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Counteraction of the Antiapoptotic Protein Survivin by Diverting Expression to Its Proapoptotic Splice Variant Survivin-2B

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Health Research Inc.
Buffalo, New York 14263

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14. ABSTRACT Abnormal glucose metabolism is associated with increased cancer risk, which is independent of body mass index for several types of cancer, with a stronger association for women than for men and for fatal cancer. The hypothesis we investigated is the following: In a proliferation-inhibited state, survivin-2B will be shifted from a rare into a major variant, and survivin will be shifted from a major into a rare variant. In order to test this hypothesis we used glucose deprivation as a model to inhibit proliferation capacity and induce apoptosis, and analyzed endogenous survivin and survivin-2B expression in a proliferation-inhibited (low glucose) verses proliferation-promoted (high glucose) state brought about by varying extracellular glucose in media in which human breast cancer cells were cultured. Further, our experiments are trying to determine the extent of tumor cell dependence on glucose for survival and proliferation, and the relationship with survivin gene regulation. To our knowledge this is the first finding to provide evidence that the survivin gene (survivin and survivin-2B) is down-regulated by low glucose. This report highlights our findings arising from my DoD Breast Cancer Pre-Doctoral Traineeship Award project.

15. SUBJECT TERMS
Survivin, Survivin-2B, Apoptosis, Breast Cancer, Inhibitor of Apoptosis (IAP), Baculovirus IAP Repeat (BIR), Alternative Splicing, Splicing motifs, Minigene Cassettes

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INTRODUCTION

Survivin is a member of the Inhibitor of Apoptosis (IAP) gene family.[2] The ubiquity of survivin in common cancers facilitates circumvention of physiological apoptosis mechanisms and regulated cell-division, creating an imbalance of cellular turnover/tissue-architecture integrity. Survivin is a biomarker associated with shortened duration of patient survival. [3] Alternative splicing of the survivin gene primary transcript generates distinct variants, including survivin-2B.[4] Unexpectedly, ectopic survivin-2B in human ovarian cancer cells (HeLa) reduced the number of mitotic cells, inhibited cell growth, induced apoptosis, and sensitized cells to taxol, thereby suggesting an antagonistic function compared to survivin. [5] Based on our previous observation, we focused on determining environmental factors (in particular, glucose starvation) that may be capable of regulating survivin variant expression and to address the possibility of inducing an endogenous transcript shift (survivin decreased, while survivin-2B increased). We searched for environmental factors instead of drugs since drug delivery is beleaguered by poor tumor vasculature, other tumor physical barriers such as overcrowding, rapid drug clearance, metabolism to inactive molecular structures, and efflux pumps[6]. We adopted glucose deprivation (grade 1 or approximately 55 mg/dL) as a possible method for the following reasons: the unambiguous clinical relevance of glucose to cancer [7, 8]; (2) glucose starvation should selectively inhibit cancer cells due to their hypoxic microenvironment, which restricts metabolic possibilities; *i.e.,* forcing the use of only non-oxidative metabolism (mainly glycolysis) [9]; (3) a deprivation of a nutrient depended on by cancer cells is simpler than drug-delivery to inaccessible tumor sites; (4) glucose restriction may be possible to harbor normal cells (checkpoint-proficient), but not cancer cells (checkpoint-deficient) in G1 and G2 phases of cell cycle[10], where they are less vulnerable to chemotherapy-associated toxicity, especially of anti-mitotic agents; (5) decreasing glucose within acceptable limits of low-normal range (~60 mg/dl) would be expected to decrease death, whatever the cause, as graded response has been demonstrated in population studies [11], and safety thresholds for hypoglycemia are described [12]; (6) mechanistic considerations pertinent to survivin inhibition, including the observation that Sp1 plays a role in constitutive expression of survivin [13] . With regard to the regulation of Sp1's ability to transactivate genes, Walsh *et al* demonstrated that glucose deprivation weakens DNA-binding ability of Sp1 to two glycolytic enzymes it activates (aldolase A and pyruvate kinase M_2) by altering Sp1's phosphorylation state; that is, glucose promotes dephosphorylation of Sp1 and increases its binding competency, while absence of glucose promotes phosphorylation of Sp1 and decreases its DNA-binding efficiency [14]. Also, glucose starvation induces proteosomal degradation of Sp1 [15]. Other positive regulators of survivin, such as mTOR[16], are also negatively regulated by low glucose. [17] Further, glucose restriction activates the longevity-associated histone and protein deacetylase, SIRT1 (Sir2 homolog), [18, 19] which is known to deacetylate histone-3 lysine-9 in the survivin promoter [20], thereby creating an epigenetic/chromatin-repressive state at the survivin locus. In addition, c-myc, which interacts with the survivin promoter and upregulates survivin transcription[21], is implicated in the Warburg Effect (glycolysis in the presence of oxygen) in breast cancer [22]. Glucose restriction inhibits proliferation of human breast cancer cell lines.[23] The relevance of glucose in cancer is attested to by the use of fluorescently labeled glucose analogues to detect and monitor many types of cancers, including breast cancer[24]. Abnormal glucose metabolism is associated with increased risk of incident and fatal cancer, independent of body mass index. [1] In summary, there is a possible relationship between survivin gene regulation/function and glucose status. Thus, in the present project we will focus on the study of this relationship.

BODY

TASK 1. Test whether expression of survivin-2B and survivin is associated with breast cancer cell statuses – proliferation vs. apoptosis (months 1-14).

a. Generate the flag-tagged survivin mini-gene construct.

Using the entire survivin gene cloned in pBS-KS vector at the Hind III site (Vector I) from the Li Lab (Fig 1)., I have digested Vector I with Sal I and Mlu I, and then re-self-ligated to derive Vector II. Vector II was digested with BamH I and re-self-ligated to derive Vector III (Fig 2)

Next, I have taken advantage of the unique Asc I and Fse I sites to insert the flag sequence in frame after the survivin ATG codon. With PCR, I amplified the AscI-FseI fragment using the following two primers: AscI-flag (5'- G CGG CGC GC C ATT AAC CGC CAG ATT TGA ATC GCG GGA CCC GTT GGC AGA GGT GGC GGC GGC GGC ATG GAT TAC AAG GAT GAC GAC GAT AAG GGT GCC CCG ACG TTG CC -3', Asc I site underlined, flag tag in bold) and FseI (5'- GGG AGG CCG GCC TGG ACC CAA -3', FseI site underlined). The resultant PCR product replaced the original AscI-FseI DNA fragment to derive Vector IV (Fig 2-3).

Vector IV was used to isolate the Kpn I-BamH I DNA fragment. The isolated DNA fragment was then subcloned into pcDNA3 at Kpn I and BamH I sites to derive pcDNA3-Sp-fE1-E2-2b-E3, which contains Sp (survivin core promoter, 268 bp), fE1 (N-terminal flag-tagged exon 1), E2 (exon 2), 2b (exon 2b), E3 (exon 3), and SpA (survivin poly A sequence) (Fig 4).

Task1a has been completed (Fig 1-4).

b. Generation of stable breast cancer cell lines that express pcDNA3-Sp-fE1-E2-2b-E3 expression vector.

