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Abstract

Title of Dissertation: Tumor Angiogenesis and the role of RUNX2 transcription factor in endothelial cell (EC) function: regulation by glucose levels and oxidative stress

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Dissertation Directed by: Dr. Antonino Passaniti, Associate Professor, Department of Pathology

The RUNX2 DNA-binding transcription factor is an important regulator of tumor angiogenesis. Its transcriptional activity is dependent on interaction with a cofactor, CBF β , which does not bind DNA, but enhances RUNX2 DNA binding. Recently, we discovered that RUNX2 DNA binding activity is regulated by glucose and the redox status of the cell. Euglycemic levels of glucose initiate a series of events leading to RUNX2 phosphorylation and DNA binding. In the presence of elevated levels of glucose (hyperglycemia), RUNX2 oxidation may reduce its ability to bind DNA. Therefore, we propose the hypothesis that the RUNX2 transcription factor regulates endothelial cell (EC) proliferation through its glucose response and redox status. To test this hypothesis, in specific aim 1, we will examine the ability of RUNX2 to associate with a target gene that regulates cell cycle progression and proliferation, the p21^{Cip1} promoter. This DNA interaction will be examined by chromatin immunoprecipitation assays and by DNA-binding assays. In specific aim 2, we will examine the effect of mutations in critical cysteine and methionine residues within RUNX2 that regulate DNA binding. The effect of these mutations on DNA binding will be examined using electrophoretic mobility shift assays. Two mutant proteins will be used: a GFP.tagged RUNT DNA binding domain fusion protein in which a methionine is converted to a valine (RUNT.M106V) and a

cysteine to serine substitution in full-length RUNX2 at position 118 (RUNX2.C118S).

These studies will help define the mechanisms through which nutrients and cellular redox status regulate RUNX2 activity and will provide opportunities for new therapeutic approaches.

Tumor angiogenesis and the role of the RUNX2 transcription factor in endothelial cell
(EC) function: regulation by glucose levels and oxidative stress.

by
Sravya Kommineni

Thesis submitted to the faculty of the Graduate School of the
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Chapter 1

Introduction

Tumor cells, like normal mammalian cells, require nutrients and oxygen for their survival, which are provided by the recruitment of blood vessels (angiogenesis). In order for tumor cells to grow beyond a certain critical size and even to metastasize to different organs they require angiogenesis,^{1,2} which is recognized as one of the seven hallmarks of cancer³. Research in how tumors can recruit blood supply has expanded in recent years because of the demonstration that therapeutic interventions to inhibit angiogenesis are also effective anti-tumor strategies⁴ (**Figure1**)

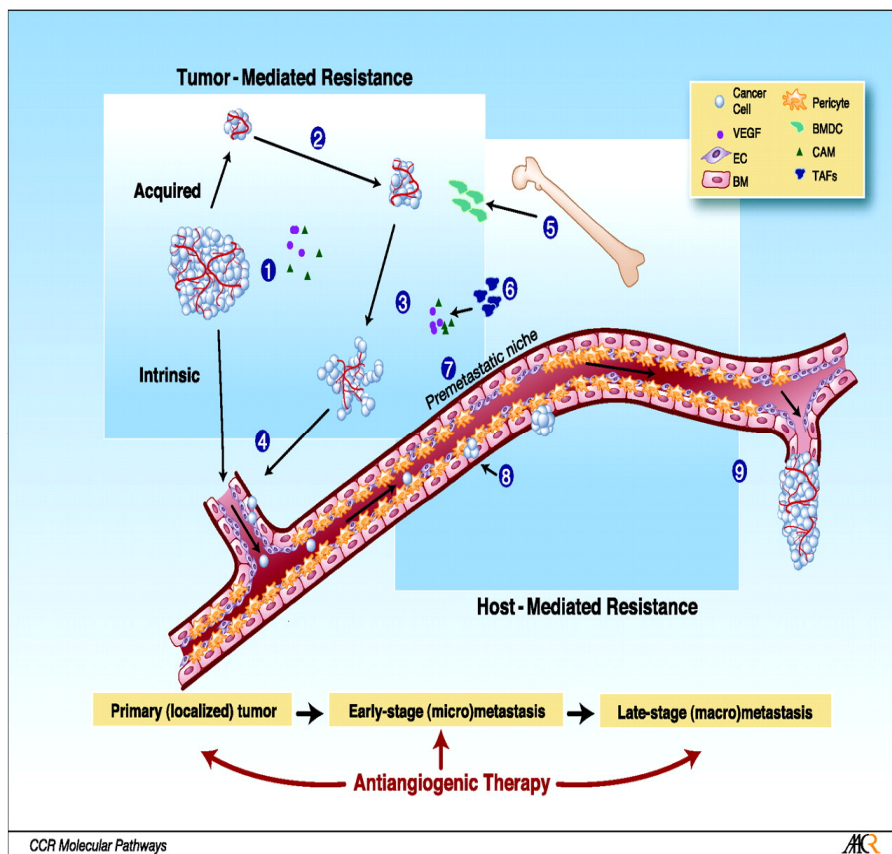


Figure 1 -Mechanisms of resistance to antiangiogenic therapy may allow for differential efficacy in different stages of disease progression⁵.

Angiogenesis is regulated by the coordinated expression of several transcription factors that control the expression of genes important for endothelial cell (EC) migration, invasion, proliferation, and survival. It is known to be regulated by several transcription factors such as p53 tumor suppressor gene, the Rb cell cycle regulator, and Foxo family of transcription factors which exhibit anti-angiogenic activity. The p53 gene inhibits angiogenesis through degradation of HIF1 α which is mediated through Mdm-2 ubiquitination and proteasomal targeting. It is also known to inhibit angiogenesis through inhibition of VEGF, bFGF and COX-2 and also via increasing synthesis of anti-angiogenic factors such as TSP1, EphrinA2, and collagen. In the case of Rb family gene, the pRb interacts with E2F and inhibits its transcriptional activity. Overexpression of E2F increases expression of angiogenic factors like VEGF, FGFR1⁶⁻⁸. The Foxo protein

inhibit EC growth⁹ because when the Foxo1 gene is silenced in cultured ECs we see an increase in tube formation which happens to be an indicator of angiogenesis. It is also known that Foxo1 destabilizes blood vessels by increasing the expression of angiopoietin-2, which is a known competitor of angiopoietin-1 for the Tie2 receptor which mediates vessel sprouting and normalization^{10,11}.

