CD44^-$ cells as previously reported (see, for *e.g.,* Li *et al.,* 2008 and Fillmore and Kuperwasser, 2008) and as reproduced herein (Fig. 11B). Additionally, it has been shown that knocking down CD44 reduces the ability of cells to form mammospheres (To *et al.,* 2010). As a functional readout of TICs the ability of MDA-MB-231 and SUM149 cells to form mammospheres in culture was measured. In line with the decreased CD44, RSK inhibition markedly suppressed mammosphere formation by 80-100% (Fig. 4D and Fig. 11C). There was such a substantial effect it was not possible to serially passage the spheres. The compound caused regression of established mammospheres (Fig. 4E). Extending these findings, individually inhibiting either RSK1 , RSK2 or both suppressed the growth of MDA-MB-231 cells in mammosphere cultures (Fig. 4F). The controls are provided to illustrate that RSK1 siRNA inhibited the protein expression of RSK1 but not RSK2 (Fig. 4G) and visa versa. The growth of MDA-MB-231 and SUM149 cells was also inhibited by silencing RSK1 , RSK2 or both in anchorage-independent growth assays (Fig. 4H). The corresponding controls are included to show that silencing RSK1 reduced RSK1 mRNA levels by >90% but had no effect on RSK2 (Fig. 4I) and visa versa).

[0083] Further, as shown specifically in Figure 4 , targeting RSK suppresses growth of TICs. Further, as shown in Figure 4, Panel A, $CD44^+$ cells express high levels of P-histone $H3^{S10}$ (observed as a grey halo around the nucleus). The scale bar is 15μm.

Further, as shown in Figure 4, Panels B-C, treatment with BI-D1870 (1-10 μM) reduces P-histone $H3^{S10}$ and CD44 protein levels respectively. Further, as shown in Figure 4, Panel D, MDA-MB-231 cells treated with BI-D1870 or RSK1/2 siRNA had a significantly reduced ability to form mammospheres after 7 days. Further as shown in Figure 4, Panel E, mammosphere number was reduced following treatment of established spheres with BI-D1870 (10 μM). Further, as shown specifically in Figure 11, Panel A, $CD44^+$ cells are more actively under going cell division and express a higher proportion of mitotic figures and P-histone $H3^{S10}$ staining when compared to non-$CD44^+$ cells. Further, as shown in Figure 11, Panel B, sorted $CD44^+/CD24^-$ SUM149 cells had increased mammosphere-forming ability compared to CD447CD24* cells. Further, as shown in Figure 11, Panel C, mammosphere formation in SUM149 cells was inhibited by both BI-D1870 and siRNA against RSK1/2. Further, as shown in Figure 11, Panel D, PI uptake was increased in $CD44^+$ compared to $CD44^-$ SUM149 cells.

[0084] Next, TICs ($CD44^+/CD24^-$ cells) that were isolated by FACS were found to be enriched for P-RSK $^{S221/227}$ and P-YB-1 ^{S102} compared to CD44/$CD24^+$ cells suggesting that this pathway may be particularly important in $CD44^+/CD24^-$ cells (Fig. 5A-B). These TICs were dependent upon RSK signaling because exposing them to BI-D1870 reduced cell growth by >90% after 72hrs at doses as low as 1μM (Fig. 5C). Apoptosis was also induced as indicated by increased PI uptake (Fig. 5D). When compared to their $CD44^-/CD24^+$ counterparts, TICs were found to have increased PI uptake after treatment with BI-D1870; perhaps, due to increased RSK expression in this population (Fig. 11D). Knockdown of RSK2 expression using siRNA similarly decreased growth and induced apoptosis in $CD44^+/CD24^-$ cells (Fig. 5E and F, respectively). In parallel with our findings in unsorted cells, RSK2 inhibition was more effective at suppressing growth and inducing apoptosis in TICs than RSK1 inhibition.

[0085] Further, as shown specifically in Figure 5, inhibiting RSK suppresses growth in $CD44^+$ cells. Further, as shown in Figure 5, Panel A, $CD447CD24^-$ sorted cells have a much higher level of activated RSK and YB-1 than CD447CD24* cells. Further, as shown in Figure 5, Panel B, quantification of the level of $P\text{-}RSK^{S221/7}$ in $CD44^+$ compared to $CD44^-$ cells along with representative images of $P\text{-}RSK^{S221/7}$ staining. Further, as shown in Figure 5, Panel C, treatment of $CD447CD24^-$ sorted cells with a single dose of BI-D1870 (1μM-10μM) results in a -90% decrease in growth after 72hrs. Further, as shown in Figure 5, Panel D, treatment of $CD44^+/CD24^-$ sorted cells with BI-D1870 induces apoptosis in TICs as indicated by PI uptake. Further, as shown in Figure 5, Panel E, knockdown of RSK2 in $CD447CD24^-$ sorted SUM149 cells resulted in a -70% decrease in growth after 96hrs. Western blot demonstrates loss of protein. Further, as shown in Figure 5, Panel F, suppression of RSK2 with siRNA led to an increase in apoptosis as demonstrated by elevated PI uptake.

[0086] An additional consideration is the effect of inhibiting RSK on normal stem cells. To address this question, the growth and differentiation of primary human hematopoietic stem cells when dosed with a range (0.015-15μM) of BI-D1870 was assessed. Treatment with BI-D1870 did not suppress the growth or differentiation of normal human hematopoietic stem cells below 5 μM (see: Table 2 herein, and Fig. 12A-B). The effect of inhibiting RSK on normal breast epithelial cells (184htert) was investigated. In accordance with the data on the hematopoeitic stem cells observed no effect on the growth of 184htert cells at concentrations up to 2μM BI-D1870 was observed (Fig. 13A), a dose which suppressed growth of TICs by 90% (Fig. 5C). Interestingly, neither SL0101 (50μM) or RSK siRNA, in particular RSK2 siRNA, had any effect on growth of normal mammary epithelial 184htert cells (Fig. 13B-C respectively).

[0087] Further, as shown specifically in Figure 12, colony formation ability of hematopoietic progenitor cells treated with increasing doses of BI-D1870 is demonstrated. Further, the IC50s for erythroid and myeloid progenitors were 6 and 4.6μM respectively. Further, as shown specifically in Figure 13, Panel A, BI-D1870 dose response on immortalized normal breast epithelial 184htert cells is demonstrated. Further, as shown in Figure 13, Panel B, SL0101 inhibited growth of SUM149 cells by 90% but had no effect on 184htert cells. Further, as shown in Figure 13, Panel C, the absence of an effect on 184htert cell growth by suppression of RSK using siRNA is demonstrated.

Table 2. Colony formation of hematopoietic progenitor cells treated with increasing doses of BI-D1870. Below 5 μM BI-D1870 did not suppress the differentiation of hematopoietic progenitor cells.

Concentration μM	CFU-E	BFU-E	Total Erythroid	CFU-GM	CFU-GEMM	Total CFC
STANDARD AND SOLVENT CONTROLS						
Standard	14+/-4	26+/-2	40+/-2	54+/-8	4+/-2	98+/-10
Solvent control	16+/-5	26+/-6	42+/-6	48+/-4	3+/-3	93+/-3
BI-D1870						
15μM	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
5μM	15+/-4	17+/-2	32+/-2	25+/-5	1+/-1	57+/-4
1.5μM	17+/-3	23+/-5	40+/-4	40+/-3	1+/-1	81+/-7
0.5μM	12+/-4	28+/-8	40+/-10	50+/-6	3+/-1	94+/-6
0.15μM	11+/-3	29+/-10	40+/-8	48+/-2	2+/-2	90+/-9
0.05μM	13+/-3	29+/-1	42+/-2	50+/-7	3+/-2	96+/-8
0.015μM	17+/-3	27+/-8	45+/-10	46+/-6	2+/-2	93+/-4

T-Test Results	
x < 0.0001	##
0.0001= x < 0.0005	**
0.0005 = x < 0.001	#
0.001 = x < 0.005	*

[0088] Example 6. ***RSK inhibition delays tumor initiation.*** To directly assess the effects of RSK knockdown on tumor formation we performed a transient RSK2 knockdown in MDA-MB-231 cells which homogeneously express high CD44 and low CD24 (Fig. 6A). RSK2 was silenced for 48hrs and loss of expression was confirmed by qRT PCR and immunoblotting (Fig. 6B). A >80% decrease in RSK2 expression (Fig. 6B)

was observed. This decrease in RSK lead to a subsequent loss of CD44 protein expression (Fig. 14A). NOD/SCID mice were injected with MDA-MB-231 cells transfected with either the scrambled control siRNA or RSK2 siRNA (1x10 ^{6} cells/MFP). Given the short half-life of siRNAs (10 days; Fig. 14B and C) high cell numbers were required. Based on prior experience in our pilot studies, it was known that this number of cells would initiate tumor formation within approximately two weeks and that the siRNAs would still be active within this timeframe. 100% of the mice (4/4) that received the MDA-MB-231 cells exposed to the scrambled control developed palpable tumors beginning at 17 days post-injection. In contrast, only 40% of the mice (2/5) developed tumors following RSK2 inhibition at the same time interval and this trend continued out until 24 days post-injection (Fig. 6C). The delay in tumor-initiation correlated with RSK2 expression in that after three weeks all of the mice eventually developed tumors (day 27) (Fig. 6C). At this point RSK2 expression in all tumors was validated and it was found to be equal to the controls (Fig. 14D). Once RSK2 was re-expressed, tumors grew at equivelant rates, however the average size of the RSK2 siRNA tumors was ~half that of the control tumors (see: Table 3). These results show that RSK inhibitors block the growth of TNBC cells *in vitro* and *in vivo* in part through the loss of TICs.

[0089] Further, as shown specifically in Figure 6, inhibiting RSK2 delays tumor initiation. Further as shown in Figure 6, Panel A, MDA-MB-231 cells are ubiquitously CD44 $^{+}$/CD24 $^{-}$ as demonstrated by flow cytometry and these cells form tumors in mice within two weeks (data not shown). Further, as shown in Figure 6, Panel B, a reduction in RSK2 transcript and protein (inset) was achieved at 48hrs in MDA-MB-231 cells. Further, as shown in Figure 5, Panel C, transient RSK2 knockdown inhibited tumor initiation of MDA-MB-231 cells (1x1 0^{6} cells/injection) in NOD/SCID mice (p=0.058).

[0090] Further, as shown specifically in Figure 14, Panel A, RSK2 knockdown, but not RSK1, resulted in a decrease on CD44 expression in the MDA-MB-231 cells at 96hrs by immunoblotting. Further, as shown in Figure 14 B-C, MDA-MB-231 cells were transfected with RSK2 siRNA for 7, 10 and 14 days. Knockdown was measured by Western blot analysis or qRT-PCR respectively. Further, as shown in Figure 14, Panel D, RSK2 mRNA was measured by qRT-PCR in tumors taken from the mice upon termination of the experiment to confirm RSK2 re-expression.

