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(54) **Title:** THERAPEUTIC USES OF MIRNAS/COMPOUNDS THAT ACTIVATE TUMOR SUPPRESSOR GENES/MIRNAS

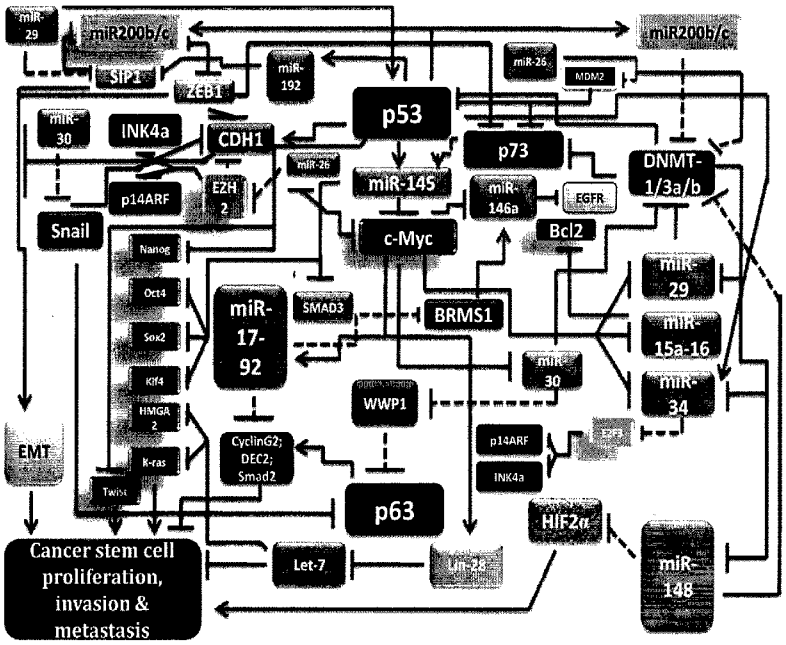

Figure 6

(57) **Abstract:** The invention illustrates how the TA-p73 and TA-p63 could function as negative regulators of invasion, metastasis, and cancer stem cells proliferation. In particular, p53 and TA-p73/ p63 appear to up regulate the expression of tumor suppressor miRNA, tumor suppressor genes and metastasis suppressors. Futher, suppressing of c-myc expression can increase the expression of tumor suppressor miRNAs/genes. Identifying small molecule compounds that simultaneously suppress oncogenes and activate tumor suppressor miRNAs/genes will aid cancer therapy.

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1. DESCRIPTION OF THE INVENTION

1. Introduction

MiRNAs (miRNAs/miRs) are small non-coding RNAs. They bind to 3'UTR of mRNAs in a sequence specific manner. They either repress translation or promote degradation of mRNAs. p53 functions as a transcription factor and it controls the expression of a number of genes to promote tumor suppression and genome integrity. It is the most frequently mutated gene in human cancer.

miRNAs have been shown to function downstream of the tumor suppressor p53. MiRNAs, such as miR-34, miR-192/215, miR-107, miR-145, are known transcriptional targets of p53. They could also be transactivated by p53 homologues, such as p73 and p63, as they are known to transactivate p53's transcriptional targets. In addition, a number of tumor suppressor miRNAs that have recently been discovered appear to play a key role in controlling tumorigenesis. However, whether they are regulated by the tumor suppressor p53/p73/p63 is not known.

Unlike p53, p73 gene is not frequently mutated in human cancer. However, TA-p73 promoter is hypermethylated in a number of human cancers [1]. A number of studies suggest that it responds to DNA damage and maintains genome integrity, suggesting that it could function as a tumor suppressor. However, how it functions as a tumor suppressor remains elusive. I have proposed previously a tumor suppressor *pathway-E2F-l/2-TA-p73/p63-p57kip2/14-3-3o/JunB-BRCA/INK4/ARF-io* explain how it functions as a tumor suppressor [1]. Remarkably, six components of-E2Fl [2], p73 [3], p63 [4, 5], p57kip2 [6], 14-3-3σ [7, 8], and INK4a/b [9]-the proposed tumor suppressor pathway appear to be either poorly expressed or hypermethylated (silenced) in transitional cell carcinoma of the bladder, suggesting the conserved nature of this tumor suppressor pathway. Furthermore, a) TSC1 (Tuberous Sclerosis 1), a putative/proven transcriptional target of p73/Fox03a, has been shown to be mutated (14. 5%) in bladder cancer [10]; b) LZTS-l/FEZl(Fasciculation and elongation protein zeta 1), a putative transcriptional target of p73/p63, protein expression is decreased in 37% of primary transitional bladder carcinoma[l 1]; c) PTEN, a transcriptional target of p53 (possibly, p73/p63), expression has shown to be decreased or absent in primary bladder cancer patients (53%) and in advanced bladder cancer patients (94%) [12]. d) AML-2/Runx-3, a putative target of p63, has shown to be hypermethylated (73%) in primary bladder cancer [13]. e) miR-145/143/200/101/29/34, a proven/putative transcriptional target of p53/p73/p63, expression is reduced or silenced in bladder cancer [14, 15]. f) miR-let-7, a putative/proven transcriptional target of E2F-l/p53/p73/p63, appears to target proto-oncogenes—PI3-K and Ha-ras—that play a key role in bladder cancer [16-18]. Based on these data, here I propose a tumor suppressor *V2Xhway--E2F-l/2-TAy73/p63y57kip2/LZTSl/TSCl/PTENl/RBs/14-3-3a/A^ L2/INK4-miR 145/143/let-7/101/29/34-XhaX* could play a critical role in the inhibition of transitional bladder

carcinoma. Additionally, this pathway could play a role in the inhibition of lung adenocarcinoma development, as some of these genes also appear to be mutated in lung cancers.

Further, in support of the notion that p73 functions as a tumor suppressor, it has recently been shown that TA-p73 knockout mice are prone to lung adenocarcinoma, suggesting that it could indeed function as a tumor suppressor gene [19]. **E2F-1/2** is a transcriptional activator of TA-p73. Remarkably, *E2Fr $^{-/-}$/E2Fr $^{-/-}$-E2F2$^{+/-}$-/E2Fr $^{-/+}$E2F2$^{-/-}$* mice are also prone to highly invasive adenocarcinomas of the lung (non-small cell lung carcinomas) [1]. Together, these data suggest that **E2F1/2** could mediate its tumor suppressor function through its transcriptional target TA-p73 [1; 19]. Further, it has been shown that increased expression of the tumor suppressor ARF co-localizes with the tumor suppressor BRCAl and thereby increases its expression [1; 20]. This data suggests that both ARF and BRCAl could play a role in p73-dependent tumor suppression [20]. Interestingly, **E2F1$^{-/-}$**, p73$^{-/-}$, and BRCA2$^{-/-}$ mice have been shown to develop lymphomas [1, 19, 21-23], suggesting that they could co-operate with each other in the inhibition of lymphoma development.

Unlike TA-p73, TA-p63 promoter is not frequently hypermethylated. However, TA-p63 expression is down regulated in bladder cancer [4-5]. I have proposed a number of years ago that p63 could increase the expression of the tumor suppressor AML-l/Runxl, which in turn could increase the expression of the tumor suppressor pl4ARF/INK4a (Boominathan, unpublished). Increased expression of pl4ARF/INK4a has been shown to promote senescence—a tumor suppressor mechanism--in a number of cell types, indicating that the *p63-AML-l-pl4ARF/INK4a* pathway could promote tumor suppression in a cell context dependent manner (Boominathan, unpublished). In addition, p63/p73, by transactivating the chronic myeloid leukemia (CML)/B-cell tumor suppressor JunB, it could increase the expression of the tumor suppressor INK4a, as JunB has been shown to increase the expression of INK4a [1; 24-27]. Evidently, p73 has shown to be hypermethylated in acute myeloid leukemia (AML), acute T-cell lymphoblastic leukemia, and Burkitt lymphoma [1, 28], whereas p63 expression appears to be mutated (11.8%) in CML [29]. Therefore, p63/p73, by increasing the AML-l/JunB-ARF/Ink4a pathway, it could function as a tumor suppressor in myeloid leukemia. To determine whether p63/p73 synergizes with JunB/AML-1 in tumor suppression, one can cross *p63 $^{+/-}$;p73$^{+/-}$ mice* with ***JunB***$^{+/-}$; AML-l/2 $^{+/-}$ mice.

Further, KAI1/CD82, a cell surface glycoprotein, has been shown to inhibit EGFR signaling, tumorigenesis and metastasis. It also appears to promote senescence. p53 has been shown to synergize with both AP-2 and JunB in the induction of KAI1/CD82 expression [30]. Interestingly, both p73 and p63 have been shown to increase the expression of JunB and AP-2, suggesting that they could also induce the expression of KAI1/CD82 [1; 31-32]. Furthermore, AP-2a, a transcriptional target of p53, has been shown to induce the metastasis suppressor KiSS [33], suggesting that p53 may increase the expression of KiSS through AP-2a. Interestingly, KiSS expression has shown to be lost in metastatic/invasive bladder cancers [34], suggesting that the *p53lp73/p63-JunB/AP-2/KAIl-KiSS* pathway could inhibit the invasive/metastatic bladder cancer development.

c-Myc has shown to be over expressed/mutated/translocated in a number of human cancers. It appears to play a key role in the development of B-cell lymphoma/leukemia/myeloid leukemia/lung adenocaricnoma. It also appears to play a role in metastasis, cancer stem cells (CSCs) proliferation, and reprogramming of differentiated cells

into pluripotent stem cells. Interestingly, it has been shown to increase a number of oncogenic miRNAs, including miR-17-92 cluster and miR221/222 [35]. Overexpression of c-myc appears to (i) repress the expression of a number of key tumor suppressor miRNAs (discussed in detail later); and (ii) suppress the expression of Angpt-2 (target scan score: 91) through its transcriptional target miRNA-221/22 and thereby increase insulin resistance.