Apart from these anti-angiogenic factors, there are several proangiogenic factors such as HIF1 α , inhibitors of the differentiation genes Id1 and Id3; Ets factor ELF-1, c-myc oncogene, PGC1 α etc. HIF1 α is known to be the potent activator of angiogenesis¹², it does so through its DNA binding domain and the heterodimerization with other factors it regulates the expression of PDGF, TGF α and VEGF. The deletion of Id1 and Id3 genes in mice resulted in poor angiogenesis and this effect was due to increased differentiation of vascular cells and decreased expression of VEGF¹³. The Ets factor ELF-1 functionally regulates angiogenesis through Tie2 receptor. The c-myc oncogene promotes angiogenesis through the downregulation of TSP-1 which is an angiogenic inhibitor. Moreover the angiogenic switch is reversed when TSP-1 and p53 are functional¹⁴. The PGC1 α regulates the process of angiogenesis through the interactions with ERR α which results in the expression of VEGF¹⁵.

One such family of transcription factors is the RUNX family, which has been shown to be regulated by nutrient status in the microenvironment. These factors are known to regulate several processes during embryonic development such as hematopoiesis and T-cell differentiation by RUNX1, bone formation by RUNX2, and gastric epithelial differentiation by RUNX3. Abnormal expressions of these factors lead

to leukemogenesis, breast and prostate cancer metastasis, and gastric cancer by RUNX1, RUNX2 and RUNX3 respectively¹⁶.

Our group and others have shown that RUNX2 DNA binding activity regulates EC migration and invasion through its control of metalloproteinase and matrix gene expression. The RUNX family is known to have DNA binding domain called RUNT which is homologous to Drosophila pair rule gene, RUNT¹⁷. Core binding factor (CBF β) is a co-transcription factor that does not interact with DNA directly and enhances the in-vitro DNA binding of the RUNT protein¹⁸.

RUNX2 exert its effects in part by modifying chromatin organization of tissue-specific gene promoters¹⁹⁻²¹. Moreover, the activity of RUNX2 is modulated by the interactions with several other co-regulatory proteins such as Groucho/TLE, Yes-associated protein (YAP), Smad that contribute to the integration of cell signaling pathways in response to physiological cues²²⁻²⁸. It resides in discrete subnuclear foci where it co-localizes with other transcriptional regulators by either activating or repressing RUNX dependent genes^{24,29-31}. The mechanism through which it gets directed to specific subnuclear domains is through its 38 amino acid segment at the C-Terminus and the nuclear-matrix-targeting signal (NMTS)³². It has been shown that RUNX2 DNA binding activity is often low in starved EC, but increases in cells treated with 5mM glucose (physiologically relevant levels). It has also been shown that RUNX2 activity is cell-cycle and nutrient dependent where it is phosphorylated by cdk4 in G2 and cdk4 in G1 and it also represses the activity of p21^{cip} promoter³³ through cofactor interactions in the carboxy terminus. It is known that glucose provides EC with the essential energy required for their proliferation and cell cycle progression. Studies have shown that

euglycemic levels of glucose regulate normal EC function, whereas hypoglycemic or hyperglycemic glucose levels result in an increase in p21^{cip} and p27 expression levels, which reduce DNA synthesis and cell cycle progression. This is due to the upregulation of the anti-proliferative effects of TGF- β .

CBF β has two subunits, the α subunit which interacts with the DNA (RUNT domain) directly and β subunit which doesn't interact directly with the DNA but associates with the α subunit to form a complex which has a greater DNA binding affinity (**Figure 2**). Functional domain analysis of both these subunits has led us to conclude that the DNA binding activity of the RUNT domain is controlled by a sensitive reduction/oxidation (Redox) mechanism where the β subunit acts to stabilize the activated state of the RUNT domain and increase its DNA binding activity.

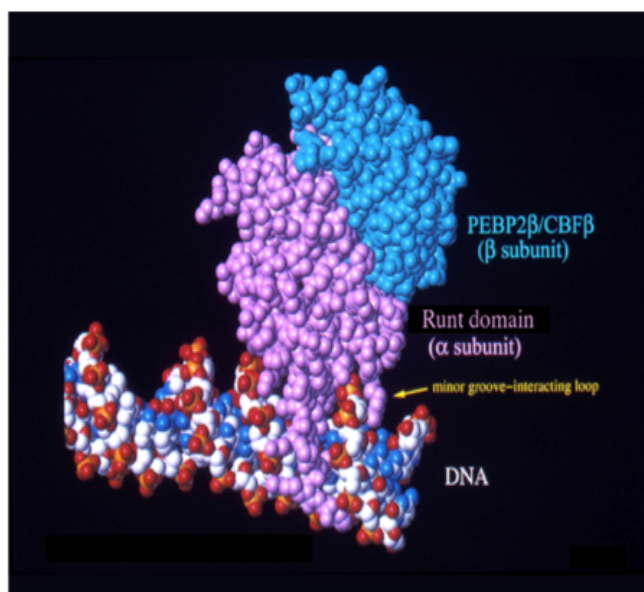


Figure 2- Crystal structure of the RUNT domain heterodimerized with the 134 amino acid region of PEBP2 β /CBF β bound to the DNA ³⁴.

RUNX2 DNA binding may be sensitive to oxidation because of the presence of two conserved cysteine residues in the RUNT DNA binding domain that are necessary for correct protein folding and interaction with DNA^{34,35} Apart from the Cysteine mutations on the RUNT domain they are several CBF α point mutations that degrade or abolish CBF α - CBF β heterodimerization have been described^{34,36,37}. Some mutations like N109D, F146Y, L148D, or Q158L impair CBF α -CBF β binding by disrupting the conformation of L5 or the overall folding architecture of CBF α , which also affects its ability to bind to DNA. Others mutations such as M106V or G108R impair CBF α -CBF β binding by specifically affecting the recognition residues at the CBF α -CBF β interface. For instance, replacement of Met106 with a b-branched valine would cause steric hindrance between the branched methyl group of valine and the methylene part of Asn63 of CBF β , while replacement of Gly108 with arginine would cause steric hindrance between the arginine and the L2 main chain of CBF β . In these cases, the mutants retain the capacity to bind to the DNA-DNA binding is also controlled by the presence of a conserved methionine residue in the Runt domain that mediates interaction with the RUNX2 binding partner, CBF β , which enhances DNA binding ten-fold³⁸⁻⁴⁰ (**Figure 3**). However, the possible role of oxidative stress in regulating the redox status of methionine residues in RUNX2 and/or RUNX2:CBF β association has not been elucidated.