Table 3. Tumor volumes following RSK2 siRNA (tumor size cutoff >60 mm)

Tumor volume (mm^3)

Days post-injection	20	24	27
Ctrl	82.28	204.26	332.67
	120.32	289.00	480.75
	74.86	94.56	243.72
	151.73	191.33	315.81
RSK2 siRNA	0.00	52.91	75.28
	0.00	40.03	62.88
	114.05	250.28	469.16
	36.00	38.65	93.08
	142.45	178.20	212.63

[0091] Example 7. ***Activated RSK is expressed in patient samples and RSK2 levels in TNBC cells.*** As RSK2 was found to have the most significant effect on TNBC and TIC survival, it was then investigated whether it was also important in patient samples. RSK2 mRNA levels were assessed in 244 breast cancer patients who had not received adjuvant therapy. Patients with high RSK2 expression had significantly worse survival outcomes (Log rank test $p=8.3x10^{-6}$; Cox proportional hazards $p=1.01x10^{-5}$) (Fig. 7A left). Analysis of the entire cohort of 771 patients showed that interestingly, RSK2 expression was highest in tumors of the basal-like subtype (also referred to herein as triple-negative breast cancers) (Fig. 7A centre) and in those with the highest grade (grade 3; Fig. 7A right). In support of this data it was demonstrated that RSK2 expression levels were consistently significantly higher in TNBC cell lines compared with non-TNBC cell lines (see: Figure 15 herein). As detailed specifically in Figure 15, a panel of breast cancer cell

lines were plated at the same density and 24 hrs later the mRNA was extracted. The RNA was then reverse transcribed into cDNA and amplified for RSK2 using a set of gene specific primers and detected with a fluorscence-labeled probe (Applied Biosystems, Assay-On-Demand). The Assay-On-Demand product numbers and their corresponding RefSeq IDs used for the qRT-PCR analysis are as follows:

RPS6KA3 (Product number: Hs001 77936_m1 ; RefSeq: NM_004586.2) - target gene PPIA (Product number: Hs041 94521_s1 ; RefSeq: NM_021 130.3)- endogenous control. The relative expression levels are detailed in Figure 15. RSK2 mRNA tended to be higher in TNBC cell lines compared to non-TNBC breast cancer cell lines.

[0092] Further, as shown in Figure 17 herein, TNBC are more sensitive to the RSK inhibitor BI-D1870 than non-TNBC. More specifically, as detailed in Figure 17 herein, breast cancer cell lines were treated with the RSK inhibitor BI-D1 870 (10 μM) for 72 hrs and viability was assessed using Hoechst staining. The number of viable cells was quantified using the ArrayScan VTI (Cellomics), and are depicted in Figure 17.

[0093] It is important to note that while the basal-like subtype is defined by gene expression, the majority of basal-like breast cancers are also triple negative in terms of expression of cell surface receptor proteins (Foulkes ei *al.,* 201 0; *The New England Journal of Medicine,* 363: 1938-1948).

[0094] A focused collection of 18 high-grade breast cancers was obtained to address whether RSK was active in TNBC and if it correlated with either P-YB-1 ^{S102} or CD44. P-RSK $^{S221/227}$ was highly expressed in 85% (11/1 3) of TNBC (Fig. 7B). Activated RSK significantly correlated with P-YB-1 ^{S102} (P=0.0002, Spearman's correlation 0.771) (see: Table 4). Furthermore, in more than half the cases CD44 expression tracks with P-$_{RSK}$ $^{s22 1/2 27}$ and $p_{-Y\ B\ -I\ S}$ ^{io2} ($P_{= 0}$.(5333, Spearmar ı's correlation 0.5031 , and P=0.01 09,

Spearman's correlation 0.5840 respectively) (Table 4). In contrast to the high frequency of RSK and YB-1 activation in TNBC, P-RSK$^{S221/227}$ and P-YB-1^{S102} were not expressed in primary normal mammary ducts (Fig. 7C). To expand this finding, ten additional normal breast tissues were examined and RSK was consistently not expressed (Fig. 7C, two examples shown). It is thus demonstrated that the RSK/YB-1/CD44 pathway is activated in primary TNBC.

[0095] Further, as specifically shown in Figure 7, activated RSK is expressed in TNBC patient samples. Further, as shown in Figure 7, Panel A, RSK2 mRNA was associated with poor survival in 244 breast cancer cases who had not received chemotherapy (log-rank p=8.3x10^{-6}; Cox proportional hazards p=1 x 10^{-5}) (left). When examining RSK2 expression in a cohort of 771 breast cancer cases representing all subtypes it was highest in the basal-like subtype (centre) (N=771 ; p<0.005) and in those of high grade (right) (N=771 ; p<0.05). Further, as shown in Figure 7, Panel B, P-RSK$^{S221/227}$ was detected in 13/18 aggressive breast tumor samples. P-YB-1^{S102} and CD44 expression also correlated (see Table 4). Further, as shown in Figure 7, Panel C), expression of activated RSK and YB-1 was not detected in normal breast tissue (note: scale bars represent 100pm).

Table 4. Correlation between P-RSK$^{S221/7}$, P-YB-1^{S102} and CD44 expression in 18 tumor samples

Variable	by Variable	Spearman ρ	Significance
P-YB-1^{S102}	P-RSK$^{S221/7}$	0.7711	0.0002*
CD44	P-YB-1^{S102}	0.5840	0.0109
CD44	P-RSK$^{S221/7}$	0.5031	0.0333

[0096] **Example 8.** ***Activated YB-1 is a marker of patient survival and relapse.*** YB-1 is activated by phosphorylation at Serine 102 which triggers this transcription factor to

translocate from the cytoplasm into the nucleus where it activates genes that promote tumor cell growth, cancer stem cell markers and drug resistance (Stratford *et al.,* 2008). A custom P-YB-1 ^{S102} polyclonal antibody was generated to address whether the activated form of the transcription factor was prognostic. Toward this goal, quantitative reverse phase protein arrays were used to assess P-YB-1 ^{S102} in 1057 breast cancer cases. The proteins are then spotted on glass slides at six different concentrations and probed for P-YB-1 ^{S102} (Cell Signaling Technologies, Polyclonal antibody). A secondary antibody is applied to produce a colored signal. The slide is then scanned and quantified. We concluded that qRPPA could be used to detect P-YB-1 ^{S102} and that high levels of it was prognostic for early relapse and death due to breast cancer. Quantitative reverse protein phase arrays (qRPPAs) are an alternative method of measuring protein expression in tissues. It is advantageous over immunohistochemistry because it is more quantitative and allows high throughput analysis of large numbers of samples (see, for e.g., Charboneau *et al.,* 2002; *Briefings in Functional Genomics and Proteomics,* 3:305-315; VanMeter *et al.,* 2007; Expert. Rev. *Mol. Diagn.,* 7(5):625-633; and Malinowsky *et al.,* 2010; *J. Cell Physiol.,* 225(2):364-370). In addition the amount of sample required is very small (VanMeter *et al.,* 2007). Because the tissues are obtained from snap frozen specimens they have preserved the activation state of the molecules in question. This is particularly important in probing signaling pathways to understand key molecular networks and for potential therapeutic interventions (Paweletz *et al.,* 2001; *Oncogene* 20(16):1981-1989 and Gonzalez-Angulo *et al.,* 2011; *Clin. Proteomics* 8(1):11). High levels of activated P-YB-1 ^{S102} scored as 2 were significantly prognostic for patient survival and relapse (Figure 16, Panels A and B, respectively).

[0097] Example 9. ***Effectiveness of inhibiting RSK pathway together with chemotherapeutic agents.*** In data depicted in Figures 17 and 18 herein, MDA-MB-231 cells (a triple-negative breast cancer cell line) were seeded into 96-well dishes. The

following day, drug treatment was administered according to the following categories: (a) Chemo or DMSO control only, or (b) Chemo (or DMSO control) with 10μM BI-D1870. After 72 hrs, the cells were fixed and stained with paraformaldehyde/Hoechst for subsequent high-content screening (HTS) analysis using an Array VI HTS machine. The data is depicted in Figure 17. The addition of BI-D1870 further reduced cell viability when combined with the anthracycline epirubicin (Epi) or the taxane paclitaxel (paclit).

[0098] With respect to Figure 18, separate 96 well plates with MDA-MB-231 cells (a triple-negative breast cancer line) were seeded at 3000 cells per well density. The cells were incubated at 37 degrees Celsius. The next day, existing media was removed and the following drug treatments were introduced: DMSO (solvent control); BI-D1870 (10μM); methotrexate (10μM or 50μM); epirubicin (1μM or 10μM); 5-fluorouracil (10μM or 50μM); paclitaxel (1nM or 10nM). Single agent treatments were delivered to Plate 1 whereas combination treatments (with 10μM BI-D1870) were delivered to Plate 2. Treatment occurred for 72 hrs at 37 degrees Celsius. Thereafter, the cells were fixed and Hoechst stain was applied. Percent viability was defined as described above. The addition of BI-D1870 enhanced the growth inhibitory effect of the chemotherapeutic agents.

[0099] Example 10. ***RSK specific effectiveness of growth inhibition.*** As summarized in Table 5 below, RSK specific siRNAs were utilized to demonstrate RSK specific growth inhibition. As demonstrated in Table 5, specific cell lines were treated for 7 days with siRNAs and their growth was assessed by Hoechst staining. The number of cells was quantified using an ArrayScan VTI high-content screening instrument. With respect to the siRNAs utilized: RSK1a correlates with SEQ ID NO:2 and SEQ ID NO:3; RSK1b corelates with SEQ ID NO:5 and SEQ ID NO:6; RSK2a correlates with SEQ ID NO:8 and SEQ ID NO:8; and RSK2b correlates with SEQ ID NO:13 and SEQ ID NO:14. RSK siRNA's had little or no effect on the growth of 184htert cells. However, inhibiting RSK1,

RSK2 or both isoforms suppressed the growth of TNBC cell lines (SUM149, MDA-MB-231, MDA-MB-468 and HCC1937). MCF-7 cell growth (non-TNBC, luminal breast cancer cells) were also inhibited.

Table 5. Percent growth inhibition in TNBC and non-TNBC cell lines with RSK-specific siRNAs after 7 days.