We used an alternative approach, which we describe in our Narrative, because during task 1b the construct from task 1a was determined to be unable to be expressed into either flag-tagged survivin or survivin-2B (Fig 5). This may have been due to the numerous Alu sequences within the survivin gene in the proximity to the survivin-2B splice site. MCF7 human breast cancer cells were transiently transfected with pcDNA3-Sp-fE1-E2-2b-E3c for 48h. We also transfected EGFP-survivin as a control to ensure transfection efficiency and carried out immunoblot. After incubating with Anti-FLAG M2 antibody (Stratagene), as well as trying a different Anti-FLAG antibody (Sigma), we only observed specific bands of expected size for our positive controls derived from cells transfected with flag-tagged proteins (Fig 5, upper immunoblot). Therefore, we also used Anti-survivin FL-142 (Santa Cruz) to try to detect f-svn or f-svn-2B (Fig 5, lower immunoblot). In MCF7 cells endogenous survivin is expressed, but we can not detect f-svn or f-svn-2b, using either Anti-FLAG M2, or Anti-survivin FL-142 antibodies, although our positive controls were detected. In addition we used lysates from several stable clones obtained in MDA-MB-231 cells transfected with pcDNA3-Sp-fE1-E2-2b-E3c and selected for using G418, but were also unable to detect the f-svn or s-svn-2b isoforms of theoretical molecular weights (data not shown). It is possible that the mini-gene is not spliced as we anticipated, is degraded due to unknown mechanisms, is modified differently from the native isoforms, which could result in a non-theoretical molecular weight. Therefore we examined the endogenous survivin variants at the mRNA levels after distinct treatments using survivin variant specific primers by semi-quantitative and real time RT-PCR, and at protein level on high resolution SDS Page Polyacrylamide gels (15-18%). This approach had the additional advantage of being less artificial compared to the minigene construct because we

could try to bring about an endogenous transcript shift and also study the endogenous gene in its native chromosome context.

Although we were not able to complete Task 1b as originally designed due to difficulty with the generated construct, we used the alternative method which had the advantage of interrogating the endogenous variants. The reason for generation of this construct was to study survivin and survivin-2B expression in relation to proliferation status in Task 1c. We used an alternative approach to accomplish this goal by studying the endogenous survivin and survivin-2B mRNA and protein levels. Since the survivin gene is regulated primarily at the transcription level, survivin protein expression is generally in agreement with mRNA levels.

c. Determine the expression of survivin and survivin-2B after distinct treatments.

We determined survivin or survivin-2B expression in model cells at a fully proliferating state: Since EGF had already been published to increase survivin expression, we used a distinct and novel treatment of culturing cells with different glucose concentrations to determine the effects of high glucose in terms of survivin and survivin-2B variant expression. First we established that high glucose induces proliferation and low glucose suppresses proliferation of various breast cancer cells (Fig 6-7). Absence of glucose (6a) or low glucose (6b) decreased breast cancer cell proliferation (ZR-75-1 and MDA-MB-231). MDA-MB-231 (p53 mutant, doubling time ~23h) is more inhibited in low glucose (0.5g/L), compared to ZR-75-1 (p53 wild type, doubling time ~80h) at the same glucose levels, at corresponding time points (Fig 6b). The more glucose-dependent cells (MDA-MB-231) were inhibited for proliferation in the short term and long term (Fig 6c and 6d). We also studied the criteria for effects of glucose deprivation on cell growth, especially the effect of cell density in both breast cancer and normal human fibroblasts (Fig 7-8). The greater the cell density or confluency, the greater the inhibition of proliferation in presence of low glucose, we think as a result of competition for glucose or its depletion (Fig 7). Only the media of breast cancer cells grown in high glucose and high density became yellow, an indication of low pH due to higher glycolysis, which produces lactic acid (Fig 7). Counting of viable cells with Trypan Blue assay indicated this low pH (yellow) high glucose media contained the highest number of cells (Fig 7). Normal human fibroblasts were not significantly inhibited until very low glucose concentration (0.25g/L) in conditions of high density (Fig 8). Further, we did not observe the pronounced color change to yellow (Fig 8), although this could be due to a slower cell division rate over the incubation period, in the various glucose concentrations, compared to breast cancer cells (Fig 8).

The hypothesis was that in a high glucose environment cells would be induced to proliferate and, in association with high proliferation state, survivin would be the major variant and survivin-2B the minor (Fig 9).

To test this hypothesis we analyzed survivin variant expression (survivin and survivin-2B) in model cells at a proliferation-inhibited state: Cells were cultured in various concentrations of glucose. Lowering glucose decreased survivin, but, unexpectedly, also decreased survivin-2B transcripts based on concentration and in absence of glucose for different durations of time (Fig 10).

We also used quantitative real time RT-PCR to study survivin and survivin-2B mRNA expression after culturing cells in varying concentrations of glucose (Fig 11). However, survivin and survivin-2B expression showed an identical pattern of decreasing with low glucose. We also studied SIRT1 because it was already reported to epigenetically silence survivin transcription and furthermore to be upregulated during glucose restriction. Indeed, SIRT1 increased with glucose restriction, in opposite manner as survivin and survivin-2B (Fig 11). No one has ever unified these discrete discoveries: that survivin could be inhibited by low glucose with a mechanism involving the longevity- associated SIRT1.

Immunoblot was used to determine endogenous survivin and survivin-2B protein expression on high percentage SDS-page acrylamide gels (15-18%). Survivin levels were decreased by lowering glucose (Fig 12a and b). The rare isoform survivin-2B did not undergo an endogenous induction in opposition to survivin as we had predicted, but instead had a very low expression that disappeared with time in low glucose (Fig 12 b).

We then studied the survivin promoter region expressed stably in MDA-MB-231 human breast cancer cells to further support our mRNA results that indicated survivin gene inhibition in low glucose conditions. This result was in agreement decreasing in low glucose and is shown is Fig 13.

Task1c is completed (Fig 6-13).

TASK 2. Test whether shifting survivin-2B from a rare transcript to a major one will automatically induce survivin going in an opposite way (months 10-26).

Work has not begun.

TASK 3. Test whether shifting survivin-2B from a rare transcript into a major constitutive transcript in breast cancer cells could effectively induce breast cancer cell death (months 18-36).

This work has not begun. We made the surprising discovery that survivin-2B was regulated similarly with survivin in a proliferation-inhibited state. The desired transcription switch would not be achievable if the transcription repression in low glucose conditions applied to both variants in identical manner. If we are permitted to follow up on the exciting finding that survivin gene is downregulated by glucose in light of the clinical importance of glucose and survivin in breast and other common forms of cancer, we would like to extend the study to discover candidates for how low glucose suppresses survivin, such as sirtuin (SIRT1), which had an inverse relationship with survivin variants in that, whereas survivin variants decreased in low glucose, SIRT1 increased (Fig 11). We would like to study the survivin core promoter luciferase constructs to identify potential candidates mediating low glucose effects on survivin and survivin-2B. How does a low glucose state inactivate survivin? Is it due to nutrient sensors like SIRT1, or is survivin itself a nutrient sensor? Addressing these questions will illuminate how chromosomal (cell) division, via survivin, is amazingly and beautifully synchronized with availability of this versatile nutrient glucose.