Chapter 2

Materials and Methods:

Cell culture- Human Embryonic cells (HEK 293) and Human Bone Marrow

Embryonic cells (HBME) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO₂.

Site-Directed Mutagenesis- The C118S mutant plasmid was synthesized using the procedure described in Stratagene Quik Change Site-directed Mutagenesis kit where oligonucleotides (C118S sense strand-5'- GTG CTG CCC TCG CAC TGG CGC **AGC** AAC AAG ACC CTG CCC GTG GCC – 3' and C118S antisense strand- 5'- GGC CAC GGG CAG GGT CTT GTT GCT GCG CCA GTG CGA GGG CAG CAC – 3') were designed in a manner such that they flank the regions around the Cysteine 118 residue which needs to be mutated to a Serine residue by the change of a single amino acid. The mutation was carried in a pCMV Tag 2A WT RUNX2 plasmid. Standard PCR was carried out Tag Pfu Polymerase. The samples were then treated with 1ul of Dpn I restriction enzyme (10U/ul) at 37°C for 1 hour. 1ul of the Dpn I treated reaction was added to 50ul of XL1-Blue supercompetent cells to a pre-chilled Falcon® 2059 polypropylene tube and the transformation reaction was incubated in ice for 30 minutes, followed by a short heat pulse at 42°C for 45 seconds and place them immediately on ice for 2 minutes. 0.5 ml NZY+ media was added to the reaction tubes and incubate them at 37°C for an hour with shaking at 250rpm. About 250ul of the reaction was spread on LB-kanamycin Agar plates and incubate at 37°C for 16 hours or more.

Transfections- For transfection purposes the cells were seeded on a 10cm dish 24 hours before the experiment needed to be carried out. The next day transfection was carried out using the effectene (Qiagen). Stably transfected cells were obtained by putting them under selection with 0.3mg/ml of Gentamycin 148 (G148).

ROS treatments - For DNA binding assays the nuclear proteins of stably transfected HEK 293 cells with C118S, Empty GFP RUNT, WT GFP RUNT, M106V GFP RUNT plasmids were treated with 100uM of peroxide and were incubated at room temperature (RT) for 30 minutes to oxidize the proteins. They were then treated with 30mM of DTT and were incubated at 37°C for 20 minutes in order to bring back the protein

Electrophoretic Mobility Shift Assays (EMSA) - This assay was used to measure RUNX2 DNA binding activity. Nuclear proteins were obtained from HEK 293 cells stably transfected with C118S, Empty GFP RUNT, WT GFP RUNT, M106V GFP RUNT plasmids using low salt/high salt extraction. Nuclear proteins (2ug) were incubated with ³²P-labelled oligonucleotide derived from the human oestocalcin promoter which has the RUNX2 consensus binding sequence 5'-CGTATT**AACCACA**ATACTCG-3'. The double stranded probe was end labeled using (α -³²P) dATP, dNTP mixture and Klenow fragment. The DNA- nuclear protein (2ug) complexes were separated using a 6% Tris Borate EDTA polyacrylamide gels. The gels were dried and placed on an X-Ray film at -80°C overnight and developed the next day.

DNA-binding enzyme-linked immunosorbent assay (*D-ELISA*) - DNA binding was quantified with a specific ELISA format method⁴² as modified for RUNX2-specific detection. Avidin-coated 96-well plates were fixed with sodium carbonate in for 2h at 24°C. After rinsing 3X with wash buffer, a 3'-biotin labeled oligonucleotide containing 3 osteocalcin RUNX2-binding sites was added for 2hr. Nuclear extracts containing RUNX2 protein were exposed to the plate with rocking for 16h at 4°C. Primary antibody (for RUNX2) was added for 1hr at 24°C and secondary antibody-HRP was allowed to react with the primary antibody for 30min at 24°C. TMB substrate (50ul) was allowed to react with the HRP in the dark. Once color development was confirmed (10-20 min), 50ul of stop solution was added to each well and a BioTrack II plate reader spectrophotometer (Amersham Biosciences/GE Healthcare, Piscataway, NJ) was used to obtain absorbance readings (A_{450nm}). For continuous (kinetic) monitoring of each reaction, automated absorbance readings were determined each minute at 635nm with equivalent results using a BIO-TEK Synergy HT (Biotek Industries, Winooski, VT) and analyzed using Gen 5 Data Analysis Software.

Glucose treatments –Stably transfected HEK 293 cells with pCMVTag 2A wild type RUNX2 were used. Sub-confluent cells were blocked with 2mM thymidine for 16hours and then replaced with full media (DMEM with 10% FBS). The cells were kept in full media for 8 hours and again blocked with 2mM thymidine for 16 hrs in-order make the cells synchronous at G1/S. They were then released with 5mM glucose at 0hrs, 2 hrs, 4hrs and 6rs respectively.

Chromatin immunoprecipitation assays- were used to check the regulation of p21 by RUNX2. Stably transfected HEK 293 with the WT RUNX2 plasmid were used for the experiment. The cells were cultured 24 hrs the day before upto 70% confluence level. The next day the cells were fixed with 270ul of 37% formaldehyde at 37°C for 10 minutes and then washed with chilled PBS twice followed by sonication of about 250,000 cells in 500ul of SDS Lysis Buffer(1%SDS,10mM EDTA, 50mM Tris-HCl pH 8.1) using the Biorupter. The cell lysates are then spun down at max speed at 4°C for 10minutes where the supernatant is dissolved to a final volume of 2ml with the ChIP dilution buffer (0.01%SDS, 1.1% Triton X 100, 1.2mM EDTA, 167mM NaCl) to proceed with the immunoprecipitation. The samples were pre-cleared with 30ul of Protein G magnetic beads at 4°C for 30 minutes with rotation. Then the samples were incubated with 2ug of the M-70 (Santa Cruz) RUNX2 antibody (protein of interest), 1ug of the nucleolin antibody (positive control), 1ug of anti-mouse IgG (negative control) at 4°C overnight with rotation. The next day the antigen antibody complexes were incubated with 30ul of Protein G magnetic beads at 4°C for 3hours with rotation in order to pull down the complexes. This is followed by the sequential washing of the beads with 1ml of Low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl) High salt buffer(0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), LiCl buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1) and twice with TE buffer (10mM Tris-HCl, 1mM EDTA). The DNA-protein complexes are then eluted with 400ul of freshly prepared elution buffer (1%SDS, 0.1M NaHCO₃). In order to reverse the cross links 16ul of 5M NaCl is added to the eluates and incubated at 65°C for 4 hours, followed

by another additional incubation at 45°C for 1 hour with 8ul of 0.5M EDTA, 16ul of Tris-HCl and 2ul of 10mg/ml Proteinase K. The DNA was then extracted from the samples using phenol-chloroform and then PCR was carried using p21 promoter primers (product size 337 bp)