		% growth inhibition compared to control					
Cell Line	Subtype	RSKIa	RSKIb	RSK2a	RSK2b	RSKIa+2a	RSKIb+2a
184hTert	Normal	15	0	5	23	ND	ND
MCF7	Luminal	73	77	53	89	63	54
SUM149	TNBC	93	87	97	94	98	89
MDA-MB-231	TNBC	87	71	85	74	79	70
MDA-MB-468	TNBC	55	ND	95	ND	91	ND
HCC1937	TNBC	67	ND	75	ND	88	ND

General Discussion of Examples

[00100] Herein, targeting RSK has been identified as a novel strategy for specifically inhibiting growth of TNBC but not normal breast epithelial cells. Herein, it was demonstrated that the growth of TNBC cells is inhibited by 90-100% when RSK is inhibited with RSK2 siRNA, SL0101 or BI-D1870. Importantly, upon subsequent treatments with BI-D1870, cells do not acquire resistance to the compound.

[00101] TNBCs have a higher percentage of TICs - as defined by CD44 expression - compared to other breast cancer subtypes, which could partially account for their increased propensity to relapse (Park *et ai,* 2010). The growth of the $CD44^{+}$ cells are notably different in that the number of mitotic figures and the levels of P-histone $H3^{S1o}$ are considerably higher in those cells compared to the $CD44^{-}$ cells. One way of explaining this growth advantage is through the activation of signaling in CD44 positive cells by way of an autocrine loop where its ligand hyaluronan is produced. The MDA-MB-231 cells are described as having an autocrine production of hyaluronan which activates cell signaling

through recruitment of Rhamm and Erk, this in turn would engage the MAPK pathway (Hamilton *et al.,* 2007; *Journal of Biological Chemistry,* 282:1 6667-16680). Consistent with this model, the MEK1 inhibitor PD098059 disrupts this pathway. While the CD44/Rhamm/Erk pathway is attributed to increased cell motility which is an important aspect of the spread of TICs from the primary site, this signaling network would also fuel cellular proliferation and drug resistance. While CD44 is used as a means to isolate tumor-initiating cells it too serves important functions in maintaining cell growth and invasion. Thus, eliminating the TIC subpopulation alongside the rest of the tumor may help overcome the challenge of relapse.

[00102] Herein it is demonstrated that inhibiting RSK, particularly RSK2, is sufficient to suppress growth in the $CD44^{+}CD24^{-}$ population within TNBC. Additionally, the ability to suppress mammosphere formation indicates an inhibition of self-renewal. It was determined that RSK inhibition demonstrated specific/increased toxicity to breast TICs when compared to normal hematopoietic stem cells. Collectively, these data indicate that RSK inhibition is effective at eliminating breast cancer TICs but unlike conventional chemotherapies, has little effect on normal stem cells. Accordingly, it has been determined that RSK is uniquely linked to promoting the proliferation of $CD44^{+}$ cells and as such targeting this pathway has important implications in the management of TNBC.

[001 03] As used herein, the term "includes" includes alternatives without limitation. While specific embodiments of the invention have been described and illustrated, such embodiments should be considered illustrative of the invention only and not as limiting the invention as construed in accordance with the accompanying claims. Other features and advantages of the invention will be apparent from the following description of the drawings and the invention, and from the claims.

CLAIMS:

We claim:

1. A method of treating TNBC in a subject, the method comprising administering an effective dose of an inhibitor against the p90 ribosomal S6 kinase (RSK) family of kinases to the subject.
2. The method of claim 1, wherein the inhibitor selectively inhibits RSK1.
3. The method of claim 1, wherein the inhibitor selectively inhibits RSK2.
4. The method of claim 1, wherein the inhibitor is a small molecule inhibitor or a small interfering RNA (siRNA).
5. The method of claim 4, wherein the small molecule inhibitor is BI-D1870 or SL0101.
6. The method of claim 2, wherein the siRNA comprises a sequence having at least 80% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.
7. The method of claim 2, wherein the siRNA comprises a sequence having at least 90% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.
8. The method of claim 2, wherein the siRNA comprises a sequence having at least 95% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.
9. The method of claim 2, wherein the siRNA comprises a sequence according to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.
10. The method of claim 3, wherein the siRNA comprises a sequence having at least 80% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID

NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

11. The method of claim 3, wherein the siRNA comprises a sequence having at least 90% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

12. The method of claim 3, wherein the siRNA comprises a sequence having at least 95% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

13. The method of claim 3, wherein the siRNA comprises a sequence according to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

14. The method of any one of claims 1-13, wherein the subject is a human.

15. A method of inducing apoptosis in a tumor cell in a subject, the method comprising administering an effective dose of an inhibitor against the RSK family of kinases to the subject.

16. A method of decreasing CD44 expression in a tumor cell in a subject, the method comprising administering an effective dose of an inhibitor against the RSK family of kinases to the subject.

17. The method of claim 15 or 16, wherein the tumor cell is a TNBC tumor cell.

18. The method of any one of claims 15-17, wherein the inhibitor selectively inhibits RSK1.

19. The method of any one of claims 15-17 wherein the inhibitor selectively inhibits RSK2.

20. The method of any one of claims 15-17, wherein the inhibitor is a small molecule inhibitor or a siRNA.

21. The method of claim 20, wherein the small molecule inhibitor is BI-D1870 or SL0101.

22. The method of claim 18, wherein the siRNA comprises a sequence having at least 80% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

23. The method of claim 18, wherein the siRNA comprises a sequence having at least 90% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

24. The method of claim 18, wherein the siRNA comprises a sequence having at least 95% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

25. The method of claim 18, wherein the siRNA comprises a sequence according to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

26. The method of claim 19, wherein the siRNA comprises a sequence having at least 80% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

27. The method of claim 19, wherein the siRNA comprises a sequence having at least 90% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

28. The method of claim 19, wherein the siRNA comprises a sequence having at least 95% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

29. The method of claim 19, wherein the siRNA comprises a sequence according to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

30. The method of any one of claims 15-29, wherein the tumor cell expresses any one or more of CD49f and EpCam.

31. The method of claim 30, wherein the tumor cell is a tumor-initiating cell.

32. The method of any one of claims 15-31, wherein the subject is a human.

33. A method of diagnosing breast cancer in a subject, the method comprising identifying phosphorylated RSK in a breast tissue of the subject.

34. A method of diagnosing breast cancer in a subject, the method comprising identifying mRNA expression of RSK2 in a breast tissue of the subject.

35. The method of claim 33 or 34, wherein the breast cancer is TNBC.

36. The method of any one of claims 33-35, wherein the subject is a human.

37. A method for diagnosing a TNBC in a human subject, the method comprising: (a) determining phosphorylated RSK protein levels in a biological sample obtained from the subject; and (b) making a TNBC determination based on the phosphorylated RSK protein levels in the subject sample.

38. A method of monitoring RSK inhibition in a biological sample, the method comprising: (a) obtaining a first expression level reading for each of phosphorylated-YB-1, phosphorylated-GSK3p, and phosphorylated-histone H3 from the biological sample; (b) introducing a compound which may or may not inhibit RSK in the biological sample; and (c) obtaining a second expression level reading for each of P-YB-1, P-GSK3β, and P-histone H3, wherein when the second expression level is lower than the first expression level, there is RSK inhibition in the biological sample as a result of the compound introduced in step (b).

39. The method of claim 38, wherein the biological sample is a TNBC.

40. A method of treating breast cancer in a subject, the method comprising administering a chemotherapeutic agent together with an inhibitor against the RSK family of kinases to the subject.

41. The method of claim 40, wherein the chemotherapeutic agent is a taxane or an anthracycline.

42. The method of claim 40 or 41, wherein the inhibitor selectively inhibits RSK1.

43. The method of claim 40 or 41, wherein the inhibitor selectively inhibits RSK2.

44. The method of claims 40 or 41, wherein the inhibitor is a small molecule inhibitor or a siRNA.

45. The method of claim 44, wherein the small molecule inhibitor is BI-D1870 or SL0101.

46. The method of claim 42, wherein the siRNA comprises a sequence having at least 80% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

47. The method of claim 42, wherein the siRNA comprises a sequence having at least 90% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

48. The method of claim 42, wherein the siRNA comprises a sequence having at least 95% sequence identity to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

49. The method of claim 42, wherein the siRNA comprises a sequence according to siRNA pairs: SEQ ID NO: 2 and SEQ ID NO: 3; or SEQ ID NO: 5 and SEQ ID NO: 6.

50. The method of claim 43, wherein the siRNA comprises a sequence having at least 80% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

51. The method of claim 43, wherein the siRNA comprises a sequence having at least 90% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

52. The method of claim 43, wherein the siRNA comprises a sequence having at least 95% sequence identity to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

53. The method of claim 43, wherein the siRNA comprises a sequence according to siRNA pairs: SEQ ID NO: 8 and SEQ ID NO: 9; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; or SEQ ID NO: 15 and SEQ ID NO: 16.

54. The method of any one of claims 37-53, wherein the subject is a human.

55. A method of diagnosing breast cancer risk in a subject, the method comprising: identifying the presence or absence of phosphorylated Y-box binding protein-1 (YB-1) in a breast tissue of said subject, wherein the presence of phosphorylated YB-1 is indicative of a higher risk of breast cancer associated relapse and/or patient survival and wherein the absence of phosphorylated YB-1 is indicative of a lower risk to breast cancer.