KEY RESEARCH ACCOMPLISHMENTS

- Lowering glucose decreases cell proliferation of human breast cancer cells studied (ZR-75-1 and MDA-MB-231 cells)

- Glucose deprivation significantly decreases survivin:
  - promoter activity
  - mRNA
  - protein

REPORTABLE OUTCOMES

Our manuscript is in preparation as a result of our DOD research fellowship.

Pharmacology Day 2009

Abstract Submission Form

Title: Regulation of survivin and its splicing variant survivin 2B by glucose

Author(s): Pasha Apontes[1,2], Kai Zhong[1], and Fengzhi Li[1]

Departments: Pharmacology and Therapeutics[1], and Molecular and Cellular Biology[2] at Roswell Park Cancer Institute

Oral Presentation ■ Poster Presentation

Abstract:

Since its initial description in 1997, survivin has gained prominence as a cancer biomarker, and as a target for inhibition in common cancers. Aberrant survivin expression contributes to the obstruction of physiological suicide mechanisms/advancement through cell-division checkpoints despite errors. In addition to survivin, alternative splicing of the survivin gene primary transcript generates distinct variants, including survivin-2B. Based on our previous observation that exogenous survivin-2B displays pro-apoptotic activity, we focused on determination of environmental factors (lowering glucose) capable of regulating survivin isoform expression, thereby increasing cancer cell death.

Previously, our lab discovered that serum deprivation results in differential effects on cell death, proliferation and survivin protein levels in breast cancer MCF7 sublines. Currently, we explored whether glucose deprivation might either promote cell death or inhibit growth. We observed that: (1) MDA-MB-231 (p53 mutant, doubling time 23h) are severely growth-retarded in the presence of low levels of glucose (0.5g/L), whereas ZR-75-1 (p53 wild type, doubling time 80h) are modestly growth-restricted at the same glucose levels at corresponding time points; (2) In glucose-dependent cells (MDA-MB-231) survivin and survivin-2B transcripts are significantly decreased after glucose starvation, as is survivin-promoter activity; (3) Glucose deprivation correlated with a substantial (MDA-MB-231) versus a modest (ZR-75-1) decrease of survivin protein. We conclude that breast cancer cells have dissimilar glucose dependency, and that survivin and/or survivin-2B promoter activity, mRNA and protein levels are negatively regulated by low levels of glucose in rapidly proliferative (MDA-MB-231), and to a lesser extent in slowly proliferative (ZR-75-1) cells.

CONCLUSIONS

We constructed a minigene which would allow us to study survivin or survivin-2B variant expression after treatment that would either inhibit or promote cell proliferation. But when the construct was tested it was not translated into the minigenes. As an alternative approach we studied the endogenous mRNA and protein levels of the variants in their native context. We tested the hypothesis that during a proliferation-inhibitory state brought about by culturing cells in the presence of low glucose media, survivin would be a shifted from a major variant to a minor variant, and survivin 2B from a minor variant to a major variant. Low glucose decreased breast cancer cell proliferation, and in high cell density, there was greater cell growth inhibition from lowering glucose. The media of cancer cells cultured in high glucose and high density became yellow, an indication of low pH and higher glycolysis rate which produces lactic acid. Cell quantification indicated this low pH/yellow high glucose media contained the highest number of cells. In contrast, normal human fibroblasts proliferation was significantly inhibited at very low glucose concentration (0.25g/L) at high density conditions, but we did not observe the pronounced color change to yellow, although it is possible that this is due to a slower cell division rate over the incubation period in the various glucose concentrations. In this study we demonstrated for the first time that glucose deprivation causes the suppression of survivin gene promoter activity, mRNA, and protein levels. Survivin and survivin-2B transcripts *both* decreased significantly in a low glucose environment (0.75 and 0.5g/L) after 48h. This observation disproved our original hypothesis and appeared to be due to the fact that survivin transcription was repressed, and the splicing events that result in survivin or survivin-2B are *post*-transcriptional. Thus, we have not observed the expected endogenous transcript switch. When taken together, these results suggest that the survivin gene is downregulated in response to glucose starvation. The control of survivin expression by glucose, to synchronize cell division with the vital nutrient glucose, is consistent with survivin's major physiological role in cellular division as the "starlet" of the Chromosome Passenger Complex.

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APPENDICES

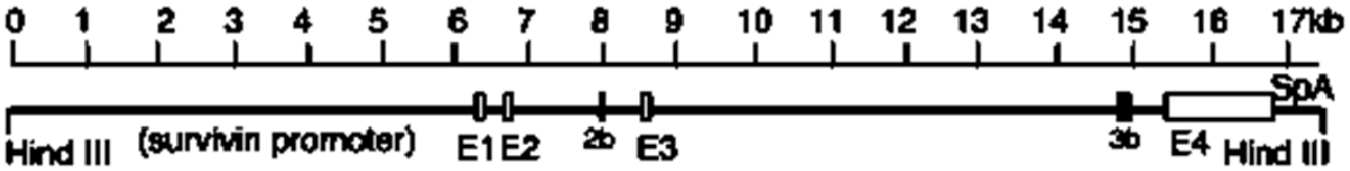


Fig 1 Survivin gene structure/cassette: E1, exon 1; E2, exon 2; 2b, exon 2b; E3, exon 3; 3b, exon 3b; E4, exon 4; and SpA, survivin poly A signal sequences

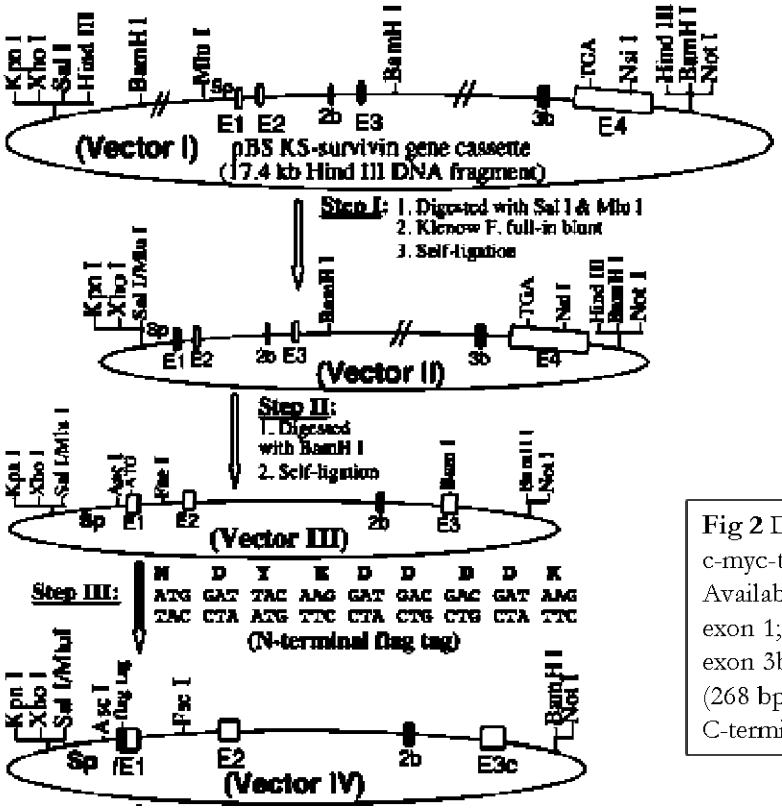


Fig 2 Diagram of the construction of flag- and/or c-myc-tagged survivin minigene constructs (I): Available restriction enzyme sites are marked. E1, exon 1; E2, exon 2; 2b, exon 2b; E3, exon 3; 3b, exon 3b; E4, exon 4; Sp, survivin core promoter (268 bp); fE1, N-terminal flag-tagged exon 1; E3c, C-terminal c-myc-tagged exon 3.