Forward primer 5'- GGG CAG AAG TCC TCC CTT AGA GTG – 3'

Reverse primer 5'- CTC CCA CTG CCT TGA AGC CCT C – 3'

Immunohistochemistry –HBME cells were plated on a 24-well plate on cover slips .The cells were fixed with 1ml of 10% formaldehyde at 4°C for 10mins and washed twice with 1ml of PBS at RT for 5minutes on the rocker. The cells were permeabilized with 1ml 0.25% Triton-X100 in PBS at RT for 10 minutes and washed twice with 1ml of PBS at (RT) for 5minutes on the rocker. They were then blocked with 1ml of %BSA/0.5%NP40 in PBS at RT for 1 hour on the rocker washed twice with 1ml of PBS at (RT) for 5minutes on the rocker. The cells were incubated with Anti-rabbit M-70 RUNX2 from Santa Cruz Biotechnology (primary antibody) at 4°C overnight and washed thrice with 1ml of PBS at RT for 10 minutes on the rocker. Incubate the cells with 250ul of (1:1000) Alexa 488 Anti-rabbit (secondary antibody) at RT for an hour on the shaker washed thrice with 1ml of PBS at RT for 10 minutes on the rocker. Mount them on a pre-labeled slide with 15ul of Fluoromount-G and let the slide set at RT for 30 minutes.

Statistical Considerations - Results are expressed as the mean (\pm SD) of at least three replicate samples per data points. Experiments were repeated at least twice. For comparison of measurements relative to control samples, the Student's t-test was used to

determine statistical significance. p values < 0.01 were considered significant. For comparison of multiple measurements, data were analyzed using Tukey's post-hoc adjustment for 2-by-2 comparisons following ANOVA. p values < 0.05 were considered significant.

Chapter 3

RESULTS

Glucose-activated RUNX2 phosphorylation and subnuclear localization

It is known that endogenous RUNX2 is localized in discrete subnuclear foci. To determine if glucose could alter the subnuclear localization of RUNX2, cells were double-thymidine blocked in G1/S and released into G2 with 5mM glucose. By immunofluorescence, focal staining of RUNX2 protein was observed within 4hr after treatment, with reduced focal staining after 8hr (**Figure 4**). These data show that glucose can activate RUNX2 re-distribution to subnuclear compartments in the G2 phase of the cell cycle. RUNX2 is known to shuttle from chromatin to sites of transcription when activated^{43,44}. Since glucose induces changes in subnuclear localization by immunofluorescence, biochemical fractionation was used to confirm these results (data not shown).

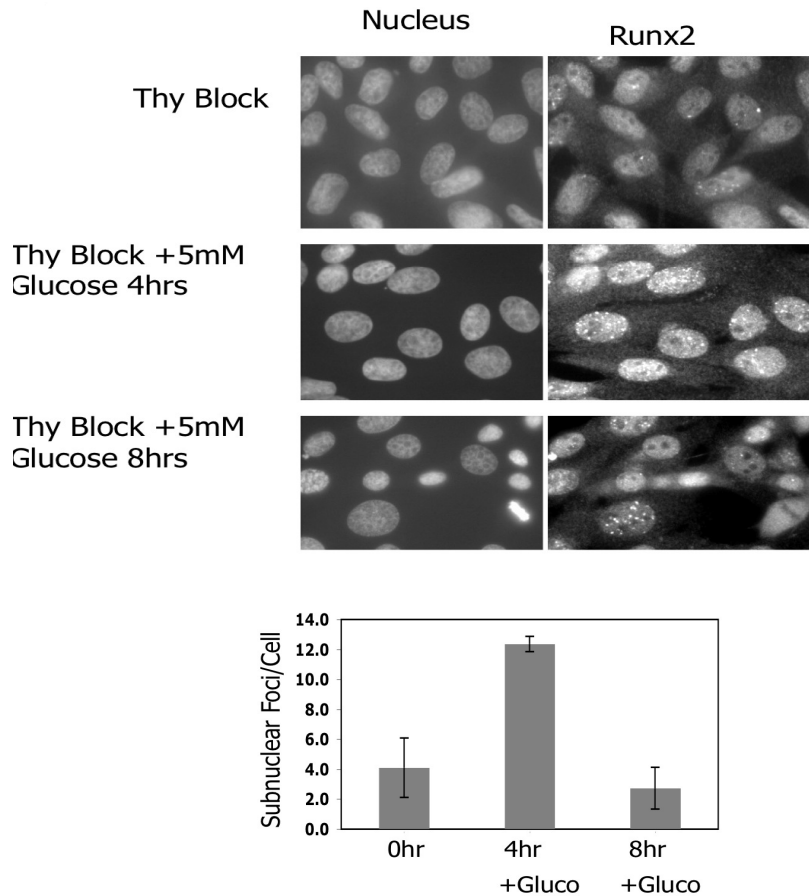


Figure 4 - Subnuclear localization of RUNX2 by Immunofluorescence. HBME cells were thymidine blocked in G1/S and released with 5mM Glucose to allow entry into G2 after 4hrs and 8hrs. Subnuclear localization of RUNX2 was detected by IF using a RUNX2-specific antibody. The number of nuclear foci was counted from 3 separate fields. DNA was stained with a Hoescht dye to visualize the nuclei. The number of nuclear foci was counted from 3 separate fields of cells. In the case of 4hrs and 8hrs treatment, p values of 0.029 and 0.365 were obtained relative to 0hrs.