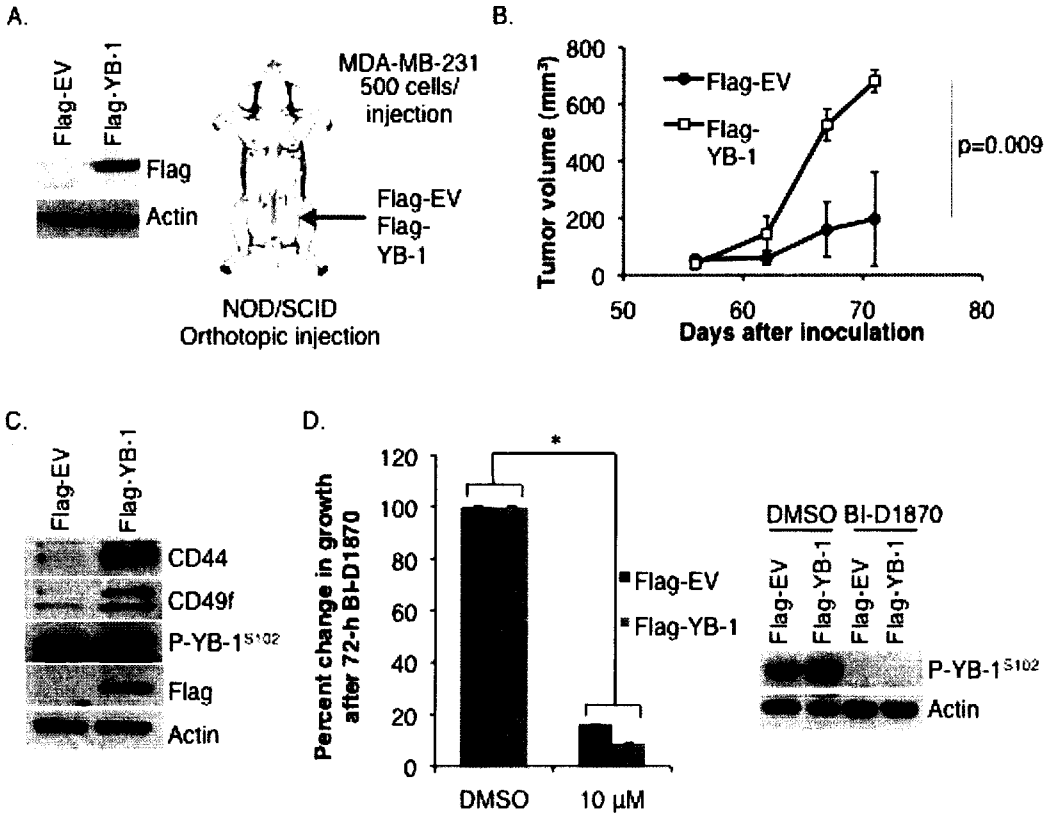


Figure 1 of 18

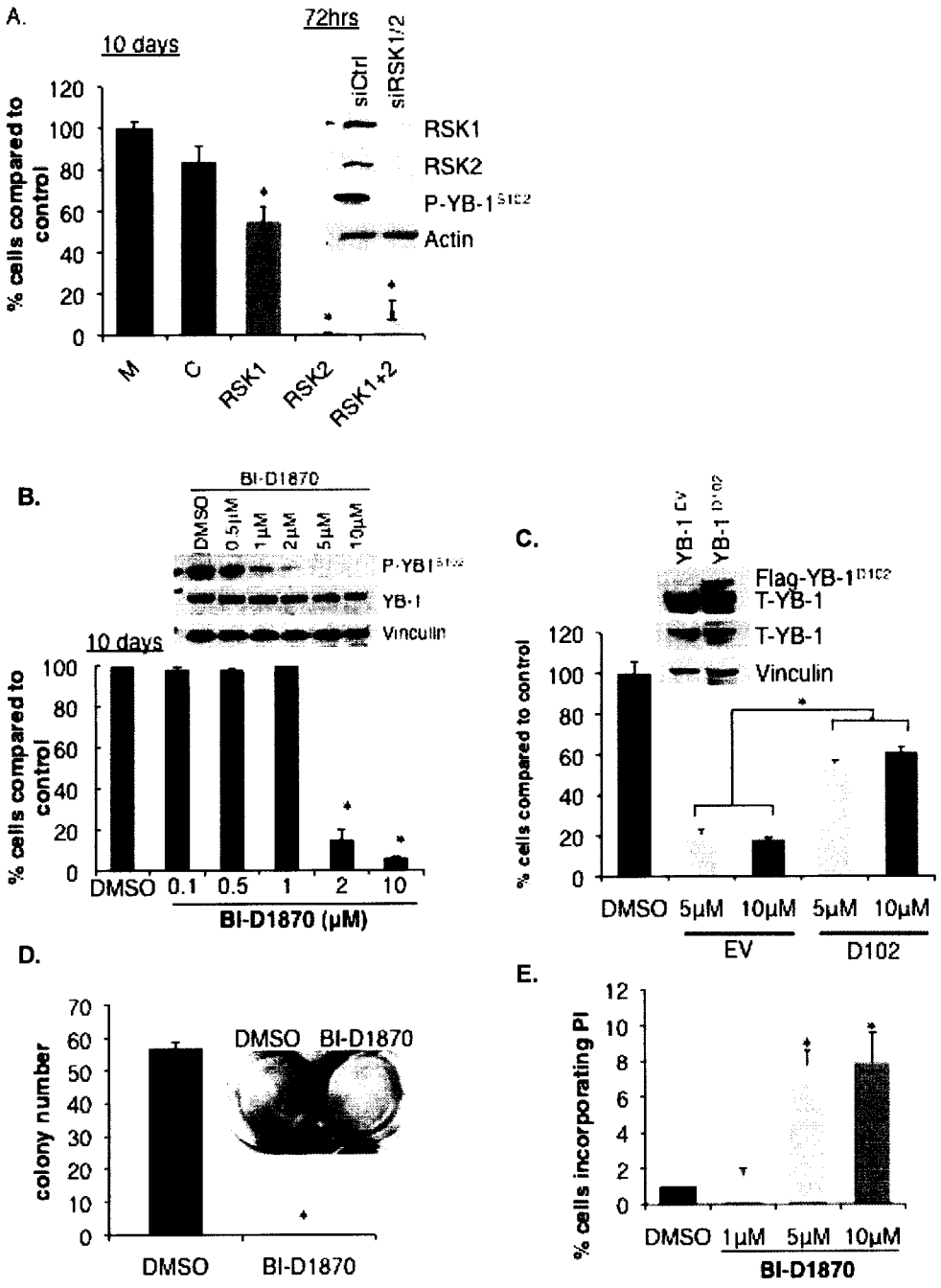


Figure 2 of 18

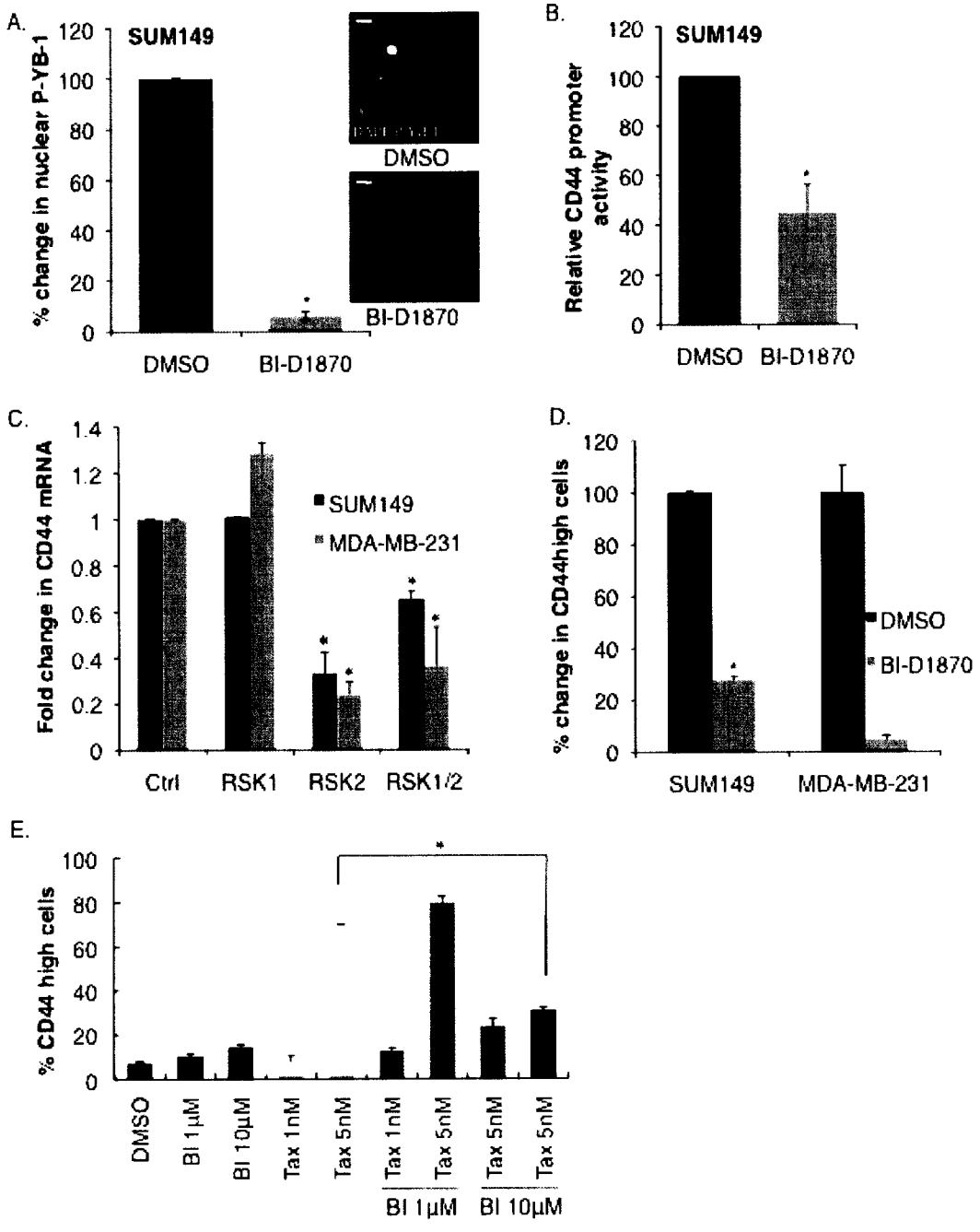


Figure 3 of 18

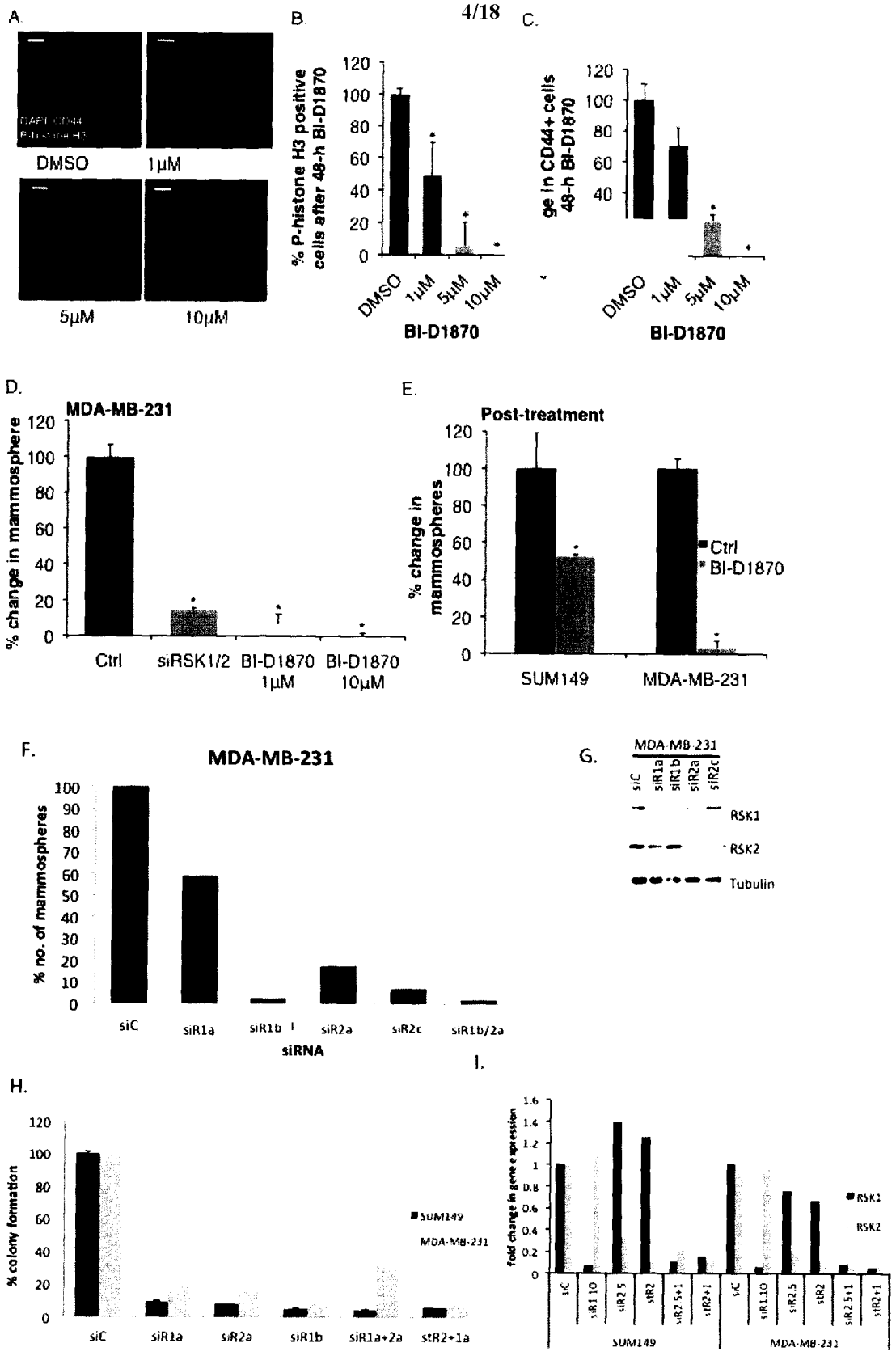


Figure 4 of 18