In this patent application, I will be discussing how the tumor suppressors p53, p73, and p63 regulate: a] tumor progression, invasion, and metastasis through their target miRNAs; b] c-myc through their target miRNAs/genes; c] tumor suppressor miRNAs network; and d] Epithelial to mesenchymal transition [EMT], migration, and CSCs proliferation; and how identifying compounds that suppress the expression of c-myc and induce the expression of tumor suppressor genes/miRNAs will be useful in a number of disease conditions, including cancer, diabetes, and hypertension.

2. The role of the "guardians of the genome and miRNAs" during tumor progression, invasion, and metastasis

p53, TA-p73 and TA-p63 have been shown to play an essential role in control of tumorigenesis, tumor progression, invasion, and metastasis. However, how they function as metastasis/invasion suppressors is just beginning to be understood. It has recently been shown that p53-induced HDM2 promotes degradation of both Slug/Snai2 and Snail/Snail, the negative regulators of the metastasis suppressor E-cadherin [36-37]. Interestingly, mutant p53 expressing non-small lung carcinoma cells have lower levels of HDM2 and higher levels of Slug. This results in increased invasiveness and metastasis. This data suggests that p53, by promoting the degradation of both Slug and Snail, it could increase the expression of E-cadherin, and thereby inhibit metastasis [Fig. 1]. Of interest, Snail 1 has been shown to impair dentric cell function and thereby promotes induction of immune suppression (suppression of tumor-specific tumor-infiltrating lymphocytes) [38]. This data suggests that p53-induced down regulation of Snail may suppress both metastasis and immune suppression. Furthermore, Snail appears to inhibit the expression of the metastasis suppressors, such as Raf kinase inhibitory protein (RKIP)/PEBP1 and Tissue metalloproteinase inhibitor (TIMP3) [39-40]. RKIP has been shown to inhibit Raf-l(a downstream target of ras), MEK1, c-Myc, HGMA2, and lin-28 proteins and increase the tumor suppressor miRNA, let-7a/g processing [41]. This data suggests that p53, by degrading Snail, it could increase the expression of RKIP and let-7 [Fig. 1]. Remarkably, this data suggests a possibility that RKIP/let-7, by negatively regulating the transcriptional activators of HDM2 (the Ha-Ras-Raf-l-MEK-ERK signal transduction cascade), it could increase the expression of p53 [41-42; 16; Boominathan, unpublished]. Interestingly, Trichostatin A, a histone deacetylase inhibitor and an inducer of E2F-l/TA-p73/p63 [43-44], has been shown to induce RKIP expression, suggesting that Trichostatin-A and its derivatives could induce the ***E2F-1-TA-p73/p63/p53-Smil-RnP-c-myc-lin-28-let-7a/g-HMGA2-ras(Ha/N/K)tu^*** or/metastasis suppressor pathway.

Further, a number of studies have shown that p53-miRNAs~such as miR-34, miR-23, miR-107, and miR-145--play a key role in control of tumor progression, angiogenesis, and metastasis. First, p53-miR-34a has been shown to inhibit the expression of c-Met, a known promoter of migration and invasion of cancer cells [45]. Second, p53-miR-23 suppresses the expression of both c-Met and Urokinase-type plasminogen activator (an invasion and a migration

promoter) [46]. Third, p53-miR-107 has recently been shown to suppress HIF-Iβ expression [47]. This in turn results in inhibition of tumor angiogenesis. Fourth, p53-miR-145 has shown to be poorly expressed in a number of cancers, including those of the lung, b-cell, liver, bladder, breast, and prostate. miR-145 suppresses Mucin expression and thereby inhibits invasion and lung metastasis in an experimental metastasis animal model [48]. In addition, it has been shown to suppress the expression of a) BCL2/adenovirus E1B protein-interacting protein-3, a transcriptional repressor of apoptosis-inducing factor and a promoter of prostate cancer progression [49]; and b) FSCNl (actin-binding protein, Fascin homologue 1), a promoter of bladder cancer and esophageal squamous cell carcinoma progression [50-51]. Further, it has been shown that suppression of p63 in squamous cell lines resulted in up regulation of genes that promote mesenchymal morphology, motility, and invasion [52]. In support of this data, I have proposed previously that TA-p73/p63/p53, by inhibiting the expression of the negative regulators of E-cadherin~such as ZEB1/2, Snail 1/2, Twist & Hey-1—through its target miRNAs, it could suppress Epithelial to Mesenchymal transition [EMT], invasion and metastasis [53; Boominathan, unpublished]. Interestingly, ZEB1 has been shown to function as a negative regulator of the tumor suppressors TA-p73 and E-cadherin expression [54], suggesting that p53-miRs, by suppressing the ZEB1 expression, it could induce TA-p73 and E-cadherin. Furthermore, a. TA-p73 has been shown to suppress notch signaling and its downstream target Hey-1, suggesting that it may increase E-cadherin, and thereby suppress the EMT, and metastasis .(TA-p73 - INotch-1/N1^{IC_D}→ Hey-l/Slug/Snaill —| E-cadherin) [55-56].

AN-p63~that lacks the NH2-terminus of full length TA-p63~has been touted to function as an oncogene. It has been shown to inhibit the functions of full-length p53/p63/p73. In addition, a number of studies provide correlative evidence for the conjecture that it may promote EMT, invasion and metastasis:

(a) ANp63-¾-STAT3 → Twist -┤E-cadherin —| EMT; TA-ρ63γ—┤EGFR—► STAT3 [57-59]
(b) ΔNρ63—► Brachury-* Slug —|E-cadherin —| EMT, Invasion & Migration [60-61]
(c) ANp63→HIF-la →VEGF— [Snail 1—| E-cadherin; TA-p63-| HIF-la [62-65]
(d) ΔNρ63—► HIF-la → Twist —|INK4a/ARF/p53 [66]
(e) ΔNρ63— |GSK3β — ISnaill —|E-cadherin [67]

Paradoxically, ΔNρ63 has shown to be lost in advanced invasive urothelial (bladder) carcinomas [68; 4-5], suggesting a possibility that the presence of ΔNρ63 may not support the invasion program in urothelial carcinomas. However, this data may prompt us to ask why it looses its expression if it can favor invasion and metastasis. Interestingly, ΔNρ63 has been shown to down regulate N-cadherin (promotes mesenchymal phenotype), matrix-metalloproteinase-9 [69], and ERK activity/expression (ΔNρ63 —┤ERK1/2 —┤Fra-1 →ZEB1/2 —┤E-cadherin), connoting that it could function as an invasion suppressor [69; Boominathan, unpublished]. Furthermore, it has been shown to induce inhibitor of differentiation-3 (ID-3) (ΔNρ63 —► ID-3 —| matrix-metalloproteinase-2 activity) [70] and vitamin D receptor (induces E-cadherin) expression [71-75], suggesting that ΔNρ63 could inhibit invasion and metastasis. In support of these data, it has previously been shown that: a) the EMT/metastasis promoter protein Snail 1 down regulates ΔNρ63 and thereby promotes invasion of human squamous cell carcinoma (SCC) in ***invitro*** [76]; b) the myeloid/lung tumor suppressor CEBP-a induces the expression of ΔNρ63 [76]; c) the metastasis suppressor protein Bone morphogenetic protein-4 induces ΔNρ63 expression [77]; d)

GATA3, a transcriptional target of ΔNp63/p63, induces the metastasis suppressors DLC1 and PAEP and thereby inhibits EMT, breast cancer dissemination and lung metastasis [78-81]; e) IKKa, a transcriptional target of ΔNp63/TAp63, inhibits SCC [82-85]; and f) p57Kip2, a transcriptional target of ΔNp63/p73, functions as a tumor suppressor in a number of human cancers [86] [Boominathan, submission in progress]. Of note, a weak transcriptional activator (ΔNp63) can become a strong transactivator when it is highly expressed, while a strong transcriptional activator (TA-p63) can become a weak transactivator when it is poorly expressed [Expression pattern in most of the tissues: AN-p63>TA-p63(e.g., keratinocytes: ΔNp63 (100): TA-p63/p53 (1) ratio; Transcriptional activator efficiency: TA-p63>ΔN-p63]. Nevertheless, a number of TA-p63/AN-p63-specific transcriptional targets have recently been identified. Evidently, ΔNp63 has been shown to transactivate cell adhesion molecules, such as BPAG1, EVL, PERP, ITA3-6, β4 INTG, and Laminin, suggesting that reduced ΔNp63 expression may decrease cell adhesion and increase migration, invasion, and metastasis [87]. ΔNp63 has also been shown to increase/transactivate the following tumor/metastasis suppressor genes: (a) p62DOK (lung cancer/leukemia tumor suppressor; 3.5 fold); (b) JunB (CML tumor suppressor; 3.7 fold); (c) PP2A-Aβ (mutations/deletions found in lung/colon/breast cancer; 9 fold); (d) APC (colon cancer tumor suppressor; 4.3 fold); (e) AML/Runxl (acute myeloid leukemia tumor suppressor; 5.2 fold); (f) HUGL (colon cancer tumor suppressor; 3.6 fold); (g) RASSF4 (hypermethylated in lung cancer; 2.5 fold); (h) AML-2/Runx3 (gastric/lung cancer tumor suppressor (ΔNp63 -*-Ets-l → AML2 → Claudinl); expression is absent in small cell lung carcinoma (50%)/adenocarcinoma (50%)/squamous cell carcinoma(33.3%); hypermethylated in non-small cell lung carcinoma (25%)/adenocarcinoma (36.1%)); and (i) Claudinl (a tight junction protein and a transcriptional target of ΔNp63; poorly expressed in metastatic breast cancers/tumor-initiating stem-cells; inhibits progression, motility and invasivity of lung adenocarcinoma) [88-96]. These data suggest that ΔNp63 could function as an invasion or a metastasis/tumor suppressor by increasing the expression of a number of tumor/metastasis suppressor genes.