Focal association of nuclear RUNX2 may indicate regulation of a specific RUNX2 target gene after glucose treatment. We⁴⁵ and others³³ showed that RUNX2 represses the cdk inhibitor p21^{Cip1} at the promoter level (using luciferase reporter constructs) and that doxorubicin-induced p21^{Cip1} protein expression is partially inhibited by overexpression of RUNX2 in EC⁴⁵. RUNX2 knockdown leads to increased p21^{Cip1} expression, as expected (**Figure 5**).

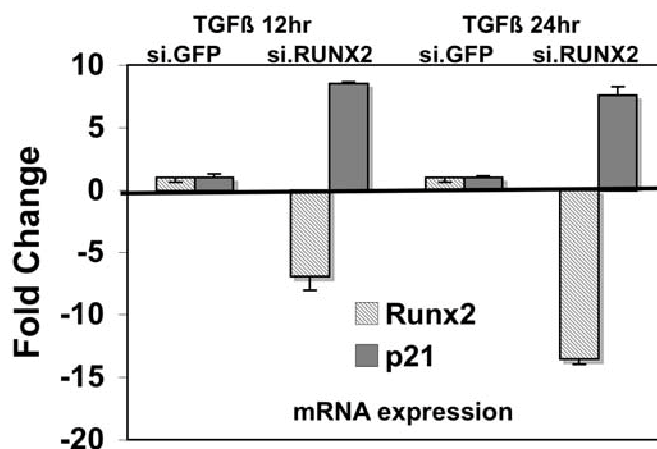


Figure 5 - Regulation of p21^{Cip1} expression by RUNX2. Cells were transfected with RUNX2 siRNA or GFP targeting duplexes (1 μ g each) and treated with TGF β (0.1ng/ml) for 12 or 24hr. Expression of p21^{Cip1} or RUNX2 mRNA was quantified using qRT-PCR and the indicated primers (**F**, forward; **R**, reverse): **RUNX2 primers: F** 5'-GCACAGACAGAAGCTTGAT-3', **R** 5'-CCCAGTTCTGAAGCACCT-3' **p21^{Cip1} primers: F** 5'-CCGAAGTCAGTTCCTTGTGG-3', **R** 5'-CCGCCATTAGCGCATCACAG-3'; **GAPDH, primers: F** 5'-CCGTCTAGAAAAACCTGCCAA-3' **R** 5'-TGTAGCCAAATTCGTTGTCATACC-3'. Expression is shown relative to GFP-transfected cells. Specific siRNA sequences included: si.RUNX2...Rx2 (5'-ccataaccgtcttcacaaa-3') + Rx3 (5'-ggacgaggcaagagttca-3') or si.GFP (5'-tctgtgtgtttgactctga-3').

To determine whether p21^{Cip1} might be regulating cell proliferation in response to RUNX2, a p21^{Cip1}-specific shRNA vector was used to knockdown p21^{Cip1} expression (**Figure 6**). p21^{Cip1} knockdown resulted in a >80% reduction in p21^{Cip1} protein and a 4.5-fold increase in cell growth by day 6 (relative to control GFP-targeted shRNA).

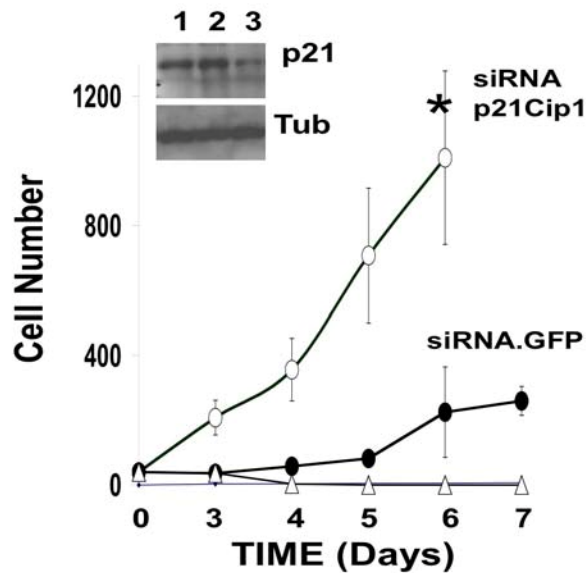


Figure 6. p21^{Cip1} expression regulates EC proliferation. A region close to the 5'-end of the p21^{Cip1} gene was targeted by a short hairpin loop RNA encoded by the Imgenex pSup expression plasmid. Control plasmid expressed short hairpin RNA targeting GFP. Western blot of p21^{Cip1} expression (*inset*): untransfected (*lane 1*), control sh.GFP (*lane 2*), or sh.p21^{Cip1} (*lane 3*). HBME cells expressing shRNA.p21^{Cip1} (*open circle*) or shRNA.GFP (*closed circle*) were replated in media containing 0.6mg/ml G418. Antibiotic resistant cell growth was quantified as cells per high-power field as a function of time (Days). *p < 0.004 at 6 days for siRNA relative to GFP controls. Sequences targeted include: p21Cip1 (5'-ttagtctcagtttgtgtcttaattatt-3') and GFP (5'-tctgtgtgtttgactctga-3').

Examination of the p21^{Cip1} promoter revealed 3 possible RUNX2 interaction sites: A, B, C (**Figure 7**). To determine which site was the most functional binding site, oligonucleotides corresponding to each site were synthesized and examined as competitors for RUNX2 DNA binding using traditional EMSA. RUNX2 binding site A exhibited the highest affinity for RUNX2 but not as high as the osteocalcin promoter site used as control (**Figure 8**). To determine whether glucose treatment promotes RUNX2 association with the p21^{Cip1} promoter, thymidine blocked cells (low RUNX2 expression)

were released from G1/S blockade with glucose to activate RUNX2 and Chromatin Immunoprecipitation assays were performed. Glucose treatment after thymidine block and release resulted in recruitment of RUNX2 protein to p21^{Cip1} site A (**Figure 9**). Control IgG precipitation in the proximal p21^{Cip1} promoter did not reveal RUNX2 association.