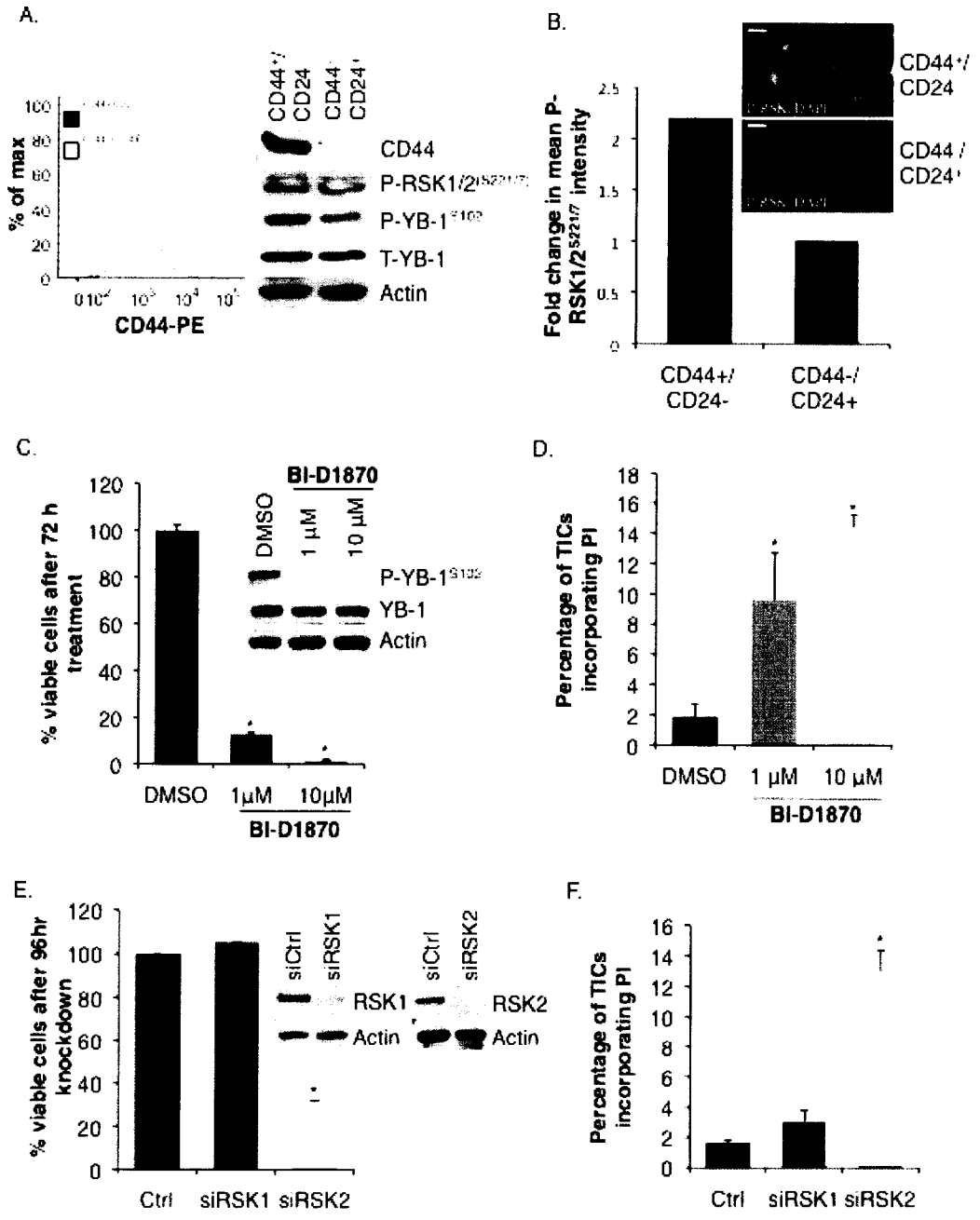


Figure 5 of 18

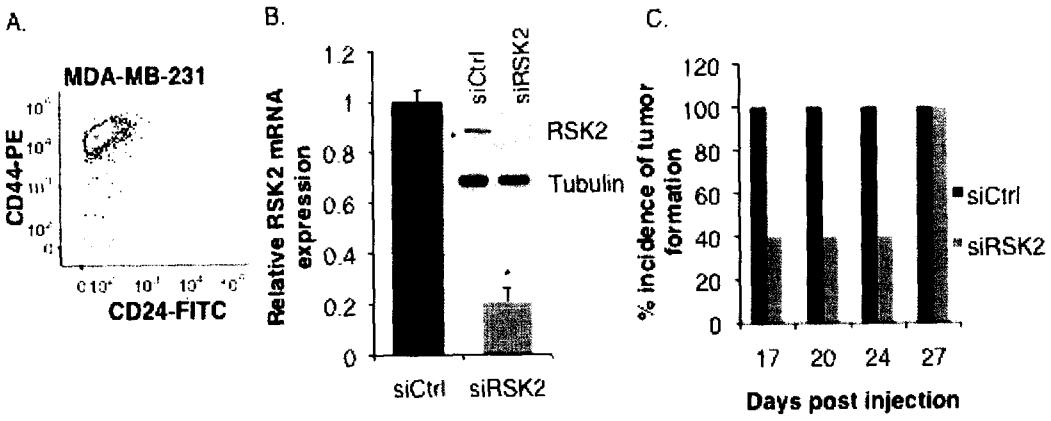


Figure 6 of 18

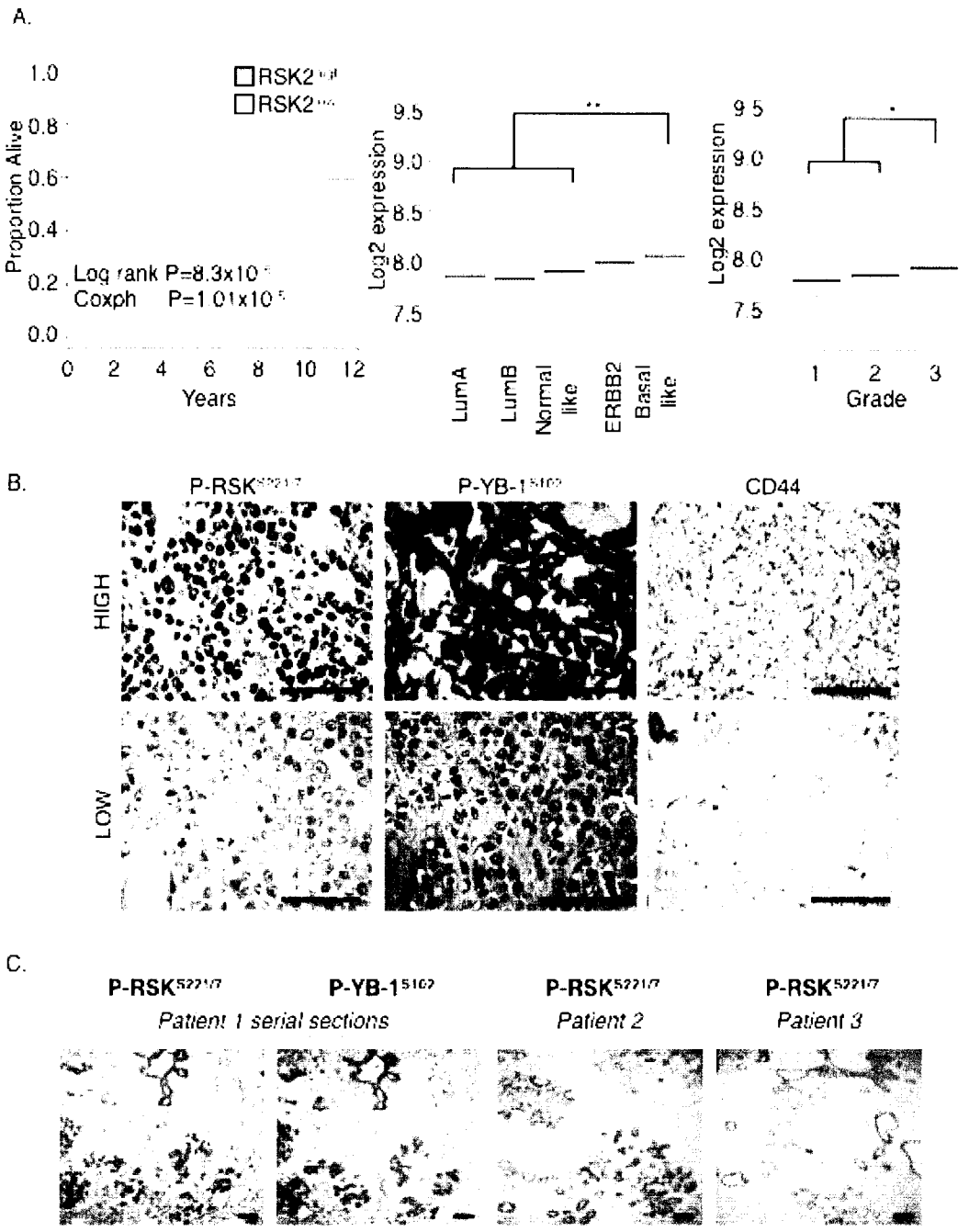


Figure 7 of 18

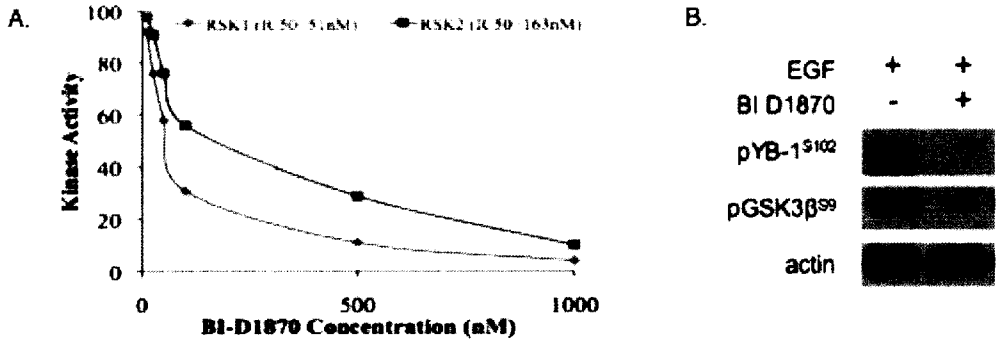


Figure 8 of 18