Fig 3 Confirmation of the insertion of flag-tag downstream of ATG translation codon in Vector IV by sequencing: The DNA was sequenced with a reverse primer that will read the opposite chain

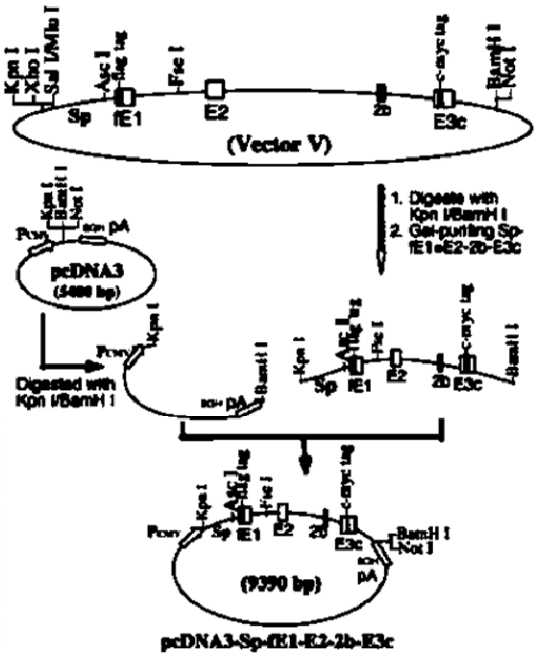


Fig 4 Construction of the flag- and/or c-myc tagged survivin minigene construct (II): Sp, survivin core promoter (268 bp); fE1, N-terminal flag-tagged exon 1; E2, exon 2; 2b, exon 2b; E3c, C-terminal c-myc-tagged exon 3; SpA, Survivin polyA signal sequence.

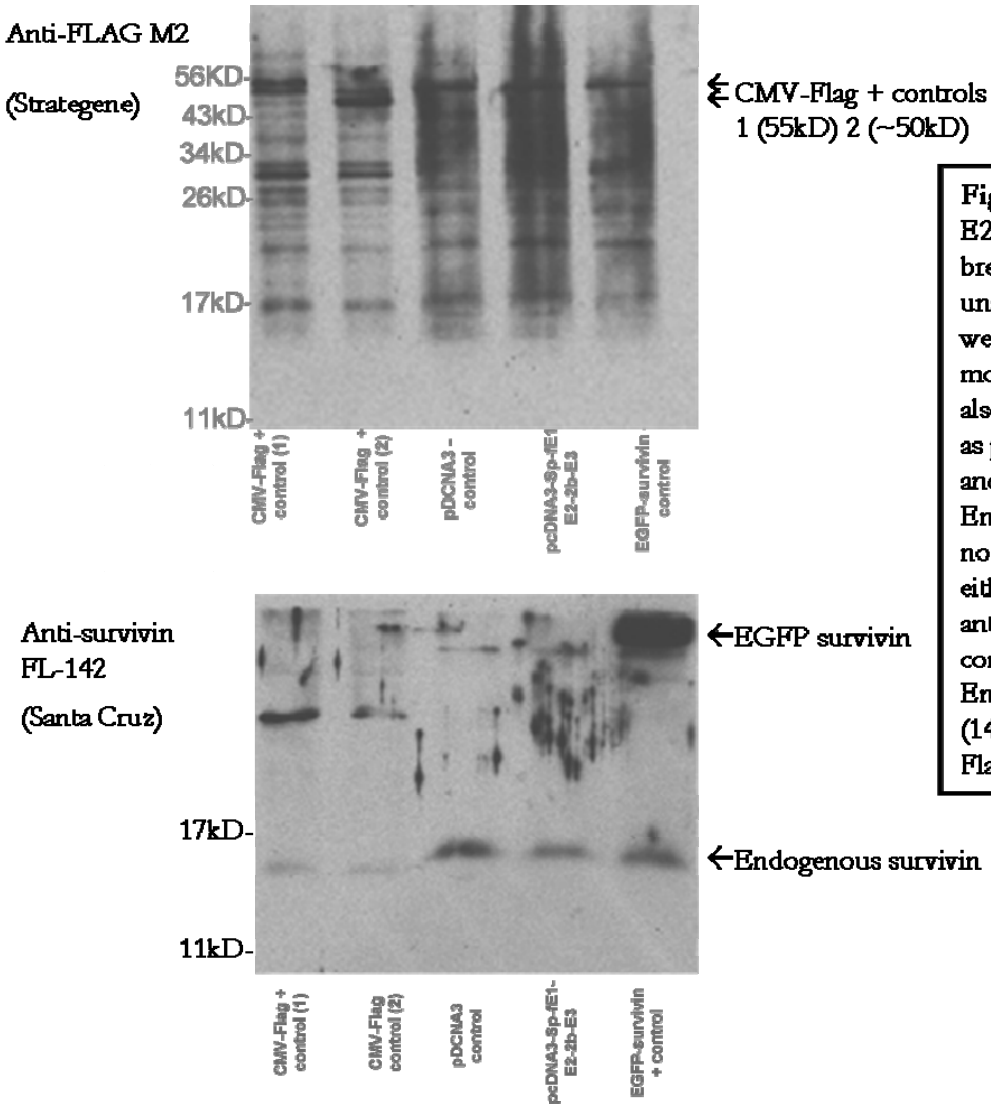
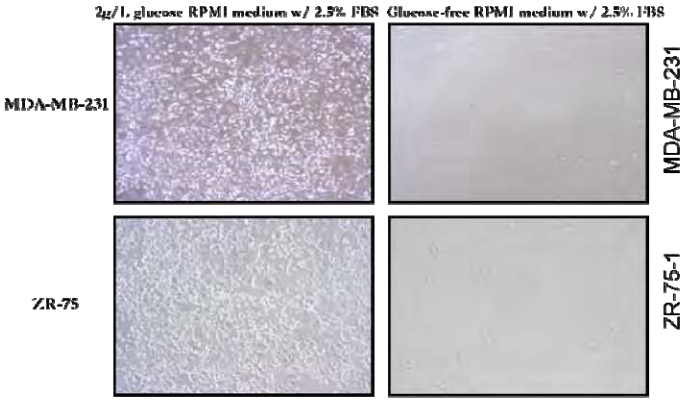