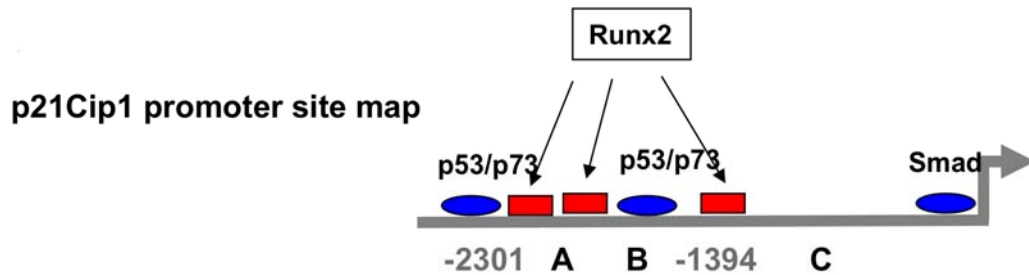


Figure 7. p21^{Cip1} promoter site map. The p21^{Cip1} promoter contains two p53/p73 consensus interaction sites and three RUNX2 consensus binding sites (ACACCAA) close to the distal p53/p73 sites. The proximal promoter contains consensus Smad binding sites.

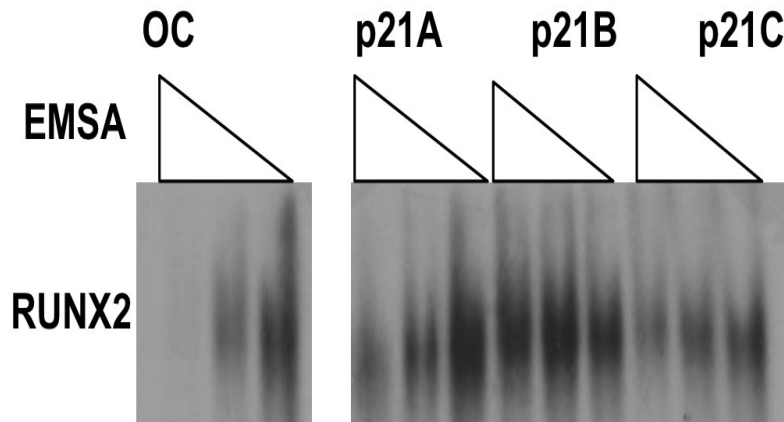


Figure 8. RUNX2 activation in response to glucose is associated with changes in RUNX2 subnuclear localization and association with binding sites in the p21^{Cip1} promoter. DNA oligonucleotides were synthesized corresponding to the p21^{Cip1} promoter sites A, B, and C. These were used as competitors in a DNA-binding EMSA

format using radiolabeled osteocalcin (OC) promoter oligonucleotide recognizing RUNX2. For each assay, p21^{Cip1} promoter site A was the most active inhibitor. Each triangle depicts variable amounts of cold oligonucleotide from 100X, 50X, and 10X the amount of labeled oligonucleotide.

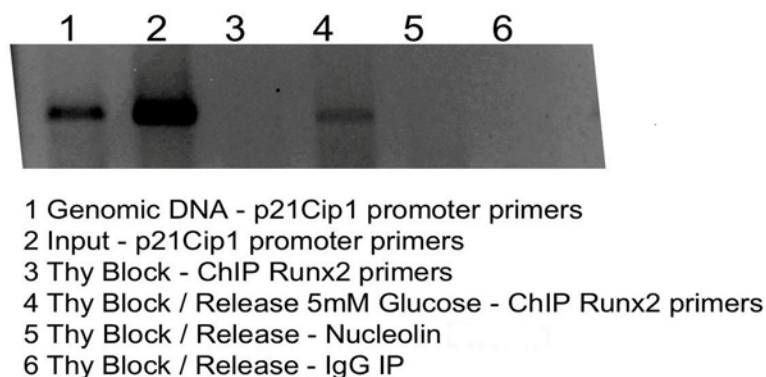


Figure 9- Chromatin Immunoprecipitation Assays in Thymidine blocked cells treated with Glucose. HEK 293 cells transfected with WT.RUNX2 were thymidine blocked to get a synchronous population of cells in G1/S phase and then released with 5mM Glucose after 2 hours. Control IgG was used as the negative control for Immunoprecipitation and M-70 RUNX2 antibody was to Immunoprecipitate RUNX2.

ROS-regulated RUNX2 and angiogenesis

Cysteine and methionine residues in proteins are sensitive to oxidative stress, which regulates transcription factor DNA binding^{46,47}. The DNA-binding RUNT domain of RUNX2 contains two cysteine residues, which mediate proper folding for Arg+/DNA interactions. A critical methionine residue within the Runt domain mediates association with its essential cofactor CBF β , which increases DNA binding 10-fold relative to Runt alone⁴⁰. To quantitatively assess DNA binding in cells expressing RUNX2 with mutant Cysteine or Methionine residues in the Runt domain, a DNA-binding assay with increased specificity for RUNX2 was used⁴⁸. In this assay, a RUNX2-specific DNA

oligonucleotide and a RUNX2-specific antibody are incubated with nuclear extracts and RUNX2: DNA binding is measured in a 96-well colorimetric format. As expected, nuclear extracts isolated from HEK293 cells transfected with wild type RUNX2 showed high DNA-binding activity, which was abrogated by H₂O₂ treatment (**Figure 10A**). Cells transfected with a RUNX2 mutant (Cys118Ser), however, exhibited RUNX2 DNA binding comparable to what is observed with a non-specific IgG antibody control. Similarly, using EMSA DNA-binding assays, a GFP-tagged RUNT domain vector mutated at position Met-143 of the full length RUNX2 form (corresponding to Met106 of the RUNT domain from RUNX1) exhibited reduced DNA binding relative to wild type GFP-tagged Runt (**Figure 10B**).

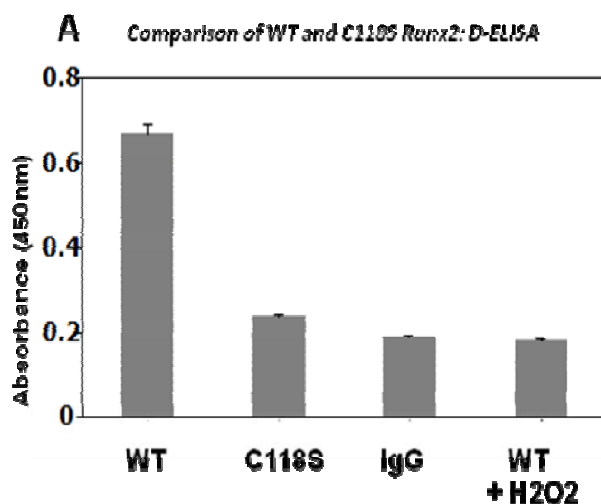


Figure 10A- RUNX2 DNA binding (measured by quantitative ELISA) is dependent on redox regulation of a specific cysteine residue in the RUNT domain (Cys118S). Comparison of wild type and RUNX2 C118S mutant DNA binding. Nuclear extracts from HEK293 cells transiently expressing WT.RUNX2 or RUNX2.C118S were compared by D-ELISA. IgG control antibody and wt.RUNX2 treated with H₂O₂ are shown for comparison. p values were between 0.0006 to 0.0007 indicating that C118S, IgG, and WT+H₂O₂ are significantly lower than WT.