Incongruously, TA-p63 isoforms, but not ΔNp63, have shown to be over expressed in primary mediastinal large (diffuse) B-cell lymphoma and in high-grade follicular lymphomas [97-98]. In addition, TA-p63 expression appears to be frequently reactivated in human squamous cell carcinoma. Reactivation of TA-p63a in chemically-induced skin carcinogenesis model accelerates tumor development and promotes EMT, spindle cell carcinomas, and lung metastasis [99], suggesting that its function may be altered in tumor microenvironment in such a way that it plays a pro-proliferative role in a cell type dependent manner. Furthermore, TA-p63 has been shown to increase the Notch receptor ligand Jagged-1/2 (and its downstream target, Hes-1); and CDH3, suggesting that it could regulate the EMT and motility in a cell context dependent manner [100-102]. Interestingly, TA-p63 has recently been shown to transactivate the E-cadherin suppressor ZEB1 in response to ischemic stress [103]. However, whether it will increase or decrease the ZEB1 expression in conditions that favor tumor progression remains ambiguous.

Considering TA-p63/p73 could induce apoptosis in a number of cell types suggests that its function is altered through post-translational modifications (or through interaction with oncoproteins) during tumor development or progression (so that it could support tumorigenesis or tumor growth). In addition, TA-p63/p73: (i) is induced in response to a number of DNA damaging agents, suggesting that it could play a role in protecting the genome integrity/stability;

and (ii) could function as a tumor suppressor by transactivating genes, such as JunB and AML/AML1/2 (other target genes/miRNAs discussed elsewhere in this patent application), in a cell context dependent manner.

Further, ΔNp63 is predominantly expressed in most of the epithelial tissues compared to TA-p63, suggesting that it may function as a *guardian of epithelial integrity.* Of importance, most of the human cancers (carcinomas) are of epithelial origin. At this point, it may be germane to discuss the role of p53 and ΔNp63 in Notch signaling. Notch- 1 functions as a tumor suppressor in skin/keratinocytes/SCC [104], while it functions as a proto-oncogene in lymphoid cells/acute T-cell lymphoblastic leukemia/lymphoma [105]. Intriguingly, p53 increases the transcription of Notch- 1, while it decreases its expression at the post-transcriptional level through its target miR-34 [106-107]. On the other hand, ΔNp63 suppresses its expression(and its target gene Hes-l-the negative regulator of the tumor suppressor PTEN) by directly binding to its promoter [108-109], suggesting that ΔNp63 could play a metastasis/tumor suppressor role in cell context dependent manner (e.g. skin, lymphoid cells/acute T-cell lymphoblastic leukemia/AML/lymphoma/lung adenocarcinoma). That is, ΔNp63, by inhibiting notch- 1 signaling cascade [ΔNp63 -blotch- 1 → Slug/Snaill/Hesl/c-myc —I E-cadherin/PTEN— → EMT, invasion & metastasis], it can increase the expression of the invasion/tumor suppressor E-cadherin/PTEN and thereby inhibit migration, invasion, and metastasis [108-109; 55]. Based on these data, I would like to propose that ΔNp*63 could function as an invasion or a metastasis/tumor suppressor* in a cbll context dependent manner. Nonetheless, one can confirm the hypothesis proposed here by a) generating isoform/tissue specific (conditional) knockouts; and b) crossing ΔNp$63^{+/-}$ mice with oncogenic/tumor/metastasis prone mouse models.

3. The p53/TA-p73/p63 → TRIM32 —1 c-myc —1 let-7 tumor suppressor pathway:

It has recently been shown that the E3 Ubiquitin ligase TRIM32 binds to c-Myc and thereby targets it for degradation [110-111]. In addition, TRIM32 has been shown to bind to Argonaute l(a component of RNA-induced silencing complex), and thereby increases the efficiency of processing of miRNAs. It appears to enhance the processing of a group of miRNAs, including the tumor suppressor miRNAs, let-7, and miR-134 [112-113]. Together, TRIM32, by promoting c-myc for degradation, it increases the expression of let-7 and miR-134, and thereby suppresses the proliferation and self-renewal of stem cell lineages [110-113].

p63 has been proposed to play a role in regulating asymmetric cell division [114]. However, how it regulates asymmetric cell division remains abstruse. Asymmetric cell division appears to be critical for stem cell self-renewal and differentiation. Deregulation of asymmetric cell division has been shown to result in cancer [115-116]. Interestingly, TRIM32 has been shown to localize asymmetrically in one of the dividing progenitor neural cells. The progenitor cell that has higher levels of TRIM32 undergoes differentiation, while the other progenitor cell that has lower levels of TRIM32 retains its ability to undergo self-renewal [110-111]. Based on this interesting observation, one may be tempted to propose that p63, by increasing the expression of TRIM32, it could regulate asymmetric cell division and differentiation of stem cells.

Further, I have recently proposed that TA-p73, by increasing the expression of miRNA, let-7, it could function as a tumor suppressor in lung cancer [117]. To find out whether let-7 cluster promoters contain p53/p63 responsive elements (p53/p63-REs), I have analyzed the let-7 cluster promoters using the TRASFAC bioinformatics tool. This analysis suggests that Let-7c contains three perfect p53-RE half-sites (-1980 to -1989: **aaacatgctt;** -3472 to -3481: **aaacttgttt;** -3615 to -3624: **gagcatgttc)** and three nearly perfect p53REs **(-1348 to -1366: (aatcatgcca)t(tatcgtgcca); -1594 to -1623 (aaacgtgtat)g(tggctggctt); -2583 to -2603: (tatcjjtgttt)t(cttctcgatc).** Other let-7 cluster miRNAs also appear to contain several p53-REs, suggesting that let-7 cluster miRNAs could be transcriptional targets of p73/p63/p53 [117]. In support of this notion, it has been shown that activation of p53 results in increased expression of let-7c (2.7fold), let-7e (2.1 fold), and let-7a (1.9 fold) [118].

To find out whether TA-p73 could also increase let-7 expression through TRIM32, I have analyzed the TRIM32 promoter sequence for potential p73/p63 binding sites. Remarkably, I have found a number of p63/p73 binding sites in human/mouse TRIM32 promoter [112], suggesting that it could be a transcriptional target of TA-p73/p63. Evidently, a microarray study suggests that TRIM32 expression is increased in response to TA-p73 expression [119]. This data further strengthens the notion that the TA-p73/p63, by increasing the expression of TRIM32, it could decrease the expression of c-myc in a cell context dependent manner. Decreased expression of c-myc may result in increased processing of the tumor suppressor let-7 [113]. Together, TA-p73/p63, by decreasing the expression of c-myc through TRIM32, it could increase the expression of let-7 and thereby function as a tumor suppressor and a key player in asymmetric cell division of stem cells [Fig.2].

The let-7 miRNA cluster appears to be highly expressed in lungs, and it has been shown to function as a tumosuppressor in lung cancer [120-121]. In particular, its expression appears to be down regulated in non-small cell lung cancer [120-122]. A recent microarray data suggests that let-7 expression *down regulates the expression of proliferation/transcription factor/replication/cell cycle/metastasis/oncogenic kinase mRNAs, such as *CCNA2, CDC34, ASK, ARKA(a suppressor ofp53), ARKAB, E2F5-8, PLAGL1 and 2, Dicer1, GMNN, NRAS (possibly K-ras/Ha-ras), HMGA2, Lin28B, CDC2, CCNB1, CCNE2, CCNF, CCNJ, SKP2, CKS1B, CDCAl-3;5,7-8; RRM1-2, CDC6, CDC45L, CDT1, ORC1L and ORC6L, MCM2/3/4/5/6/7/8/10, RFC2/3/4/5, MAD2L1, and CDC23* [Fig.2] [123]. This data suggests that TA-p73, by increasing the expression of let-7, it could decrease the expression of these proteins and thereby function as a tumor suppressor in lung cancer [Fig.2].

In addition, TA-p73, by regulating the expression of BUB1, BUB1B/BUBR1 [spindle assembly proteins], and CDC20 through let-7 [123], it could inhibit the function of anaphase promoting complex and thereby promote proper alignment of chromosomes during mitosis/meiosis to maintain genomic integrity. Of interest, BUBRl is also targeted by p53/p73/p63-miRs, such as miR-34c-3p and miR-130a/b* (Target scan; *-Putative target) [113]. Furthermore, BUBRl has been shown to function as a negative regulator of INK4a expression [124], suggesting that p73/p63/p53-miRs, by regulating BUBRl expression, it could regulate INK4a. Intriguingly, crossing BUBRl hypomorphic mice with INK4a$^{-/-}$ mice predisposes them to lung adenocarcinoma, suggesting that both BUBRl and INK4a co-operate in the inhibition of adenocarcinoma development [35]. Remarkably, TA-p73$^{-/-}$, E2F-1$^{-/-}$ and p63$^{+/-}$ mice are also prone to adenocarcinoma of the lung, suggesting a lung specific tumor suppressor network

involving E2F1, TA-p73, p63, let-7, BUBR1 and INK4a proteins[l, 19]. This notion is in concordance with the tumor suppressor pathway-E2F 1-TA-p73-JunB-INK4a/BRCA-that I have previously proposed [1]. Of interest, INK4a promoter is inactivated by hypermethylation in "metastatic lung cancers [125-126]. Further, a. p63/p73's putative target gene Dicerl has been proposed to function as a metastasis suppressor [113] [Fig.l]; and b. p73/p63's target gene BRCAl/2 has been shown to promote kinetochore localization of BUB1 and BUBR1 and thereby increases their mitotic checkpoint function [1; 127]. Therefore, it would be interesting to cross TA-p73$^{+/-}$/TA-p63$^{+/-}$/p63$^{+/-}$/p73$^{+/-}$/p73$^{-/-}$ mice with *E2Fl*$^{+/-}$, *JunB*$^{+/-}$, *INK4a*$^{+/-}$, *INK4b*$^{+/-}$, PTEN$^{+/-}$, *Dicer*$^{+/-}$, *BUBRl*$^{+/-}$, *TSC*$^{+/-}$ *and BRCA-l/2*$^{+/-}$ mice to determine whether they synergize/cooperate in tumor suppression. Together, TA-p73, by promoting genomic integrity/stability through let-7, it could function as a tumor suppressor gene.