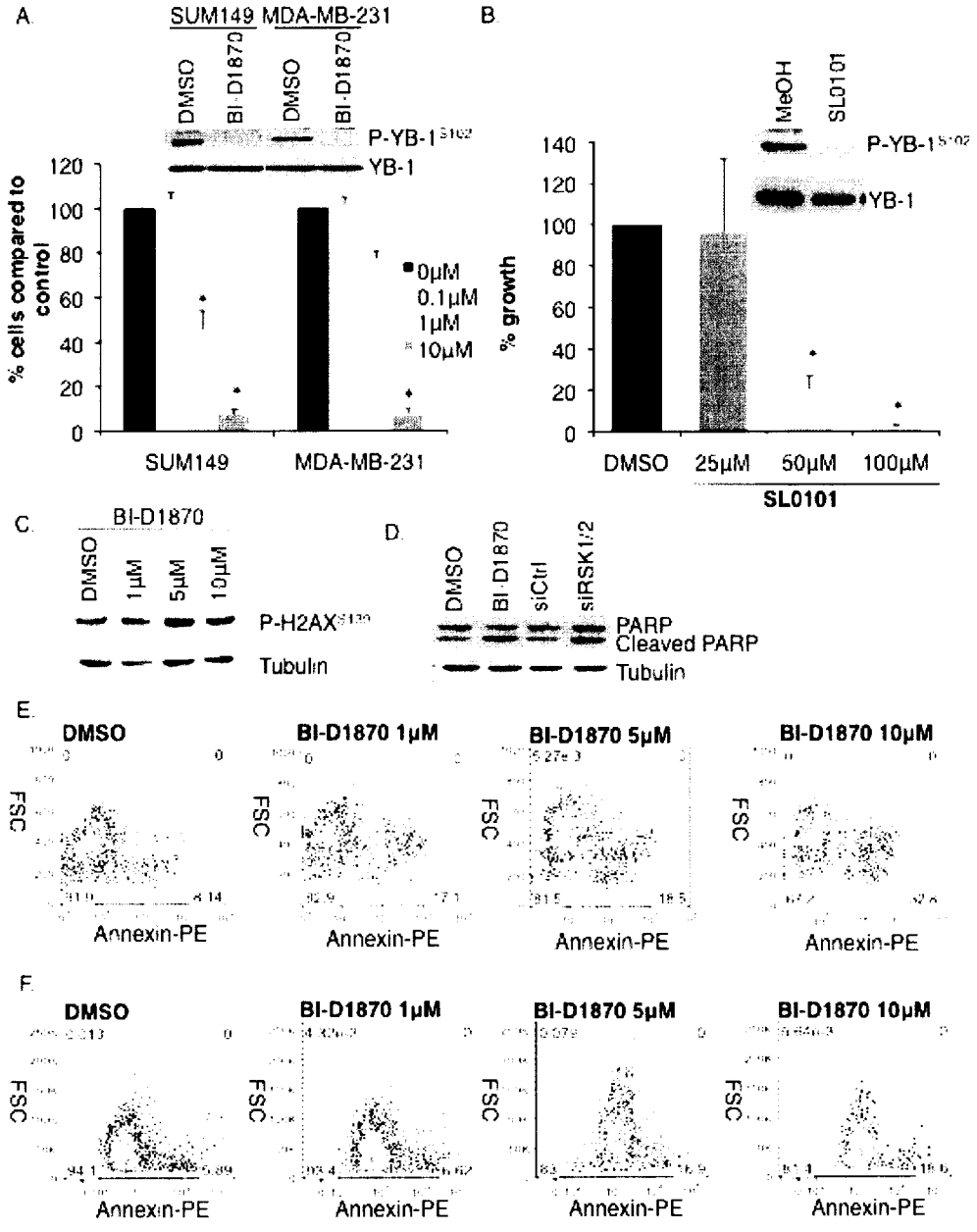


Figure 9 of 18

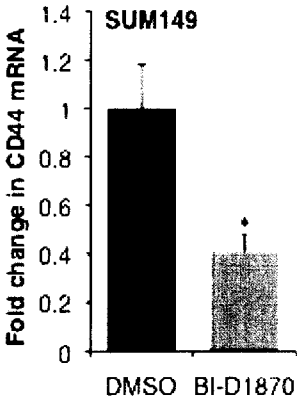


Figure 10 of 18

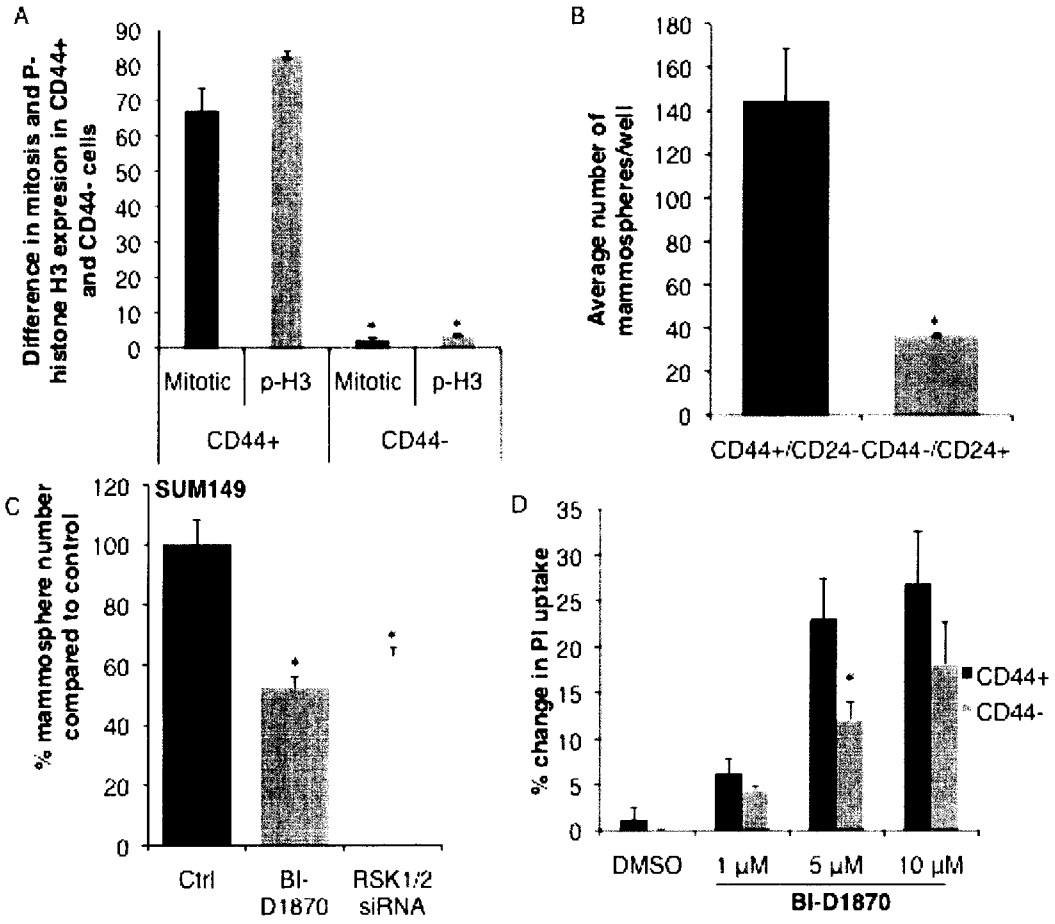


Figure 11 of 18

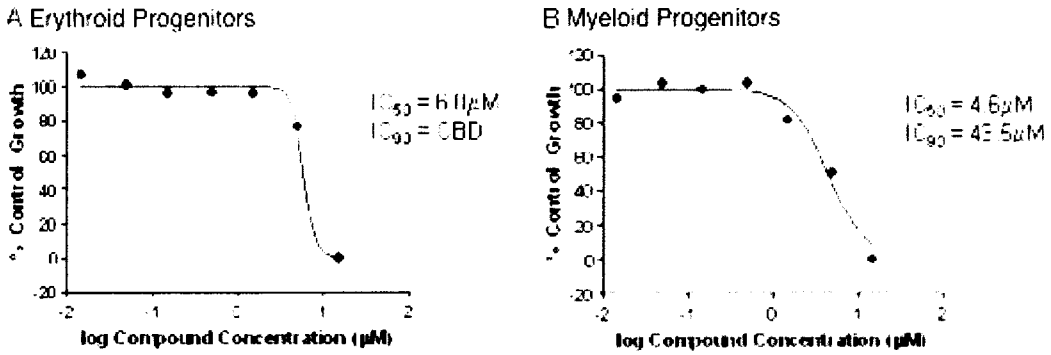