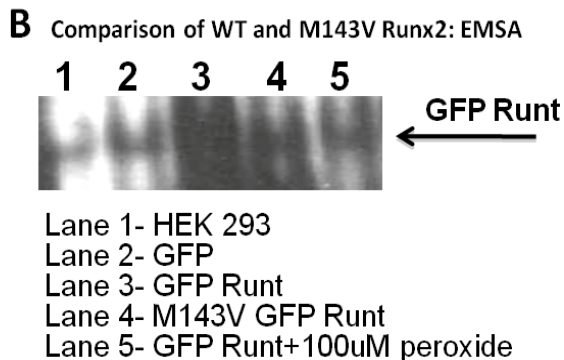


Figure 10B - RUNX2 DNA binding (measured by EMSA) is dependent on redox regulation of a specific methionine residue in the Runt domain (Met106V) . Comparison of WT.RUNX2 DNA binding domain RUNT.GFP and mutant RUNT.M143V.GFP by DNA binding EMSA.

To provide further evidence of redox regulation of RUNX2 activity, the HEK 293 cells transfected with empty GFP RUNT, WT GFP RUNT and the M106V GFP RUNT plasmids individually and were observed under fluorescence microscopy we saw that there was a cytoplasmic localization of the protein expressing the M106V mutation, which strengthens our hypothesis that this particular residue may affect the nuclear localization of RUNX2 in order to bind to the DNA (data not shown). Further, using the Cys-selective oxidant diamide³⁴, we observed a dose-dependent inhibition of endogenous RUNX2 DNA binding in nuclear extracts from EC, which was completely reversed by the addition of the reducing agent DTT at 24°C (**Figure 10C**). Treatment of nuclear extracts with H₂O₂, which oxidizes both Cysteine and Methionine residues⁴⁹, inhibited RUNX2 activity as well. However, this inhibition was not reversible when nuclear extracts were incubated at 24°C with DTT (**Figure 10D**)

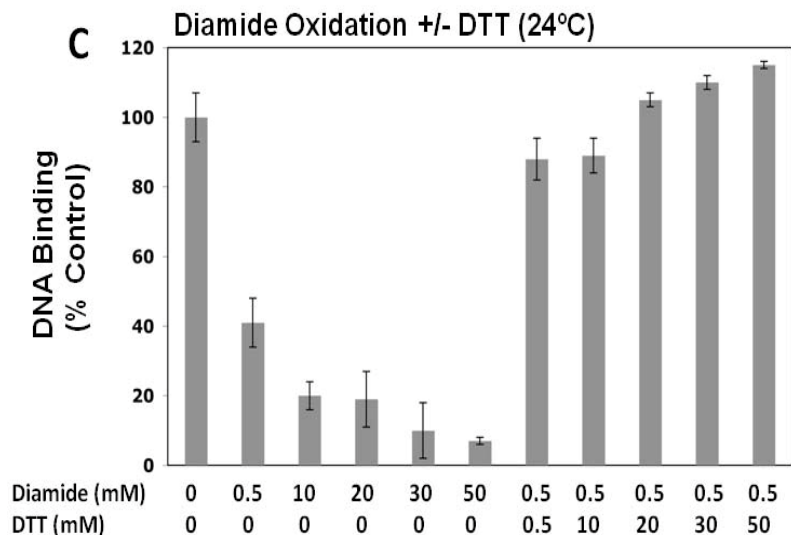


Figure 10C- RUNX2 DNA binding (measured by quantitative ELISA) is dependent on redox regulation: Diamide treatment oxidizes cysteine residues. Diamide oxidation (15min) was followed by reduction with DTT (15min) at 24°C. Diamide oxidation of cysteine residues inhibited RUNX2 activity, which was reversible by DTT treatment at 24°C. Treatment with diamide (0.5mM) was significantly different than control (no treatment) with $p < 0.0015$. Treatment with diamine (0.5mM) + DTT (20mM) was not significantly different than control ($p < 0.06$).

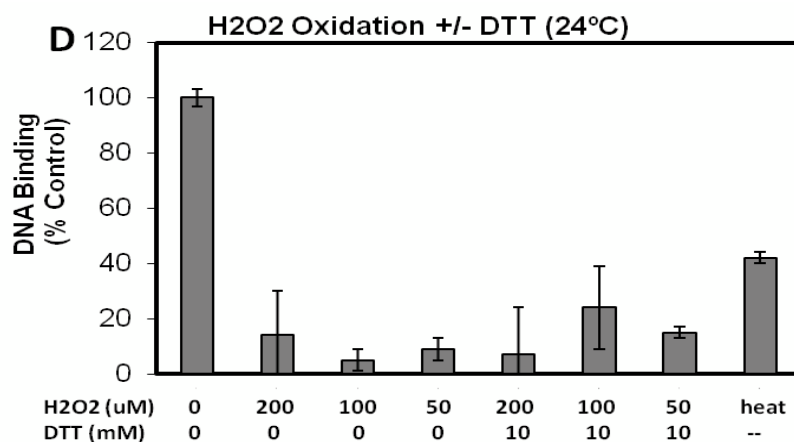


Figure 10D- RUNX2 DNA binding (measured by quantitative ELISA) is dependent on redox regulation: RUNX2 DNA-binding activity as a function of H₂O₂ oxidation. Nuclear extracts containing RUNX2 protein were treated with hydrogen peroxide (H₂O₂) for 15min alone, which oxidizes cysteine and methionine residues or with H₂O₂ followed by reducing agent (DTT) for 15min at 24°C. Heat (55°C) was used as a positive control to inhibit activity. Oxidation of cysteine and methionine residues inhibits RUNX2 activity, which was not reversible by DTT treatment at 24°C. Treatment with H₂O₂

(200uM) was significantly different from control ($p < 0.0032$) as was treatment with H₂O₂ (200uM) + DTT (10mM) ($p < 0.0038$).