Further, let-7 promoter contains PAX-5 responsive elements, suggesting a possibility that PAX5 could regulate its expression. Pax5 has recently been shown to be mutated [31%] in acute lymphoblastic leukemia [128], indicating that it could function as a tumor suppressor gene. Of interest, Pax5 promoter contains a number of p63-REs [-149 to -173: **(ggccgcgacc)cccaa(gcgcatgtct);** -807 to -829: **(gaacagggag)ggg(aggcttgagt);** -2494 to -2515: **(gcacatgtat)ct(gtgcttgcaa)**; -2526 to -2548: **(tctctggcgg)tgt(ctgcgtgtgt);** -4024 to -4045: **(gcgctggaaa)ct(agtcgtggaa)** ; -4052 to -4073: **(acacttgacg)tc(taccatgtgt);** -5865 to -5895: **(gtacatgagt)(ctacgtgcaa)a(ttgcatgaga);** -6473 to -6495: **(gagcatgacc)cca(ccccttgcca):** -8921 to -8940: **(gggcatggtg)(gctcatgcca)],** suggesting that it could be a direct transcriptional target of TA-p63. In support of this notion, p63 has been shown to bind to the Pax5 promoter [129], suggesting that it may increase let-7 expression through Pax5, and thereby it could function as a tumor suppressor gene. Interestingly, let-7 may also increase p63 protein level by suppressing the expression of its negative regulator RNPC1 (Context percentile score: 61-63), and thereby share a positive feedback loop (Target scan) [130] [Fig.2]. The Pax-5 promoter also contains activator protein-1 responsive elements [-6567 to -6573: **tgactca;** -8017 to -8923: **tgaatca],** suggesting that TA-p73 could also increase the expression of Pax-5 through its ability to increase JunB/activator protein-1 responsive element containing promoters [1; 131].

As discussed, ΔN-p63 binds to the tumor suppressor p53/TA-p73/p63 protein and thereby inhibits its functions. miR-203 has recently been shown to negatively regulate the expression of ΔN-p63 [132], suggesting that it could increase the functions of p53/TA-p73/p63. Interestingly, miR-203 promoter also contains Pax5 responsive elements, suggesting that TA-p63 could down regulate the expression of ΔN-p63 by regulating the Pax5 ⟶ miR-203 pathway. In addition, miR-203 could be a direct transcriptional target of TA-p73/p53/p63 [53], as it appears to contain a number of p53/p63-REs [-171 to -197: **(cggctgggat)cccccag(cgccaggcga);** -200 to -226: **(cagcgaggac)gcggcg(gggctgggct);** -406 to -442: **(gagcaggtcc)ccg(ggccgtggag)gatc(agtcgcggga);** -558 to -600: **(gcccgagcac)ccccggccc(agacgagacg)gttc(gggcgtggcc);** -926 to -949: **(gagcgaggct)cag(gcccttgctg);** -2552 to -2582: **(agacaggctt)ggagc(gttcgtgtcc)tg(cgccgcgttg);** -4566 to-4587: **(ggacgtgact)t(ggccaagtgg)]** [53]. This data further strengthens the notion that p53/TA-p63/p73 could down regulate the expression of ΔN-p63 by inducing the expression of miR-203 in a cell context dependent manner (e.g. DNA damage response). Further, miR-203 appears to suppress the expression of Snail 1*/Slug* (N-cadherin*, TGFp2*, Src*, VEGFA*), which has been shown to inhibit the expression of the metastatic suppressor E-cadherin/PTEN [53; Target scan; *- predicted]. Down regulation of E-cadherin has been shown to promote

EMT, invasion, and metastasis. Together, TA-p73/p53/p63-dependent miR-203 expression may result in down regulation of Snail 1/Slug and increased expression of E-cadherin *[TA-p73/p53/p63-JunB-miR-203-Snaill/Slug/E-cadherin/PTEN].* This in turn will result in inhibition of metastasis. Correspondingly, both TA-p73 and TA-p63 have recently been predicted to function as negative regulators of EMT and metastasis by suppressing the expression of ZEB1/2 [53-54]. Interestingly, knockdown of ZEB1 results in increased expression of the tumor suppressor INK4B, which in turn promotes senescence. This data suggests that TA-p73 and TA-p63, by suppressing ZEB1 expression, they could increase the expression of CDKN1 A & INK4B and thereby promote senescence [53]. Of interest, miR-203 is predicted to target the expression of the negative regulator of INK4a/ARF, BMI *[TA-p73/p53/p63-JunB-miR-203-BMI- }NK4O/ARF]* [Target scan].

Further, let-7 dependent down regulation of S-phase kinase associated kinase (Skp-2) may: a) promote senescence by inducing the expression of Atf-4, CDKN1A, and CDKNlB/p27Kipl [123; 133]; and b] inhibit the c-Myc-Skp2-Mizl-p300-RhoA cascade and thereby inhibit cell migration, invasion, and metastasis [134] [Fig.4]. Interestingly, p27Kipl has been shown to inhibit RhoA activity, suggesting that the *let-7-Skp-2-p27Kipl* pathway may inhibit cell cycle progression by inhibiting cyclin-CDKs. Remarkably, inhibition of the RhoA-mDIA (Mammalian Diaphanous 1) pathway up regulates the expression of CDKN1-A and -B, while inhibition of the RhoA-ROCK pathway induces the expression of INK4-A, -B, -C, and -D [135] [Fig.4].

CKS1B, a key regulator of Skp-2, also appears to be negatively regulated by let-7 (miR-145/194*{*-a putative transcriptional target of p53} Target scan) [Fig.l]. Let-7-dependent down regulation of Skp2 and CKS1B (transcriptional targets of c-myc) may result in up regulation of tumor suppressors such as p53, p27Kipl, CDKN1A, p57kip2, and pl30 [136-137]. Increased expression of p27Kipl may impair Stathmin (a microtubule destabilizing protein) activity and thereby inhibit sarcoma progression, mesenchymal cell motility, and metastasis [138]. Of relevance, p63/p73/p53 appears to inhibit EMT and sarcoma progression/development [53]. Importantly, loss of CKS1B has been shown to inhibit c-myc-induced lymphomagenesis [139]. Furthermore, the tumor suppressor pi30 has been shown to inhibit k-ras induced lung carcinoma, suggesting that the *let-7-Skp2-pl 30* tumor suppressor pathway may inhibit lung carcinoma [140]. Together, the *p53/p73/p63-let-7-c-myc/Skp-2/CKSlB/CDKl-pl30/RhoA-CDKNl-A/-B/p57Kip2/INK4-a,-b-,c,-d* pathway may promote senescence and inhibit tumorigenesis, motility, invasion, and metastasis.

Yet another target that has shown to be suppressed by let-7 is CDC6 [123]. CDC6 is over expressed in a number of human cancers, including lung carcinomas. It appears to bind to repressor elements present in INK4-ARF gene locus (containing INK4B/A & ARF) and thereby inhibits its expression [141]. Based on this data, I have proposed previously that suppression of CDC6 in human cancers may result in increased expression of the tumor suppressors INK4a, INK4b, and ARF [Boominathan, unpublished]. Remarkably, let-7, by stifling the expression of CDC6 [123], it could increase the expression of the tumor suppressors INK4a, INK4b, and A*RF [Fig.2]. Evidently, CDKN2B/INK4B, a component of the INK4A-ARF locus (inhibits CDK4/6; deleted/hypermethylated in a number of cancers including transitional cell carcinoma), has shown to be induced by let-7 [123]. In addition, it is

possible that the tumor suppressor p53/TA-p73/p63, by suppressing the expression of CDC6 through let-7, it could induce the expression of INK4a, INK4b, and ARF.

Further, expression of let-7 appears to increase p53-inducible ribonucleotide reductase (a transcriptional target of p53 and a metastasis suppressor) and CycG2 (a transcriptional target of TA-p63/p73 and a metastasis suppressor) [123; 142], connoting that it suppresses the expression of repressors of p53/p63/p73 and thereby induces the expression of p53/p63/p73. This in turn induces the expression of its transcriptional targets, such as Cyc G2 (p63/p73) and p53-inducible ribonucleotide reductase (p53) [Fig.2]. Furthermore, let-7 has been shown to increase the expression of EIF2C2/Ago2 (promotes the miRNA processing in a dicer-dependent/independent manner), and the Myc antagonist, MXI1 (Max interacting protein 1) [123], suggesting that let-7 could promote the efficiency of miRNA/small RNA processing. Additionally, by increasing the expression of the MXI1, it could suppress c-Myc-dependent oncogenic functions (discussed in detail later on). This data further suggests an intriguing possibility that both TA-p73/p63/p53 and let-7 could share a regulatory feedback loop [Fig.2] [113].