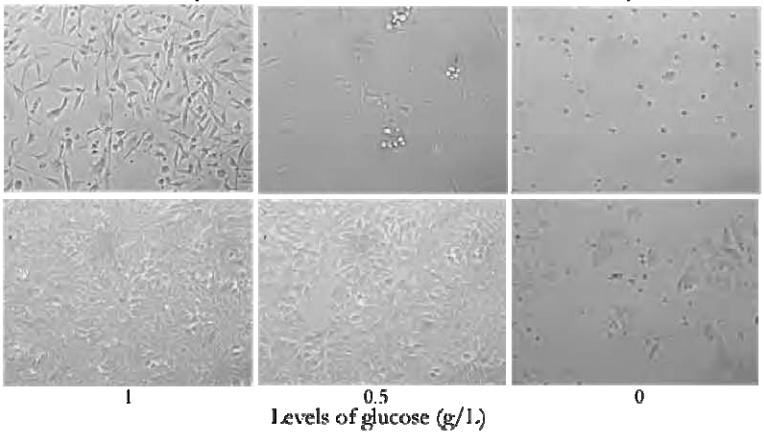
Fig 5. Immunoblot to test pcDNA3-Sp-fE1-E2-2b-E3. 48 h transient transfection in MCF7 breast cancer cells. The construct appeared unable to be expressed into f-svn or f-svn-2b as we did not observe bands of expected molecular weight upon ectopic expression. We also transfected flag-tagged and EGFP-survivin as positive controls to ensure antibody worked and for transfection efficiency, respectively. Endogenous survivin is expressed, but we can not detect truncated f-svn or f-svn-2b, using either Anti-FLAG M2, or Anti-survivin FL-142 antibodies (Santa Cruz), although our positive controls were detected.
Endogenous survivin (16.5kD); Flag-svn (14kD); Endogenous survivin-2B (18.6kD); Flag svn-2B (16kD)

Unpublished Data

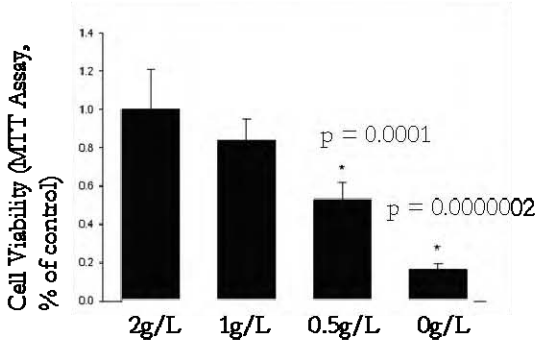
A. Glucose-dependent growth of MDA-MB-231 and ZR-75-1 human breast cells after 5 days



B. Differences in degree of cell-growth inhibition in response to glucose deficiency in human breast cancer cells (MDA-MB-231 and ZR-75-1, 96h)



C. Short-term effects of lowering glucose



D. Long-term effects of transiently lowering glucose

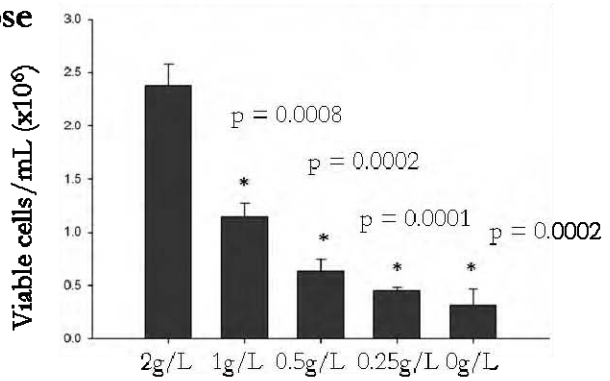
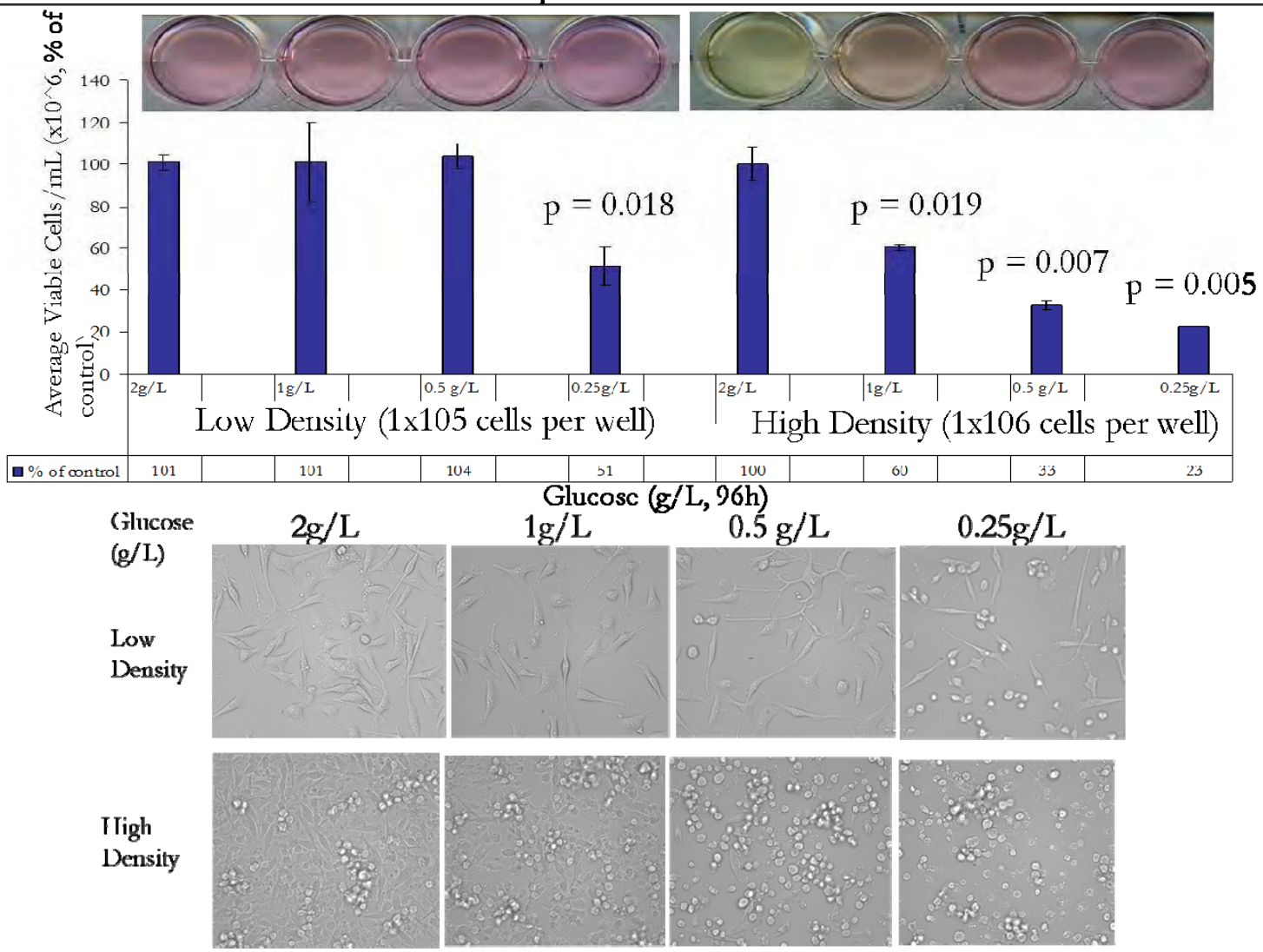