Chapter 4

DISCUSSION

RUNX2 is a nuclear, DNA-binding factor that is sensitive to glucose levels and regulates cell cycle progression and expression of a variety of target genes in osteoblasts, tumor cells, and EC^{44,50}. Glucose increased RUNX2 phosphorylation and promoted changes in subnuclear localization of RUNX2. The focal nuclear distribution of RUNX2 in these cells may indicate an association with transcriptional complexes⁵¹. Chromatin immunoprecipitation assays supported a role in promoter occupancy after glucose treatment. RUNX2 can repress p21^{Cip1} transcriptional activity^{33,45}. We now find that p21^{Cip1} protein levels are regulated by Runx2. These results suggest that induction of p21^{Cip1} expression may restore sensitivity to growth factor inhibitors in proliferative EC and may be a good anti-angiogenic strategy. TGFβ treatment is known to activate p21^{Cip1} expression while RUNX2 expression represses p21^{Cip1} at the transcriptional level, which implies that the anti-proliferative activity of TGFβ in EC may depend on the presence of p21^{Cip1} and RUNX2. However, in tumor epithelial cells that have progressed to a mesenchymal phenotype, TGFβ exhibits transforming activity in the presence of RUNX2 (*preliminary data, unpublished data*). We have observed that tumor cells lacking p21^{Cip1} expression proliferate in response to TGFβ and increase their vimentin expression, a marker of the mesenchymal phenotype⁵², suggesting that p21^{Cip1} can act as a key regulator of the cellular response to TGFβ in EC and tumor cells. Thus, TGFβ and RUNX2 may exert their negative and positive effects, respectively, on cell proliferation through control of p21^{Cip1} levels. TGFβ promotes p21^{Cip1} expression through pSmad-phosphorylation and nuclear localization. Cellular pathways other than RUNX2 can

repress p21^{Cip1} expression by antagonizing TGF β signaling to restore cell growth including the PI3K/Akt signaling pathway, induction of c-myc, expression of the hepatitis C virus core protein, activation of the androgen receptor, and activation of interferon-gamma/Stat signaling⁵³. Since the RUNX proteins interact with Smad factors, and RUNX/Smad complexes colocalize within the nucleus, it is possible that regulation of RUNX/Smad binding may control the expression of p21^{Cip1} in some cells⁵⁴.

RUNX2 is predominantly localized to the nucleus in proliferating EC⁴⁴ and its activation by glucose through an autocrine IGF1 signaling pathway does not depend on new mRNA or protein synthesis⁴¹. IGF1 can alter the subnuclear localization of RUNX2 from a chromatin to a DNA-associated fraction⁵⁵. We took advantage of chemical nocodazole blockade to trap cells in the G2/M phases of the cell cycle and maximize RUNX2 DNA binding^{41,44}. RUNX2 activity in nuclear extracts (**Figure 11**) from nocodazole blocked cells was very sensitive to low doses of diamide (a cysteine selective reagent) or H₂O₂, which oxidizes both cysteine and methionine residues in proteins⁴⁹. Previous reports had shown that diamide treatment of recombinant or ectopically expressed RUNX1 or RUNX2 DNA-binding domain (RUNT homology domain), which is conserved in all three RUNX proteins, could inhibit DNA binding activity^{34,35}. This inhibition could be partially or fully reversed by treatment with the reducing agents DTT, GSH, Trx, or Ref1. We observed similar results with wild-type (full-length) endogenous RUNX2 DNA binding using DTT as a reducing agent to reverse diamide-induced oxidation and restore RUNX2 activity. However, H₂O₂-induced oxidation of RUNX2 DNA binding could not be reversed by treatment with DTT at any dose. We suspected that one reason why DTT might not be able to reverse oxidative damage in response to

H_2O_2 was because of possible methionine oxidation, which is not reversible by DTT alone⁵⁶. The RUNX2 DNA-binding domain contains a critical methionine residue, which is essential for binding of RUNX2 cofactor CBF β to increase DNA-binding³⁹. Therefore, oxidation of a specific RUNT domain methionine residue could be responsible for reducing RUNX2 DNA binding. Consistent with this observation is the result showing that a Met-Val mutation in the Cbf β -binding domain of RUNX2 could inhibit DNA binding.

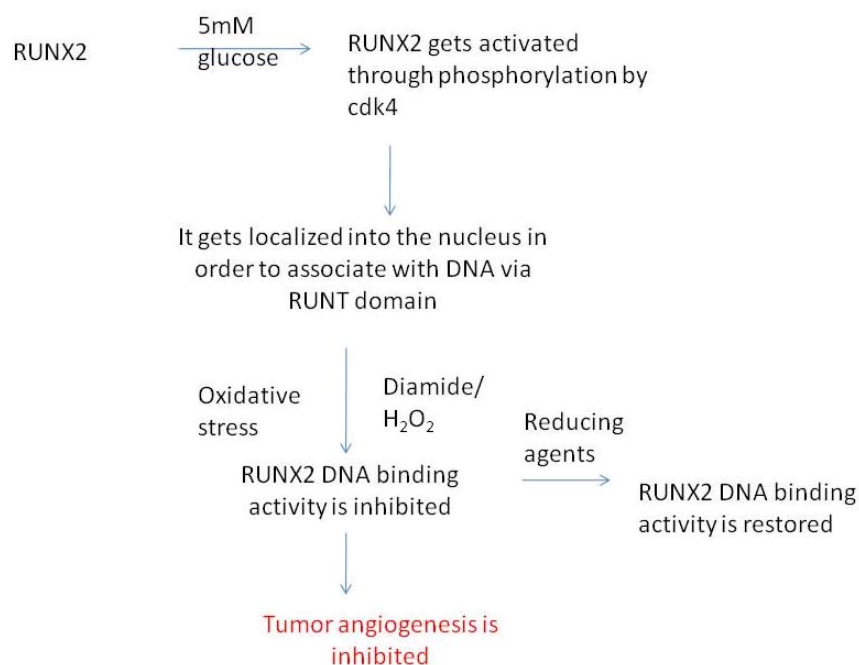


Figure 11- Overview on how the glucose levels and oxidative stress of the cells regulate RUNX2 transcriptional factor activity in tumor angiogenesis.

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