Over expression of LIN28, EGFR, k-ras, c-Myc, and HMGA2 has been shown to result in lung adenocarcinoma/CML [143]. In particular, LIN28 appears to be over expressed in both BC-CML (42.8%) and accelerated-CML (40%) than CP-CML. HMGA2 also appears to be over expressed in CML [143]. Interestingly, let-7 has been shown to repress the expression of LIN28, k-ras, c-Myc, and HMGA2 mRNAs [144; 120], suggesting that TA-p73/p63, by increasing the expression of let-7, it could suppress the expression of LIN-28, HMGA2, k-ras, and c-Myc proteins [Fig.3]. The let-7-mediated repression of HMGA2 will result in up regulation of the tumor suppressors pl4ARF and INK4a, as HMGA2 has previously been shown to suppress the expression of pl4ARF and INK4a [145] [Fig.2]. Additionally, E-cadherin will be up regulated, as HMGA2 has been shown to suppress the expression of E-cadherin's negative regulators, such as Snail, Slug, and Twist [146] [Fig.2]. Thus, TA-p73/p63, by suppressing the expression of these key oncogenic proteins, it could function as a tumor suppressor in lung adenocarcinoma/CML.

Further, TA-ρ63γ has been shown to suppress the expression of EGFR [58]. Evidently, a recent study suggests that inhibition of EGFR results in down regulation of TWIST, a known suppressor of E-cadherin expression and a promoter of EMT [Fig.3] [53; 59]. These data suggest a possibility that TA-ρ63γ/ρ73β, by inhibiting the expression of oncogenic EGFR, it could inhibit the TWIST expression, and thereby increase the E-cadherin expression [53; 147]. This in turn will result in inhibition of EMT. Remarkably, let-7 has shown to be down regulated in CSCs/tumor-initiating breast cdncer cells, suggesting that it could negatively regulate the proliferation of CSCs [16; 144-145]. Furthermore, let-7 suppresses the expression of stem cell factors, such as Lin-28 and Log-2/6; 4; 5-12, and thereby inhibits the generation of CSCs [Fig.3] [148-149]. Of importance, Lin-28 is one of the components required for the generation of the induced pluripotent stem cells [ilPs] from differentiated cells [150]. Together, TA-p73/p63/p53, by increasing the expression of let-7 ***(guardian against pluripotency and cancer progression)*** [148], it could inhibit the EMT, metastasis, and CSCs generation.

4. The p53/TA-p73/p63 ⟶ miR-145/FBXW7 ⟞ c-myc tumor suppressor pathway.

We have showed previously that the tumor suppressor p53 suppresses the expression of c-myc [Boominathan & Rotter, unpublished]. However, how it suppresses or degrades c-myc

remained elusive. FBXW7, an F-box subunit of SCF-type ubiquitin ligase complex and a transcriptional target of p53/E2F-l, has shown to be mutated in 30% of acute T-cell lymphoblastic leukemia/lymphoma [151-152]. It appears to degrade c-myc/N-myc, Notch, mTOR, ΔN-ρ63, c-Jun and Cyc E, suggesting that p53 could decrease the expression of c-myc, Notch, mTOR, c-Jun and Cyc-E through FBXW7 [153]. Interestingly, both *FBXW7*$^{+/\sim}$ and *E2Fl/2* $^{+/\sim}$ *(p53*$^{+/}$*V TAp73*$^{+/\sim}$*/TAp63* $^{+/}$*)* mice are prone to leukemia/lymphoma [154; 1 (references therein)], suggesting that they may co-operate in tumor suppression. In addition, like p63, FBXW7 appears to play a role in stem cell maintenance [155; 113]. Together, these data suggest a tumor suppressor network involving *E2Fl/2-p73-ARF-p53/p63-FBW7-Notch-c-myc-N-myc-mTOR-CycE proteins.* This tumor suppressor network could play a role in the inhibition of leukemia/lymphoma development. ,

Further, a recent study showed that p53 binds to miR-145 promoter and increases its expression, which in turn, targets c-Myc for degradation [156]. Nevertheless, miR-145 also appears to be induced in a p53-independent manner [156], suggesting that p53 homologue, TA-p73/p63 could increase its expression. Interestingly, miR-145 expression has shown to be suppressed in lung cancer [157]. Remarkably, reintroduction of miR-145 suppresses the growth of lung cancer in mice [157], suggesting that the *TA-p73/p63-miR-145* pathway may mediate tumor suppression in lung cancer. Of relevance, the E2F-l/2-TA-p73-JunB-INK4a tumor suppressor pathway has previously been proposed to inhibit leukemia/lymphoma and lung cancer [1]. This notion is supported by previous studies that showed both E2F-1$^{-/-}$ and TA-p73$^{-/-}$ mice are prone to lung adenocarcinoma [158, 19]. Remarkably, miR-145 appears to inhibit the expression of factors—Oct-4, Sox-2, c-myc, fibronectin (FN1)*, CDH2*, Hey-1*, and PTK2*—required for the generation of CSCs/Stem cell self-renewal/reprogramming/migration/metastasis [Fig.4] [112; Target scan; *-predicted]. Together, it appears that induction of the tumor suppressor *TA-p73/p63/p53-miR-145-c-myc* pathway may inhibit lung cancer development, progression, metastasis, and CSCs.

c-Myc has recently been shown to increase the transcription of Lin-28 [159], which in turn, inhibits the processing of let-7. This data suggests that TA-p73/p63/p53, by down regulating the expression of c-Myc through miR-145, it could increase the expression of the lung cancer tumor suppressor let-7[Fig.4]. This data further suggests that in the absence of TA-p73/p63/p53, c-Myc expression could be augmented. This in turn will result in increased expression of its oncogenic target miRNAs, such as miR-17-92; 106 cluster [160] [Fig.5]. Increased expression of miR-17-92 cluster may suppress the expression of metastasis/invasion/migration/tumor suppressors, such as *PTEN, E2F-1/2, CDKN1A, BIM, AML-1/2*, p38a** (inhibits the proliferation of branchioalveolar stem cells, the putative initiators of adenocarcinoma of the lung), *DMTF1 *, TSC1*, DOK2*, CDKN1C** (miR-92b), *SMAD2*, BRCA1*, Retmoblastoma(pl05*)/pl07*/pl30, PPP2R2A *, TSP-1*, TA-p63 */AN-p63-α/β* (miR-92b), *CycG2*, DEC2*, RhoB*, BRMS-1*, MEK4*, CD82/Kail*. DLC1*, CTGF*, CLU*, SIK1*, HICl*, and Dicer1** [Fig.5], and thereby promote tumorigenesis, EMT, angiogenesis, invasion, metastasis, and CSCs proliferation [160-163; Target scan/Mami/Diana; Boominathan, In preparation; *-predicted].

Remarkably, miR-17-92 cluster appears to inhibit a number of *components—p63/p73-CDKNlC/CDKNlA/CycG2/DEC2/AMLl/DOK2/pl05/CD82/Dicerl/DMTF—* within the p63/p73-tumor suppressor pathway, suggesting the conserved nature of miR-17-92 cluster to

target the p63/p73-tumor suppressor pathway [Boominathan, unpublished]. As discussed, TA-p63/p73 has been shown to function as a metastasis suppressor by inducing the expression of CycG2 and DEC2 [142]. This data suggests a possibility that increased expression of c-myc and its target gene miR-17-92 may suppress TA-p63/p73's ability to function as a metastasis suppressor. Additionally, increased expression of miR-17-92 will result in down regulation of the tumor suppressor PTEN protein [163]. This in turn may result in increased expression of p53 and PTEN loss dependent senescence (PICS) in a cell context dependent manner [164]. Interestingly, a number of p53-miRs, such as miR-23, miR-26, miR-29, miR-25, miR-32, miR-92, miR-200, miR-130*, appear to target the expression of PTEN (Target scan; Boominathan, unpublished; *-putative). Further, anti-sense oligonucleotides that suppress miR-17-92 expression promote apoptosis of lung cancer cells [165], indicating that TA-p73/p63/p53, by suppressing the expression of c-Myc, it could down regulate the expression of miR-17-5p. In addition, miR-17-5p appears to be over expressed in a number of cancers, including B-cell lymphoma and Myeloid leukemia. Increased expression of miR 17-92 may result in down regulation of the *E2F-l-p73/p63-AML-l/JunB-ARF/INK4a* and the *PTEN-PML-PP2A/p73-Foxo3a/BIM/FasL/CDKNlb* tumor suppressor pathways; and up regulation of β-catenin expression, as E2F-1 has recently been shown to suppress the expression of β-catenin [166; Target scan]. Increased expression of β-catenin may contribute to the generation of CML-specific stem cells [167-168]. Interestingly, miR-17-92 cluster appears to target the expression of the lp36 tumor suppressor and the positive regulator of INK4a/ARF, CHD5 (lp.36.31) [Target scan], suggesting that tumors that over express miR-17-92 may down regulate INK4a/ARF levels [Boominathan, in preparation]. This data further suggests that p53/p73/p63/let-7/miR-34/miR-145, by suppressing the expression of c-myc, it could down regulate the expression of miR-17-92. This in turn will result in increased expression of the lp36 tumor suppressor CHD5. Remarkably, p53 has recently been shown to suppress the expression of miR-17-92 cluster in response to hypoxia [169], suggesting that p53/TA-p73/p63, by suppressing the expression of miR-17-92 cluster, it could increase the expression of a number of tumor suppressor genes [Fig.5]. When this patent application was about to be submitted for review, it has been shown that the upstream activator of p73, E2F-1 increases the expression of let-7 [170]. This data suggests an interesting possibility that E2F-1 may also increase the expression of let-7 through p73. Interestingly, c-myc is also a transcriptional target of E2F-1, suggesting a tumor suppressor network involving *E2F-1/2, p73, c-myc, and let-7*. Together, high levels of c-myc may result in increased expression of miR-17-92 cluster in cancer cells. This in turn may degrade the transcriptional activator of p73/let-7, E2F-1 and thereby disrupt the *E2F-l/2-p73/let-7-JunB-INK4a/ARF/p53/PTEN* tumor suppressor network in Myc-induced B-cell lymphomas/lung cancer/glioblastoma [171].