Fig 6. Human breast cancer cells MDA-MB-231 and ZR-75-1 require glucose for cell proliferation but have pronounced differences in dependency **A.** Glucose is necessary for MDA-MB-231 and ZR-75-1 cell proliferation by 5 days; **B.** MDA-MB-231 cells are more dependent on glucose than ZR-75-1 as shown photographically **C.** Quantitative analysis of more sensitive MDA-MB-231 cells by Short-term cell viability MTT assay (96h incubation) **(C)** and long term cell viability using trypan blue exclusion method (72h incubation in various glucose levels, followed by 2 week incubation in RPMI media containing 2g/L glucose with 10% FBS) **(D)**

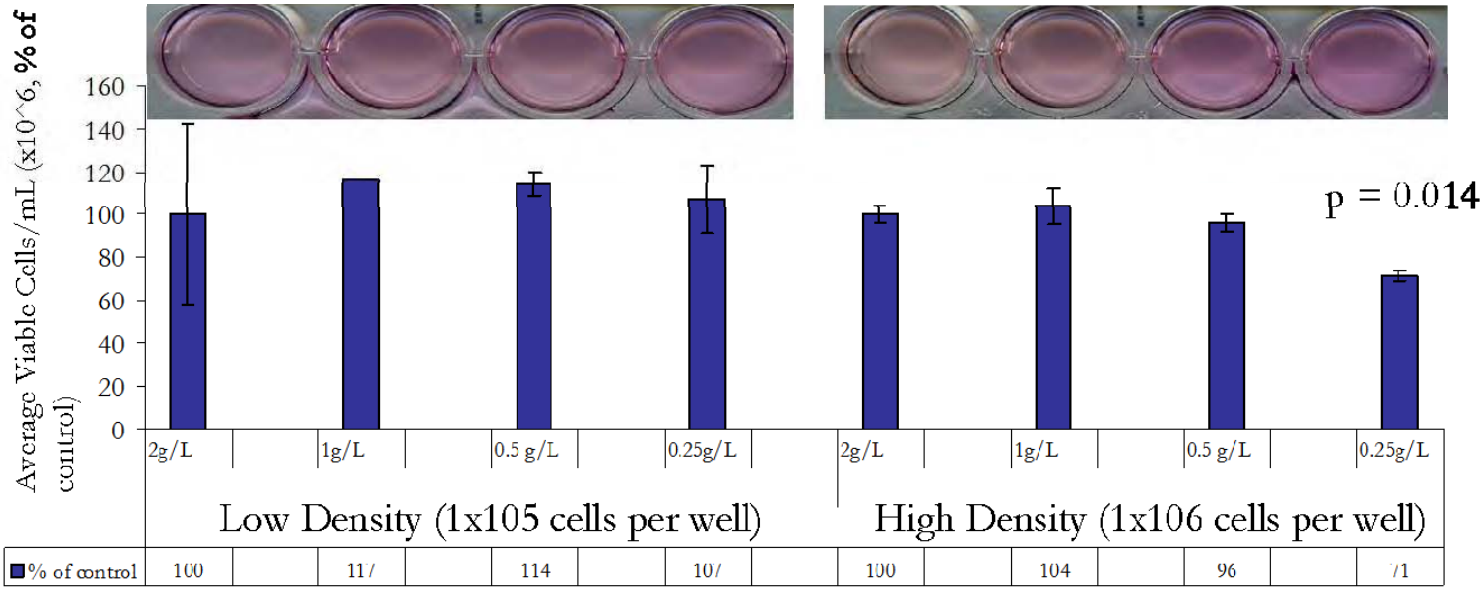
Unpublished Data

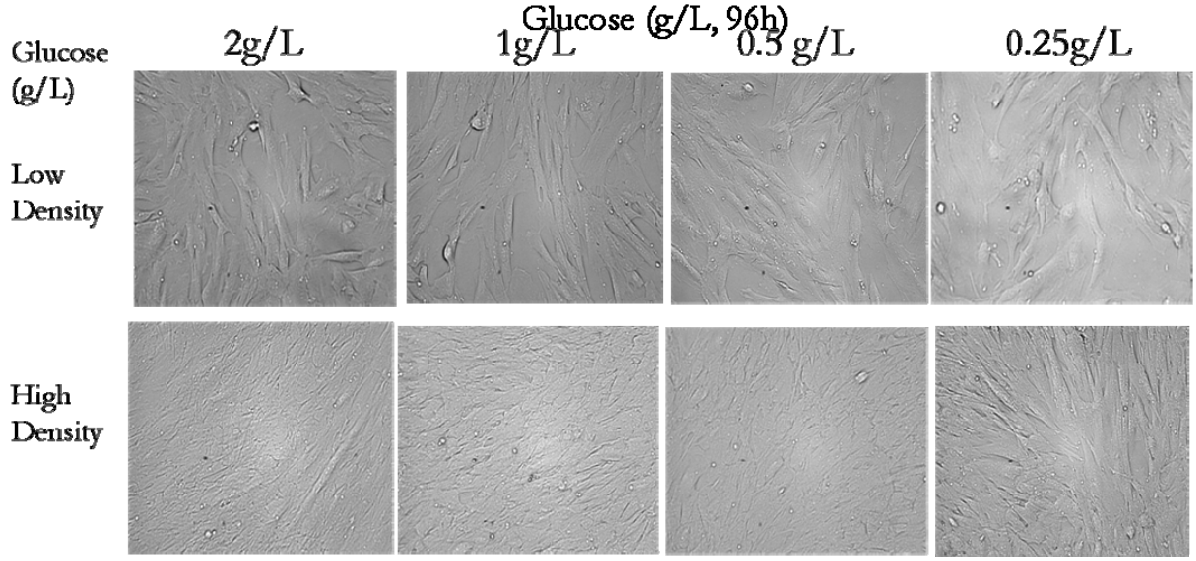
Fig 7. Effect of cell density and glucose on human breast cancer cells (MDA-MB-231) proliferation



Unpublished Data

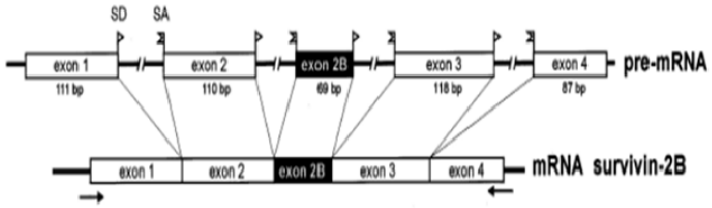
Fig 8. Effect of cell density and glucose on normal human fibroblast (WI-38-htert) proliferation