Figure 12 of 18

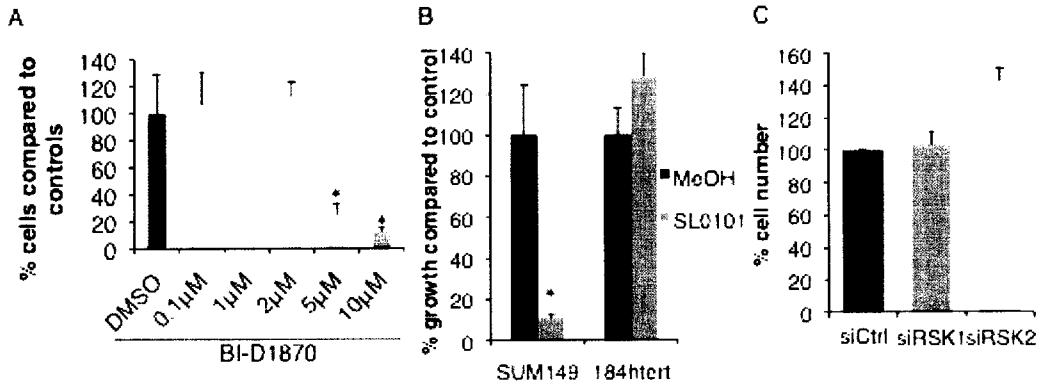


Figure 13 of 18

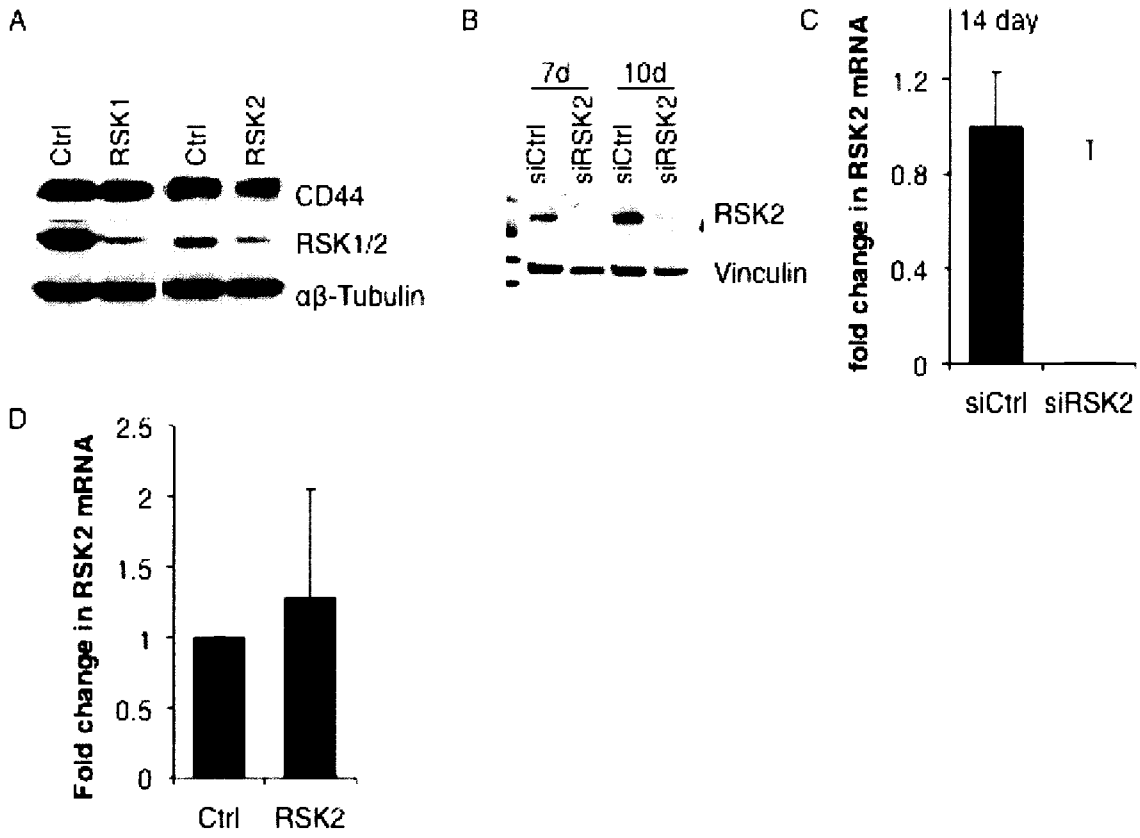
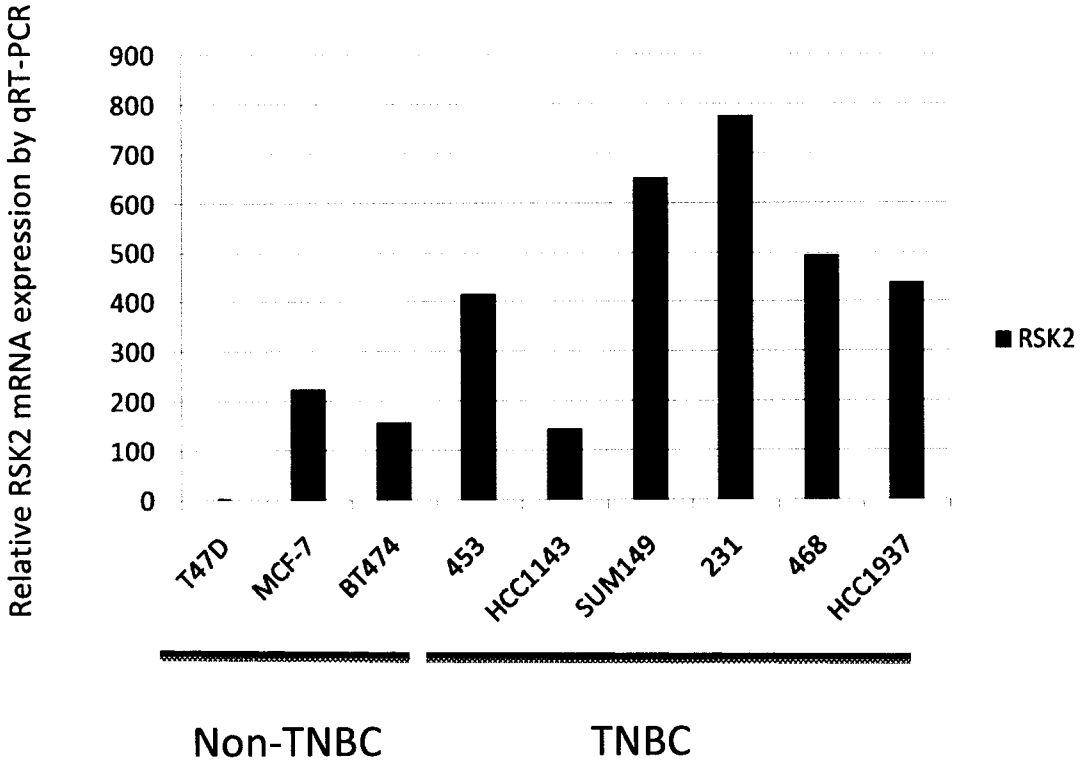


Figure 14 of 18



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A.