Further, E2F-2 has recently been shown to inhibit c-myc induced lymphomagenesis, suggesting that the *E2F-2-p73-let-7-c-myc* pathway may suppress lymphomagenesis [172]. Evidently, either E2F2 (lp.36) or p73 (lp.36.3) appears to be poorly expressed in a number of haematopoietic (Non-Hodkin lymphoma {Burkitt lymphoma, and Diffused large B-cell lymphoma}, Natural killer cell lymphoma, and acute lymphoblastic lymphoma) malignancies [1], non-small lung carcinoma, and neuroblastoma. Additionally, E2F-2 gene is deleted in neuroblastoma, breast cancer, and pheochromoctyoma, while E2F-1 expression is decreased in oral SCC, colon cancer and gastric adenocarcinoma [2]. Insulin-like growth factor 2 mRNA binding protein 1(IGF2BP1) has been shown to bind to c-myc mRNA, and thereby promotes its

stability [113]. Interestingly, let-7 appears to suppress its expression [113]. This data suggests that let-7 could regulate c-myc stability through different mechanisms. Together, these studies provide mechanistic insights into p53, TA-p73, and p63's ability to function as tumor/metastatic suppressors.

Further, it has been shown that high levels of c-Myc bind to the tumor suppressor miRNAs, such as *let-7a/d/g, miR-29a/b/c, miR-15/16a, miR-34, miR-26a/b, miR-30b/c/d/e, miR-150, miR-146a, miR-22,* and thereby suppress their expression [173] [Fig.3]. Importantly, c-myc-mediated repression of these tumor suppressor miRNAs in mice promotes B-cell lymphoma growth [173]. In addition, it has recently been shown that c-myc suppresses the expression of miR-23 [174]. Interestingly, c-Myc has been shown to interact with p73a protein and thereby suppresses its transcriptional activity [175]. Of note, p73a has been shown to suppress MYCN mRNA stability [176]. However, how it suppresses MYCN remained elusive. Here I propose that p73a may decrease MYCN mRNA stability through its ability to increase p53-miRNAs, such as let-7, miR-34, miR-200, miR-145, miR-29, and miR-101 [Target scan; Boominathan, unpublished]. Remarkably, c-Myc-inactivation has been shown to suppress tumorigenesis in a wild-type p53 dependent manner [177], suggesting that c-myc inactivation activates a p53-dependent tumor regression [177]. Together, these data suggest that p53/TA-p73/p63, by increasing the expression of miR-145/34/let-7/TRIM32/FBXW7/PTEN, it could suppress the expression of c-myc. This in turn will result in increased expression of the c-myc-suppressed tumor suppressor miRNAs *(miR-29a/b/c, miR-15/16a, miR-34, miR-26a/b, miR-30b/c/d/e, and miR-146a/miR-22)* and inhibition of tumorigenesis, invasion, motility, angiogenesis, CSCs, and metastasis [173-174] [Fig.3-5].

5. *The p53/TA-p73/p63 —| c-myc -^ miR-29 tumor suppressor pathway:*

The tumor suppressor miR-29, which appears to function as a tumor suppressor in lung cancer, chronic lymphocytic leukemia (CLL), AML, rhabdomyosarcoma and nasopharyngeal carcinoma, has been shown to negatively regulate the expression of DNA methyl transferases, such as DNMT3a, DNMT3b and DNMT1 [Fig.3/6] [178-179]. Its expression is down regulated in a number of cancers, including lung cancer, CLL, AML, rhabidomyosarcoma, and nasopharyngeal carcinoma. Remarkably, ectopic expression of miR-29 increases the expression of the tumor suppressors FHIT and WWOX (inhibits growth of lung cancer *invitro* and *invivo)* and thereby reduces the proliferation of lung cancer cells [179]. miR-29 has also been shown to suppress the expression of B/T-cell oncogene, Tcl-l/Mcl-1, which is over expressed in CLL/AML. Interestingly, miR-29 appears to target the expression of c-fos [Target scan], which has been shown to be required for the increased self-renewal of hematopoietic stem cells [HSCs] [180]. The fact that deregulation of HSCs/stem cell self-renewal results in leukemia suggests that miR-29 could play a role in the inhibition of leukemia development. Interestingly, miR-29 is predicted to target the expression of Inhibitor of DNA binding-1 [Target scan], the negative target of ΔN-ρ63 [-2.2 fold] [96]. Interestingly, over expression of Inhibitor of differentiation-1 appears to a) promote oncogenesis in a number of cancers, including T-cell lymphoma, oral SCC, AML, breast, prostate, and bladder cancer; and b) suppress the expression of CDKN1A, INK4a, and PTEN proteins. Furthermore, miR-29's promoter has shown to be epigenetically silenced [Fig.3/6] by activated NFKB-YY1 circuit in rhabdomyosarcoma; and reintroduction of miR-29 suppressed the growth of

rhabdomyosarcoma in mice [181], indicating that it functions as a tumor suppressor gene in rhabdomyosarcoma. This data suggests a possibility that miR-29, by negatively regulating DNMTs, it could increase the expression of the tumor suppressor TA-p73, as its promoter is hypermethylated (silenced) in several cancers, including acute lymphoblastic leukemia, AML, natural killer cell lymphoma, B-cell lymphoma and lung cancer [1]. Remarkably, it has recently been shown that miR-29 increases the p53 protein levels by suppressing the expression of p85 [the regulatory subunit of PI3K] and CDC42 [182]. Of importance, miR-29 promoter contains a number of p53REs (Boominathan, unpublished observation), suggesting a possibility that it could be directly regulated by p53, TA-p73, and TA-p63. In support of this notion, it has been shown that activation of p53 increases the expression of miR-29a (2.8 fold) [118; 183].

Further, it has been shown that treatment of lymph node metastatic cancer cell line with a DNMTs inhibitor increases the expression of miR-148, and miR-34 [184], suggesting that their expression is silenced and they can be reactivated to inhibit metastasis. This data also suggests a possibility that miR-29, by down regulating the expression of DNMTs, it could increase the expression of miR-148, and miR-34[Fig.3]. This in turn will result in decreased expression of their oncogenic target mRNAs, including HIF-2a, and E2F-3 [Target scan] [Fig.3].]. Decreased expression of HIF-2a may result in down regulation of its transcriptional targets, such as Oct-4, Sox2, Lin-28, c-Myc (known to play a role in the generation of CSCs), klf-4 (required for the generation of iPS cells), and Twist (required for metastasis progression) [Fig.3] [Boominathan, submitted], and inhibition of tumorigenesis, CSCs proliferation, invasion, and metastasis [Fig.3]. Interestingly, miR-148 appears to suppress the expression of DNMT3a/b and DNMT1 [185; Target scan; Boominathan, submitted], suggesting a double negative feedback loop [Fig.3]. Finally, miR-148 has shown to be down regulated in hypoxic tumors, suggesting a possibility that DNMT-l/3b will be up regulated in these tumors [Boominathan, submitted]. This in turn could result in inactivation of key tumor suppressor genes/miRNAs, including TA-p73, INK4a, PTEN, BRCA1, & miR-34 [Fig.3].

Next, miR-29 is predicted to target the expression of the metastatic promoter SMAD1 interacting protein, *SIP1/ZEB2* and the negative regulator of p53, p73, LKB1, miR-134 and Fox03a, *SIRTl* [Fig.6; Target scan]. Interestingly, miR-192, a transcriptional target of p53 (possibly, TA-p73/p63), also appears to inhibit the SIP1 expression [186-188]. p53/TA-p73/p63, by increasing the expression of miR-192/miR-29, it could suppress the expression of SIP-1. This in turn could result in inhibition of EMT and metastasis [Fig.4]. Further, it has been shown that the negative regulator of EMT and the positive regulator of epithelial phenotype, miR-200 represses the* expression of ZEBl [Fig.4] [189], which functions as a negative regulator of TA-p73, INK4B, CDKNIA, and E-cadherin and a positive regulator of EMT [Fig.4]. Interestingly, it has recently been reported that ZEBl represses the expression of miR-200, suggesting that both ZEBl and miR-200 could share a double negative feedback loop [161] [Fig.4]. miR-200 also appears to suppress the expression of SIP1/ZEB2, which in turn suppresses the expression of miR-200, suggesting that both ZEB2 and miR-200 could share a double negative feedback loop [190] [Fig.4]. Additionally, miR-200 is predicted to target the expression of DNMT3b [Fig.6] [Target scan]. Considering the fact that TA-p73/INK4a/PTEN/miR-34 promoter is hypermethylated in a number of human cancers, it is tempting to speculate that reintroduction of miR-200/29 in cancer cells may reactivate its expression by suppressing the expression of DNMTs. Remarkably, miR-200 promoter appears

to contain a number of p53REs, suggesting that it could be a transcriptional target of p53/TA-p63/p73 [53]. This data further suggests that p53/TA-p73/p63, by increasing the expression of miR-200, it could inhibit the suppressor of TA-p73/E-cadherin/INK4B/CDKNlA expression, ZEB1 [Fig.4]. Taken together, these interesting data from a number of laboratories buttress the notion that increasing the expression of miR-200 in human cancers may increase the expression of the tumor suppressors TA-p73, INK4B, and E-cadherin, and thereby prevent invasion and metastasis.