Unpublished Data

A. Survivin and Survivin-2B



B. Hypothesis

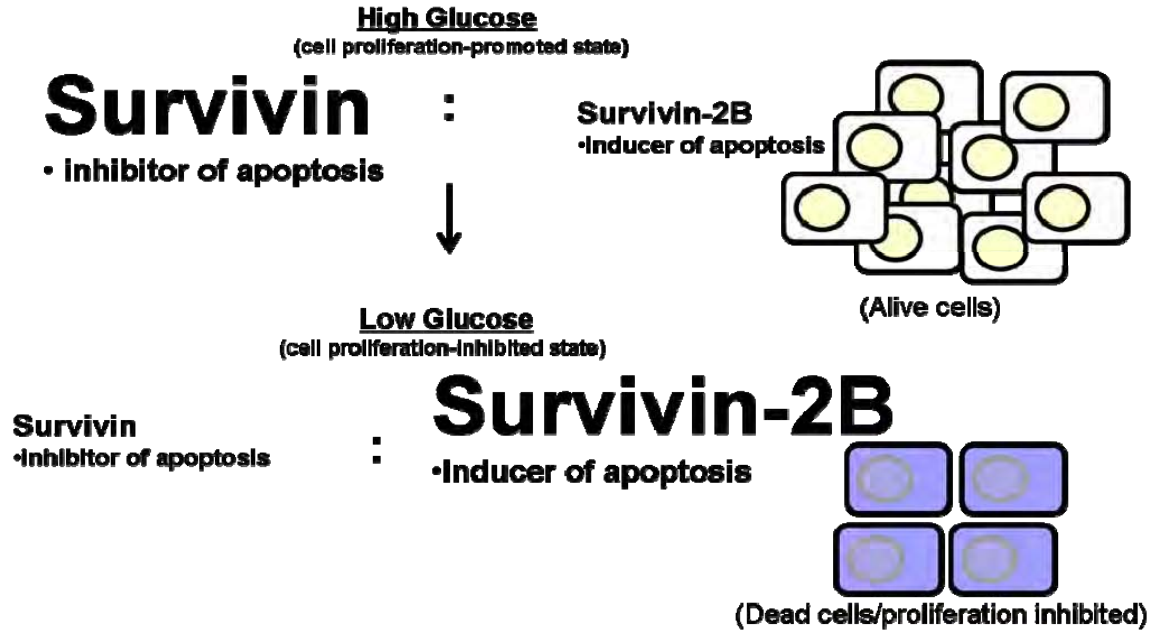


Fig 9 Survivin pre-mRNA splicing generates mutually exclusive survivin and survivin-2b transcripts **(A)** and working hypothesis **(B)**

Unpublished Data

Low glucose decreases both survivin and survivin-2B mRNA levels in MDA-MB-231 cells

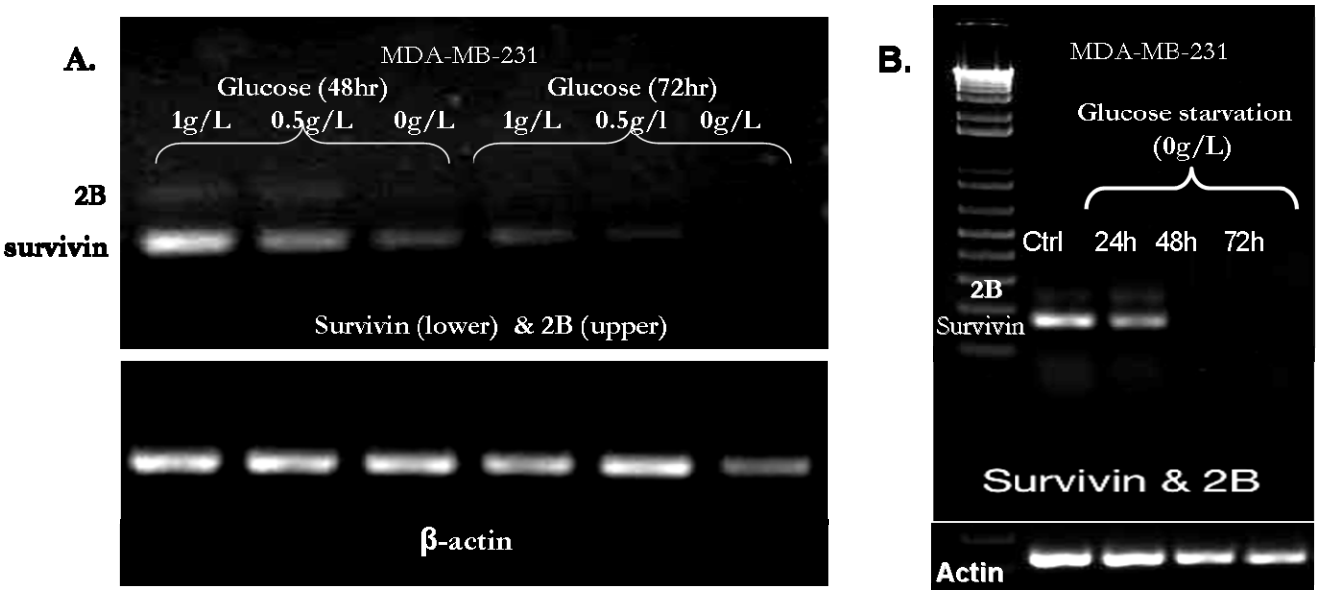


Fig 10. A. Constant time , while varying glucose concentrations. **B.** Constant glucose concentration, while varying time points. After overnight attachment, cells were washed 3x with PBS and media with the specified glucose concentrations was added, supplemented with 10% FBS and antibiotics. 24-72h RNA later, was isolated and used for cDNA first-strand synthesis. Semi-quantitative RT PCR was carried out with common survivin/survivin2B primers.

Unpublished Data

Effect of glucose on survivin, survivin-2B, and SIRT1 cDNA levels (human breast cancer cells, MDA-MB-231)

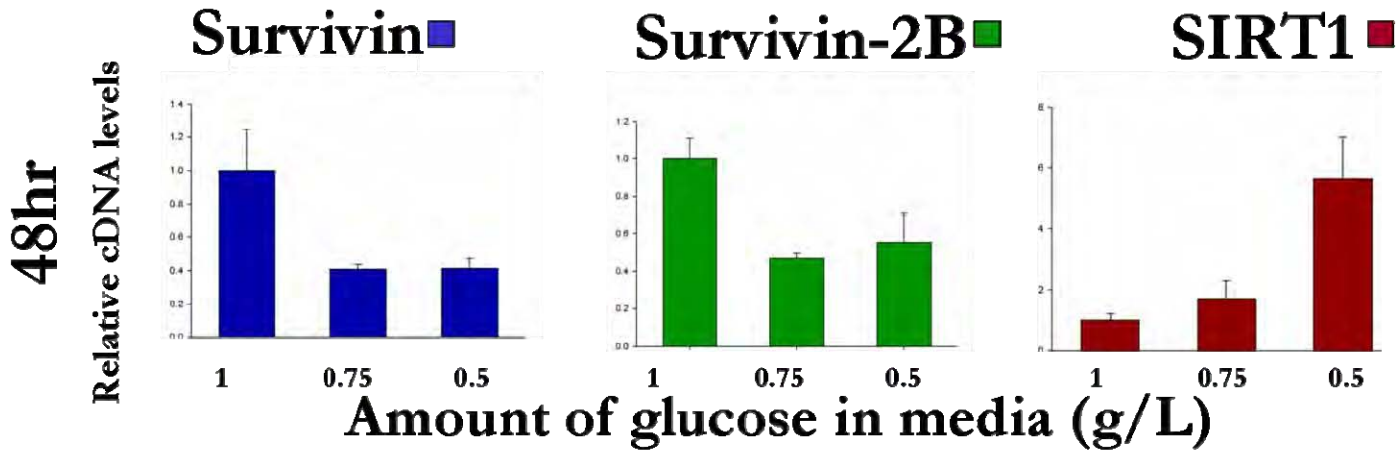
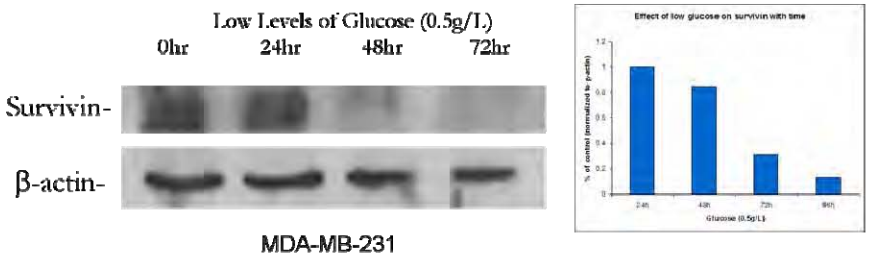