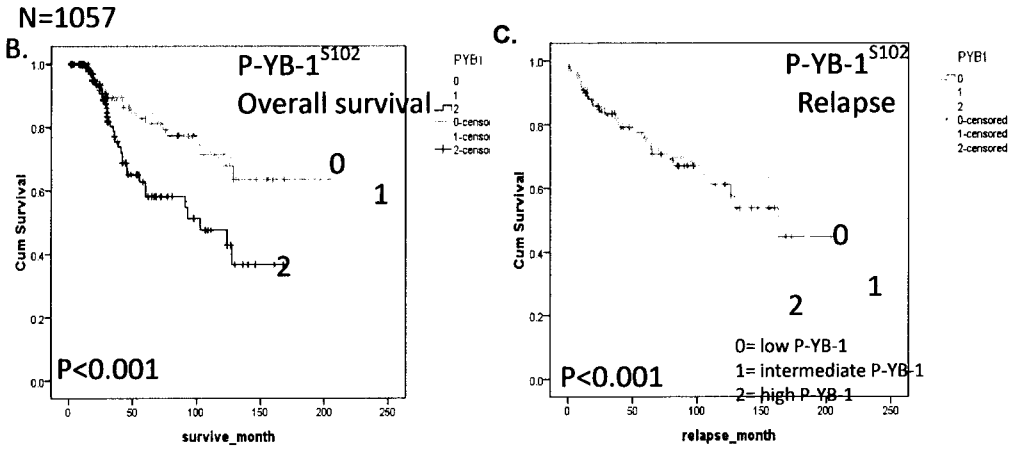

Figure 16 of 18

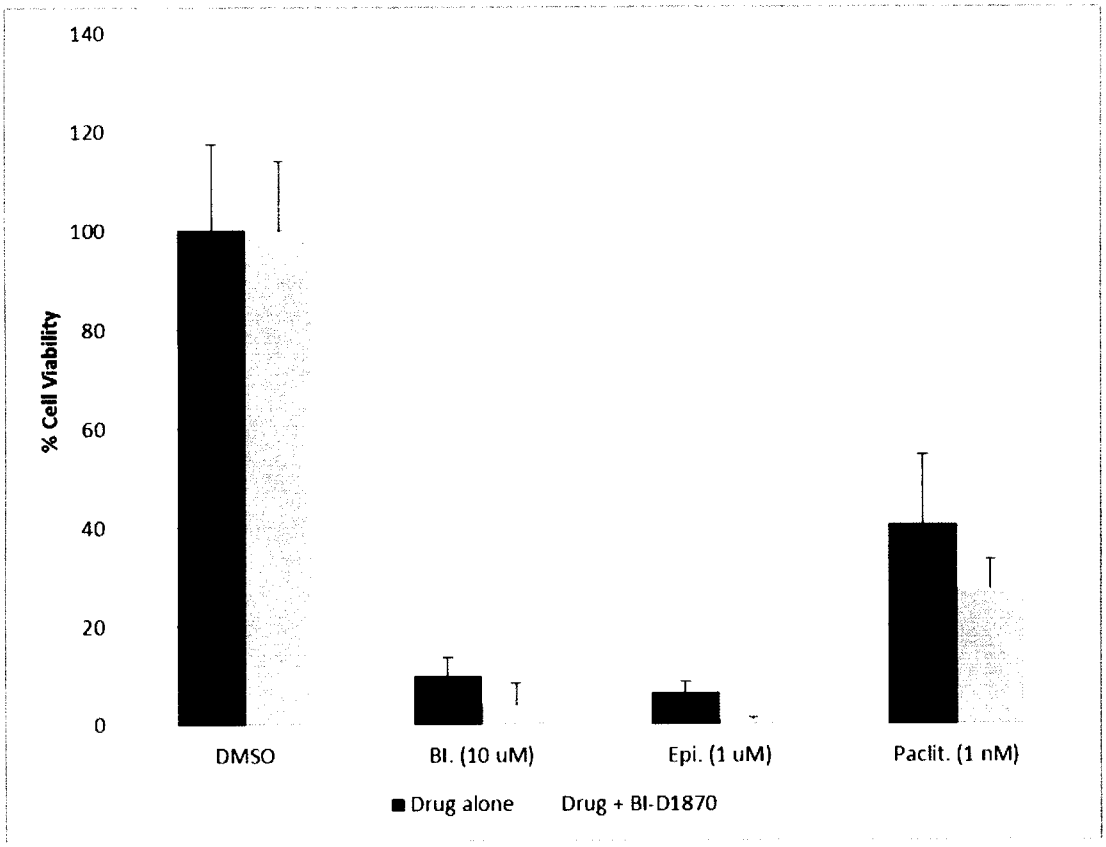

Figure 17 of 18

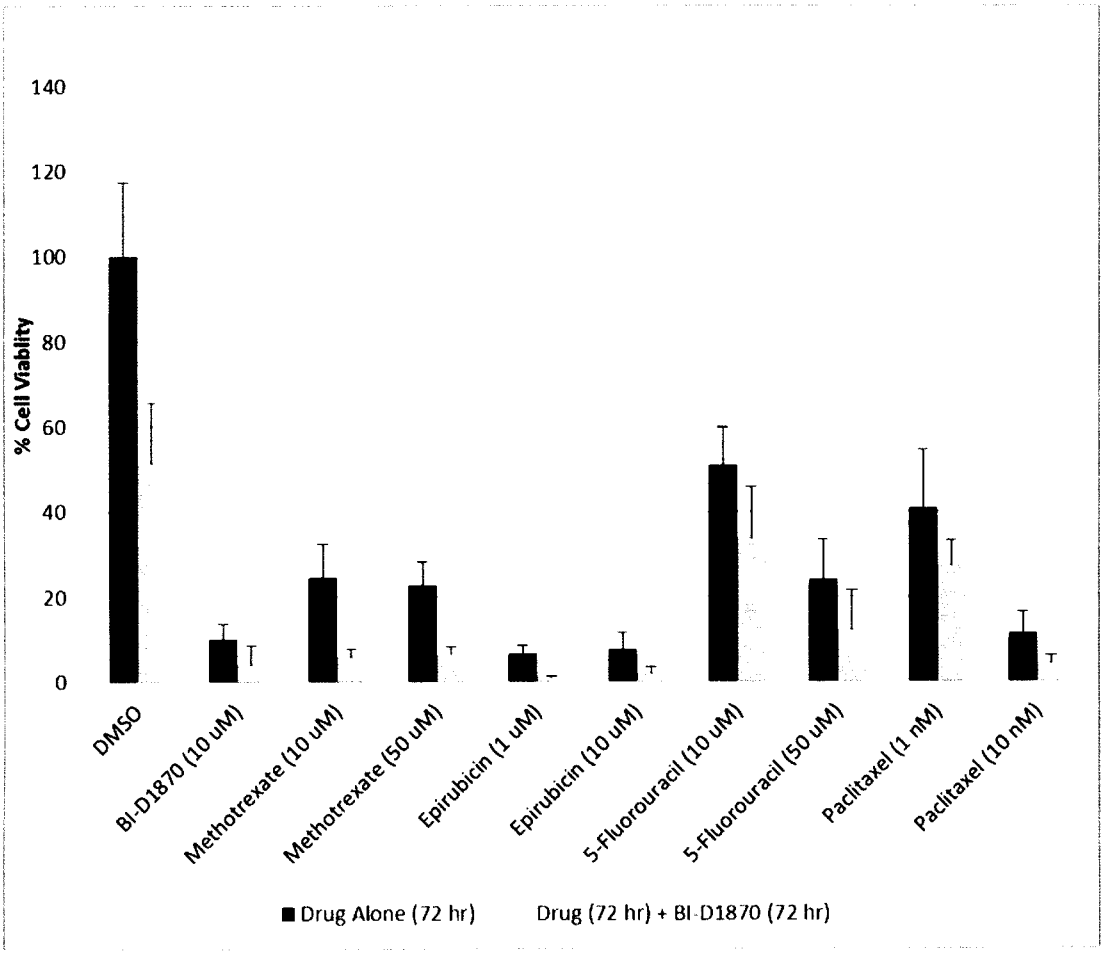

Figure 18 of 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA20 13/000541

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***A61K 31/713*** (2006.01) , ***A61K 31/519*** (2006.01) , ***A61K 31/7048*** (2006.01) , ***A61P 35/00*** (2006.01) , ***G0m 33/48*** (2006.01) , ***G01N 33/68*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: ***A61K 31/713*** (2006.01) , ***A61K 31/519*** (2006.01) , ***A61K 31/7048*** (2006.01) , ***A61P 35/00*** (2006.01) , ***G0m 33/48*** (2006.01) , ***G01N 33/68*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Biosis, CAplus, Medline, EPOQUE (epodoc, cl txte), GenomeQuest (SEQ ID NO: 2, 5, 8, 11, 13, 15 using GQPat, GeneSeq), Canadian Patent Database; Keywords: ribosomal S6 kinase, RSK, BI-D1870, SL0101, siRNA, (triple negative) breast cancer, TNBC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEELE, A.J. et al. The p90RSK inhibitor BI-D1870 induces apoptosis in CLL cells. Blood, issue of November 18, 201 1 (18-1 1-201 1), Volume 118, No. 21,	15, 20, 21, 32
A	page 1067 (abstract), ISSN 1528-0020. - the whole document	1-14, 17-19, 22-31, 40-54
X	WO03105766A2 (SMITH, J.A. et al) 24 December 2003 (24-12-2003) - the entire document, especially pages 7, 8	40, 41, 44, 45, 54
A		1-15, 17-32, 42, 43, 46-53
Y	STRATFORD, A.L. et al. Y-box binding protein-1 serine 102 is a downstream target of p90 ribosomal S6 kinase in basal-like breast cancer cells. Breast Cancer	1-14
A	Research [online], 2008 [retrieved on 2013-07-17], Volume 10, No. 6, pages R99 (1-12), ISSN 1465-542X. Retrieved from the Internet: <URL: http://breast-cancer-research.com/content/10/6/R99>. - the entire document; cited in the description	15, 17-32, 40-54

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 October 2013 (07-10-2013)	16 October 2013 (16-10-2013)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, Cl 14 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Michael W. De Vouge (819) 997-2952

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 1-15, 17-32 (all partial), 40-53 and 54 (partial)

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 1-15, 17-32 (all partial), 40-53 and 54 (partial) are directed to methods for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effect or purpose/use of an inhibitor targeting a member of the p90 ribosomal S6 kinase family as in claim 4 or 5.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

- as indicated on **Extra Sheet**

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

1-15, 17-32 (all partial), 40-53 and 54 (partial)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	SHAHBAZIAN, D. et al. The mTOR/PDK and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO Journal, issue of 21 June 2006 (21-06-2006), Volume 25, No. 12, pages 2781-2791, ISSN 0261-4189. - page 2789, right column	1-14 15, 17-32, 40-54
Y A	GLUZ, O. et al. Triple-negative breast cancer - current status and future directions. Annals of Oncology, issue of December 2009 (12-2009), Volume 20, No. 12, pages 1913-1927, ISSN 1569-8041. - the whole document	1-14 15, 17-32, 40-54
P, X P, A	STRATFORD, A.L. et al. Targeting p90 ribosomal S6 kinase eliminates tumor-initiating cells by inactivating Y-box binding protein-1 in triple-negative breast cancers. Stem Cells, issue of July 2012 (07-2012), Volume 30, No. 7, pages 1338-1348, ISSN 1549-4918. - the whole document	1, 3-15, 17, 19-32 2, 18, 40-54
P, X P, A	REIPAS, K.M. et al. Luteolin is a novel p90 ribosomal S6 kinase (RSK) inhibitor that suppresses Notch4 signaling by blocking the activation of Y-box binding protein (YB-1). Oncotarget, issue of February 2013 (02-2013), Volume 4, No. 2, pages 329-345, ISSN 1949-2553. - the whole document	1, 2, 4, 14, 15, 17, 18, 20, 30-32, 40-42, 44, 54 3, 5-13, 19, 21-29, 43, 45-53

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO03105766A2	24 December 2003 (24-12-2003)	AU2003251513A1	31 December 2003 (31-12-2003)
		AU2003251513A8	31 December 2003 (31-12-2003)
		CA2488864A1	24 December 2003 (24-12-2003)
		EP1539781A2	15 June 2005 (15-06-2005)
		EP1539781A4	02 December 2009 (02-12-2009)
		US2005233985A1	20 October 2005 (20-10-2005)
		US2007049539A1	01 March 2007 (01-03-2007)
		WO03105766A3	11 March 2004 (11-03-2004)

...continued from **Box No. Ill:**

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-15, 17-32 (all partial), 40-53 and 54 (partial) are directed to:

methods of treating triple-negative breast cancer (TNBC) in a subject by administration of an inhibitor against the p90 ribosomal S6 kinase (RSK) family of kinases;
methods of treating breast cancer by administering a chemotherapeutic agent together with an inhibitor against the RSK family of kinases;
methods of inducing apoptosis in a tumor cell by administering an inhibitor against the RSK family of kinases;

Group B - Claims 16 and 17-32 (all partial) are directed to:

methods of decreasing CD44 expression in a tumor cell by administering an inhibitor against the RSK family of kinases;

Group C - Claims 33, 35 (partial), 36 (partial), 37 and 54 (partial) are directed to:

methods of diagnosing breast cancer or TNBC in a subject on the basis of identifying of phosphorylated RSK in breast tissue or a biological sample obtained from a subject;

Group D - Claims 34, 35 (partial) and 36 (partial) are directed to:

methods of diagnosing breast cancer or TNBC in a subject on the basis of identifying RSK2 expression in breast tissue of a subject;

Group E - Claims 38, 39, 54 (partial) and 55 are directed to:

methods of monitoring RSK inhibition in a sample on the basis of reduced expression of phosphorylated Y-box binding protein-1 (YB-1) , phosphorylated GSK3p and phosphorylated histone H3;
methods of diagnosing breast cancer in a subject on the basis of identifying phosphorylated Y-box binding protein-1 (YB-1) in breast tissue of a subject.

A preliminary search of the prior art has revealed that inhibitors of RSK kinase, effective for the inhibition of breast cancer cell proliferation, are known from the following document:

SMITH J.A. et al, Cancer Research, issue of 1 February 2005 (01-02-2005), Volume 65, No. 3, pages 1027-1034, ISSN 0008-5472

Consequently, the recitation of a generic RSK kinase inhibitor as a therapeutic cannot serve as the special technical feature that unifies the claims of Groups A and B. Additionally, the special technical features of diagnostic method claims of Groups C-E are different indicia by which breast cancer or RSK inhibition is identified. Thus, the claims of Groups A-E are considered to be directed to different inventive concepts.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.