In support of the notion that miR-29 functions as a negative regulator of metastasis, it has recently been shown to suppress the expression of tristerapolin, which functions as a negative regulator of EMT, metastasis, and epithelial polarity [191]. Furthermore, miR-29 has been shown to inhibit the expression of extracellular matrix proteins, such as collagens, and laminin γ (PTP4A1*-a positive regulator of metalloproteinase enzymes 2/9{promotes CLL survival}), and thereby it functions as a suppressor of metastasis in nasopharyngeal carcinoma [192; Target scan;-*predicted]. AIB1/SRC3, a transcriptional co-activator of PEA3, E2F-1, AP-1 and nuclear receptors, has shown to be over expressed in 30% of human breast cancer. It synergizes with PEA3 in increasing the expression of matrix metalloproteinase enzymes 2 and 9, and thereby promotes EMT, migration, invasion, and lung metastasis [193]. Interestingly, miR-29 is predicted to target the expression of AIB1, suggesting that the *p53/p73-miR-29-AIB1* pathway may inhibit breast cancer invasion and lung metastasis [Target scan]. Further, $p63^{+/-}$, $p73^{+/-}$ and $E2F\text{-}1^{-/-}$ mice have been shown to develop sarcoma—which is derived mainly from mesenchymal cell type—and metastatic tumors, suggesting that p63, p73 and E2F-1 could function as negative regulators of EMT and positive regulators of mesenchymal to epithelial transition (MET). In support of this notion, p73/p63/p53 has been hypothesized to increase the MET/EMT ratio by increasing the expression of tumor suppressor miRNAs, such as *miR-200, let-7, miR-34, miR-183, miR-203, miR-145, miR-141, miR-29, and miR-148* [53-54]. Together, p73/p63/p53, by increasing the MET/EMT ratio through its target miRs, it could function as a negative regulator of metastasis [Fig.6].

6. The p53/TA-p73/p63 ⊣ c-myc ⊣ miR-34 tumor suppressor pathway:

The tumor suppressor miR-34 (1p36.22) has shown to be down regulated in cancers such as CLL, glioma, adenocarcinoma of the lung, and Nasopharengial carcinoma [194]. A number of groups have shown that it is a direct transcriptional target of the tumor suppressor p53 (possibly, p73) [195-196]. Interestingly, c-myc has been shown to suppress the expression of miR-34 [123], suggesting that reactivation of p53 in c-Myc over expressing human cancers may restore its expression. miR-34 appears to inhibit (or, target) the expression of — *Cyc D1, CDK6, Bcl2, SIRT1, Jagged1, Notch1/2/3, E2F3/1, SRC-1*, ROCK-1*,-2*, mTORC1*, TMPRSS4*, Src*, TGFβ3*, TBX1*, FN1*, Hey-1*, DLL-1**— a number of key genes required for survival, cell cycle regulation, proliferation, invasion, and metastasis [197-199; Target scan; *-predicted]. Interestingly, E2F3, a key component in cell cycle progression, has been shown to repress the expression of the tumor suppressor p14ARF [198], suggesting that p53/p73-dependent up regulation of miR-34 may result in down regulation of E2F3 and up regulation of p14ARF [Fig.6]. Further, Steroid Receptor Coactivator-1(SRC-1), a co-activator of nuclear hormone (estrogen & progesterone) receptors, has been shown to increase the expression of c-myc through Ets-2 [200]. It also appears to induce the expression of TWIST (a negative regulator of E-cadherin) in conjunction with PEA3 and thereby promotes breast

cancer progression and lung metastasis [201]. Interestingly, miR-34/130*, a direct (*-putative) transcriptional target of p53/p73* [113], appears to target the expression of SRC-1 [Target scan], suggesting that the *p53/p73/p63-miR-34/130-SRC-l-ETS-2-c-myc-Twistl—E-cadherin* tumor suppressor pathway may inhibit EMT, breast cancer progression and lung metastasis. It has recently been shown that LEF1 is required for the invasion of lung adenocarcinomas to brain and bone [202]. Interestingly, miR-34 is predicted to inhibit the expression of LEF1 (Target scan), suggesting that p73/p53-dependent up regulation of miR-34 may down regulate the expression of LEF1, and thereby inhibit the metastasis of lung adenocaricinomas to brain and bone.

7. *The p53/TA-p73/p63 ⟞ c-myc ⟞\ miR-15a/16-l tumor suppressor pathway:*

The tumor suppressor miR-15a/16-l has shown to be deleted/down regulated in B-cell CLL, non-small cell lung cancer, and prostate cancer [203-204]. It appears to suppress the expression of cell cycle progression/proliferation/survival/metastasis/stem cell renewal promoting genes, such as *Bcl-2, Wnt-3a, Cyc D1, Cyc D2, Cyc D3, Cyc El, CDK6, Mcl-1, MCM5, c-Myb, BMI-1, HMGA2* (promotes neural stem cell renewal), c-Jun*. NFKB*,* IKKβ*, *VEGFA*, SMAD3*, mDIAl*, Raf-1*, TGFβR3*, Notch2*, DLL4* (Delta-like 4 Notch ligand), DLL1 *, and Ets-1 *[203-206; Target scan/Mami/Diana-*-predicted; Boominathan, unpublished], [Fig.6]. Interestingly, IKKβ, an NFKB activating kinase, has been shown to degrade ΔN-ρ63 [207], suggesting that the tumor suppressor miR-15a/16-1, by targeting its expression, it could control the expression of ΔN-ρ63. Considering that ΔN-ρ63 could function as a metastasis/tumor suppressor in a cell context dependent manner, this supposition is of great significance. Further, miR-15a/16-l has been shown to suppress the negative regulator of CDKN1A/INK4a/PTEN expression, BMI-1 [Fig.5], suggesting that increased expression of miR-15a/16-l may increase the expression of tumor suppressor CDKN1A/INK4a/PTEN [208]. Of interest, BMI-1 is a transcriptional target of c-myc [Fig.5]. Remarkably, BMI-1 has been shown to promote expansion of bronchiolalveolar stem cells, the putative initiators of the adenocarcinoma of the lung [209], suggesting that the tumor suppressor miR-15a/16-1, by suppressing the expression of BMI-1, it could hamper the expansion of bronchiolalveolar stem cells and thereby inhibit lung adenocarcinoma development. This is a significant finding considering TA-p73 and E2F-1 null mice are prone to lung adenocarcinoma. The fact that BMI-1 is transcriptional target of c-myc suggests that uncontrolled expression of c-myc may promote the expansion of bronchiolalveolar stem cells in TA-p73 null mice and thereby cause lung adenocarcinoma. Remarkably, knockdown of miR-15a/16-l promotes survival, proliferation, and invasiveness of normal untransformed prostate cells, suggesting that it could also function as a tumor suppressor in prostate cancer [203]. Together, this data suggests a tumor suppressor pathway involving TA-*p73, p53, p63, c-myc, miR-15a, 16-1, BMI-1 and INK4a, PTEN* proteins/miRs.

Further, E2F-1/3 has recently been shown to bind to miR-15b/16-2 promoter and thereby increases its transcription [210]. Interestingly, E2F-l/3-dependent up regulation of miR-15b/16-2 inhibits S-phase progression by targeting multiple cell cycle regulators and E2F targets. Based on these interesting data, I hypothesized that miR-15/16-1 could be a transcriptional target of p53/p63/p73. Evidently, bioinformatics analysis of miR-15/16-1 cluster promoter *(miR-15a* {-1724 to -1743: (ag gcatgg tg)(gct cttg cct); -2598 to -2623: (ggc cgagg ca)ggcgga(tca cgagg tc); -2654 to -2674: (atcctgggcf)(gg gcatgg tg); -4432 to -4463:

ttgcatgctaXcaacatggat)g(aatcttgaaa)}; and *miR-16a*{(-1864 to -1884: **(aggcatggtgXgctcttgcct)**; -2737 to -2763: **(ggccgaggca)ggcgga(tcacgaggtc);** -2794 to -2813: **(atcctgggct)(gggcatggtg);** -4573 to -4594: **(ttgcatgcta)(caacatggat)g(aatcttgaaa)»** revealed a number of p53REs, suggesting that it could be a putative transcriptional target of p53/TA-p73/p63. In support of this data, it has been shown that activation of p53 results in increased expression of miR-15a(5.2 fold), miR-15b(8.2 fold) and miR-16 (2.9 fold) [118], suggesting a possibility that they could be direct transcriptional targets of p53/TA-p73/p63. Taken together, these data suggest that the *E2F-l-p73/p53/p63-miR-145/let-7/miR-34;PTEN/FBXW7-c-myc-miR-15a/16-1/miR-15b/16-2 tumor suppressor network,* by suppressing the expression of genes that promote cell cycle progression, invasion, metastasis, survival, self-renewal, and CSCs proliferation, it could promote tumor suppression. In particular, the *E2F-l/2*-TA-p73*/p63*-let-7#/miR-l5a/l 6-l#/PTEN#/INK4a#* tumor suppressor pathway (*lung cancer phenotype/#inhibits lung cancer) may play a critical role in the inhibition of adenocarcinoma of the lung.