Fig 11. Effect of Glucose on survivin, survivin-2B and SIRT1 mRNA expression 48h. Low glucose (o.75g/L and 0.5g/L) significantly decreases survivin and survivin-2B mRNA levels at 48h, at which time SIRT1 mRNA is significantly increased.

Unpublished Data

A. Effect of low levels of glucose on survivin protein levels (human breast cancer cells)



B. In higher glucose environments survivin protein levels are higher (human breast cancer cells)

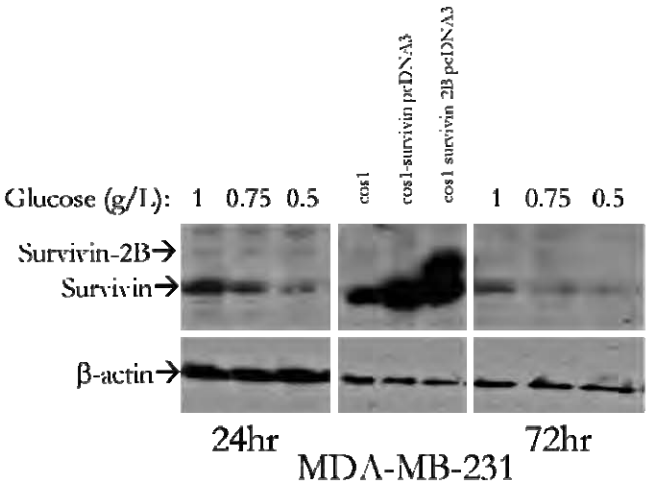


Fig 12. Cells were seeded in 10 cm^2 tissue culture dishes. Protein were separated on a 15% SDS Page gel and transferred to a nitrocellulose membrane. The membrane was incubated in survivin polyclonal antibody or **β**-Actin (Santa Cruz) overnight, blocked and incubated in secondary anti-rabbit antibody (Santa Cruz) the following day. After washing, chemoluminesence was used to visualize the proteins by autoradiography. **A**. Low levels of glucose decrease survivin protein by 48 hours in MDA-MB-231 cells. **B.** Deceasing glucose concentrations results in decrease of survivin protein in MDA-MB-231 cells at 24 h and 72 h

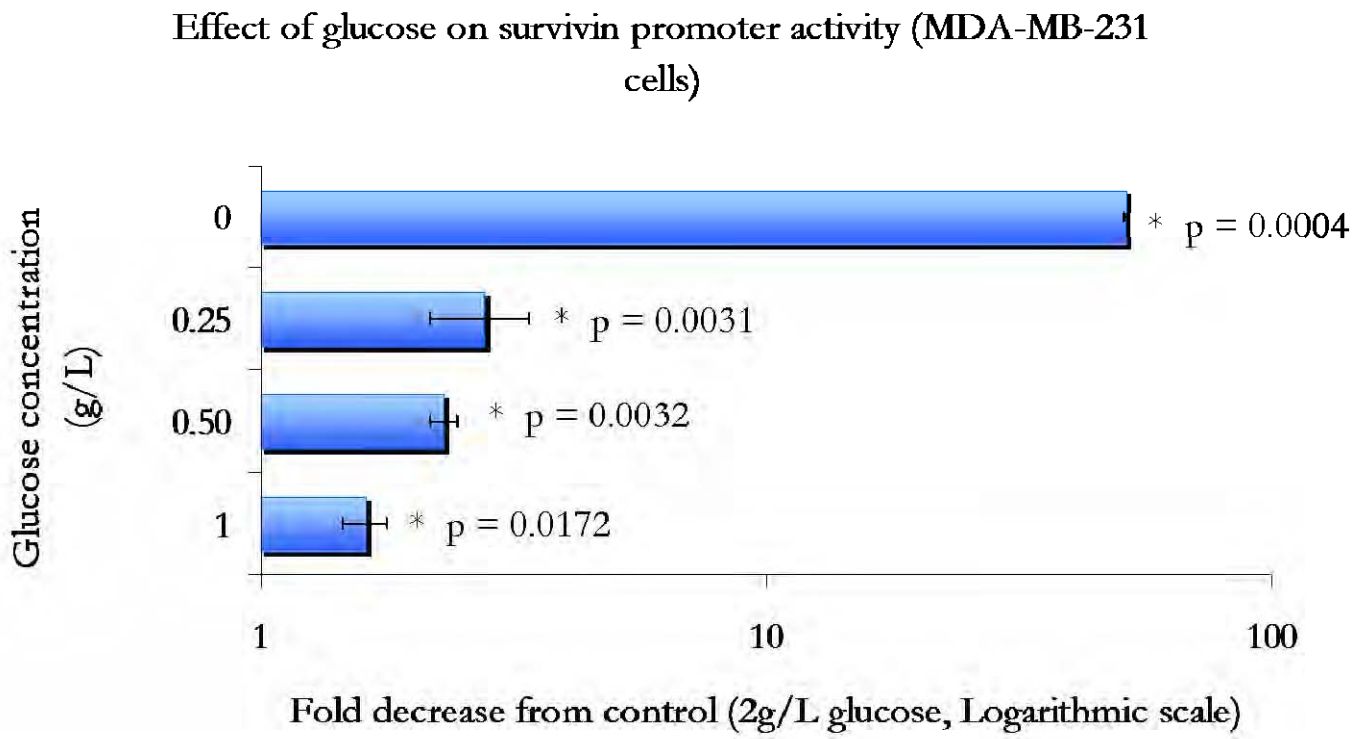


Fig 13. MDA-MB-231 stable cell line containing the entire survivin promoter cloned into Firefly Luciferase was cultured in the presence of G418. Prior to the experiment, cells were washed 3x with PBS and RPMI media with varying concentrations of glucose was added, supplemented with 10% FBS and antibiotics. 36h later cells were lysed using Promega Luciferase Assay and luciferase activity was measured according to the manufacturer's instructions. ANOVA tests were performed