8. The p53/TA-p73/p63 ——I c-myc ——I miR-26 tumor suppressor pathway:

The tumor suppressor miR-26 appears to be consistently suppressed by c-myc in a number of tumors [21 1], suggesting that it could play a role in myc-induced lymphomagenesis. It has recently been shown to suppress the expression of Enhancer of zeste homologue 2[EZH2], a histone methyl transferase and a component of polycomb repressive complex 2 [21 1] [Fig.6]. EZH2, by mediating methylation on histone H3 at lysine 27 (H3K27me3), it represses the transcription of a number of genes. It has shown to be over expressed in a number of human cancers, including human Burkitt lymphoma and Rhabdomyosarcoma. It appears to promote hypermethylation; and increase pluripotency in stem cells. Remarkably, EZH2 appears to suppress the expression of key tumor suppressor genes, such as E-cadherin, AML-2/Runx-3, INK4A, INK4B, CDKNlC/p57Kip2, and PSP94 [189; 211-218]. This data suggests that c-Myc-mediated suppression of miR-26 may result in increased expression of EZH2 (H3K27me3 mark on prorhoters) and decreased expression of its target genes (E-cadherin, AML-2, ΓNK4A, ΓNK4B, CDKNlC/p57Kip2, and PSP94). Further, it has been shown that systemic administration of miR-26a-adeno associated virus in a mouse model of hepatocellular carcinoma results in inhibition of tumor progression and induction of tumor specific apoptosis [219]. Interestingly, miR-26 is predicted to target the negative regulator of a) p53, *HDM2*; b) INK4a and ARF, *HMGA2;* and c) p53/CDKNlB/C/pl30, *Skp2* [Fig.6] [Target scan, Mami, & Diana]. This data suggests a possibility that miR-26, by down regulating the expression of HDM2, HMGA2, and Skp2, it could increase the expression of tumor suppressors p53, INK4a, ARF, pi30, and CDKN1B/C. Given that correcting pathway-specific defects is essential for better management of cancer therapy, designing miR-26 mimics will be helpful.

Further, miR-26b has shown to be induced (5.8 fold) in response to p53 activation [118]. In support of this data, bioinformatics analysis of its promoter revealed a number of p53REs (Boominathan, unpublished), suggesting that it could be a transcriptional target of p53/TA-p63/p73. When this manuscript was under preparation, miR-101 has shown to inhibit the expression of EZH2 [220]. Interestingly, miR-101(down regulated in transitional cell carcinoma) also appears to be a transcriptional target of p53, suggesting that p53 could inhibit the expression of EZH2 through both miR-29 and miR-101.

Further, miR-26b is predicted to suppress the expression of DNMT3b, Klf-4, HOXA9, HMGA2, Jagged- 1, Hes-l(over expression induces TCL; a negative regulator of the tumor suppressor PTEN; and a negative target of ΔNρ63: ΔNρ63 —iHesl —|PTEN), HIF-2a, AIB, and Prostasin [Target scan/mami]. Interestingly, HOXA9/HIF-2a has shown to be required for the survival of HSCs [221]. Remarkably, suppression of HOXA9 results in apoptosis of MLL-rearranged leukemias [221], suggesting that TA-p73 by increasing the expression of miR-26, it could hamper leukemia development. Loss of p53/TA-p73/p63-mediated c-myc suppression may result in decreased expression of miR-26 and increased expression of DNMT3b and EZH2. This in turn may result in epigenetic inactivation of tumor suppressor genes. Interestingly, it has recently been shown that systemic administration of miR-26 in a mouse model of hepatocellular carcinoma results in down regulation of Cyc D2 and Cyc E2 and inhibition of cancer cell proliferation and apoptosis [222]. Next, Klf-4 has shown to play a role in the generation of induced pluripotent stem cells from differentiated cells. Interestingly, it appears to suppress the expression of tumor suppressor p53 in a context dependent manner [223]. This data suggest a possibility that miR-26, by suppressing the expression of Klf-4 (Target scan), it could increase the expression of p53. Taken together, the *p53/p73/p63-c-myc-miR-26-EZH2-INK4a/ARF/pl30/CDKNlB/C-DNMT3b/Klf-4/HOXA9/ HMGA2/Jagged- 1/HIF-2α/AIB* tumor suppressor pathway may play a critical role in the inhibition of lymphoma, rhabdomyosarcoma, and hepatocellular carcinoma.

9. The p53/TA-p73/p63 —| c-myc —\ miR-30b/c/d tumor suppressor pathway:

Another miRNA that appears to be suppressed in response to high levels of c-myc is miR-30b/c/d [123]. Analysis of miR-30's predicted targets suggests that it may suppress: a. Lin-28, the negative regulator of the tumor suppressor miRNA, let-7 processing; b. DNMT3a; c. Skp2, which targets CDKNlB/C/pl30 for degradation; d. AIB1; e. DLL-4, Jagged-2 & Notch-1; f. PTP4A1; g. SMAD2; h. SIRT1 and i. WWP1 (Target scan) [Fig.6].

It has recently been shown that WWP1, a WW domain containing protein, binds to TA-p63 and ubiquitinates it. By ubiquitinating TA-p63, WWP1 targets TA-p63 for degradation through proteasomes [224]. This data suggests that miR-30, by down regulating the expression of WWP1, it could increase the expression of the tumor suppressor TA-p63 in a cell context dependent manner [Fig.6].

In addition, miR-30 appears to target the positive regulators of EMT/migration, such as Snaill/Slug, PTP4A1, and Vimentin-l (mesenchymal marker) [Target scan]. This data suggests a possibility that miR-30, by negatively regulating the expression of Snail 1/Slug, it could increase the expression of E-cadherin, and thereby inhibit EMT transition and metastasis. As discussed, Snail 1 negatively regulates the expression of ΔN-ρ63, and thereby promotes the invasive property of human SCC. This data suggests a possibility that p63/p73/p53-induced miR-30 expression may result in down regulation of Snail 1 and up regulation of ΔN-ρ63/E-cadherin and inhibition of invasion and metastasis. Remarkably, it has recently been shown that miR-30 reduces self-renewal of breast tumor-initiating cells (BT-ICs) by suppressing the expression of Ubc9 and Integrin β-3 [225]. In addition, over expression of miR-30 in BT-ICs xenografts reduces tumorigenesis and lung metastasis in immunodeficient mice [225]. Further, it has recently been shown that expression of miR-30 in thyroid carcinoma-derived cells promotes mesenchymal to epithelial transition by reducing the expression of TGFpRI. This in turn reduces the invasive potential of thyroid carcinoma-derived cells [226]. Together, these

data suggest that expression of miR-30 may inhibit EMT, self-renewal of tumor-initiating cells, invasion, and metastasis. These promising findings present us with a therapeutic opportunity. That is, by reintroducing miR-30b/c/d into cancer cells, one could suppress the expression of WWP1, Lin-28, Snail 1, and DNMT3a, and thereby increase the expression of the tumor suppressors p63, let-7, TAp73, PTEN, CDKN1C, and E-cadherin [Fig.6]. Therefore, this data suggests a possibility that miR-30 mimics will be useful in cancer therapy. Remarkably, activation of p53 has been shown to increase the expression of miR-30c [227] and miR-30a-3p (1.6 fold) [118]. In support of this data, bioinformatics analysis of miR-30 cluster promoters revealed a number of p53REs, suggesting a possibility that they could be transcriptional targets of p53/p63/p73 [Boominathan, unpublished]. Taken together, the *p53/p73-c-myc-miR-30-p63/let-7/CDKNlB/C/pl30/E-cadherin* tumor suppressor pathway may play a critical role in the inhibition of EMT, invasion, metastasis, and breast cancer stem cells.

10. The p53/TA-p73/p63 ⟶ c-myc ⟶ miR-23 tumor suppressor pathway:

c-Myc has recently been shown to suppress the expression of miR-23 [174]. Interestingly, miR-23 has been predicted to suppress the expression of HIF-2a* [Target scan; Boominathan, submitted; *predicted]. HIF-2a is over expressed in non-small lung carcinoma, renal carcinoma, and glioblastoma. Interestingly, HIF-2a co-operates with K-ras mutant to promote more invasive lung cancer [228]. This cancer is characterized by increased EMT, angiogenesis, and mobilization of endothelial progenitor cells [228]. Further, HIF-2a has shown to be predominantly expressed in glioma stem cells compared with non-stem tumor cells and normal neural progenitors [229]. In glioma stem cells, it appears to co-localize with the stem cell marker CD133 [229]. Interestingly, suppression of HIF-2a in glioma stem cells inhibits self-renewal, proliferation, survival, and tumor initiation potential [229]. These data suggest that p63/p73/p53-dependent up regulation of miR-23 may result in down regulation of HIF-2a and its target genes, such as Oct4, c-myc, Lin-28*, Esrrb*, klf4*, Sox-2/4* and telomerase* [230-232; ""-putative; Boominathan, submitted]. In addition, down regulation of HIF-2a may result in decreased expression of VEGFA, lysil oxidase, and Twist-1 [231-232]. Interestingly, HIF-dependent expression of Twist-1 and miR-lOb [that increases the expression of metastatic promoting gene such as Rho-c by down regulating the expression of HB10D] has been shown to promote EMT and metastasis [Fig.4]. Furthermore, miR-23 has been shown to target the expression of a) ZEB1*, TGFpR-2* & -3* mRNAs, (negative regulators of the metastasis suppressor E-cadherin); b) Skp2*, which appears to promote the c-Myc-Miz-1-p300-RhoA metastasis cascade [134] [Fig.4]; c) proteins that play a role in invasion and metastasis, such as Urokinase-type plasminogen activator and c-Met [46]; d) SIRT1*; and e) the metastasis promoter SRC-1*, which appears to increase the Ets-2-c-myc-Twist metastasis cascade [200-201; *predicted].

Remarkably, activation of p53 has been shown to increase the expression of miR-23a (3.5 fold) and miR-23-b (1.7 fold) [118]. In support of this data, bioinformatics analysis of miR-23a/b promoter revealed a number of p53REs, suggesting that it could be a direct transcriptional target of p53/TAp73/p63. Thus, p53/TA-p73/p63, by activating the *let-7/miR-145-c-myc-miR-23-HIF-2a/ZEBl/CDHl/INK4b/Skp2* tumor suppressor pathway, it could inhibit CSCs proliferation, EMT, and metastasis [Fig.4].

11. The p53/TA-p73/p63 ——\ c-myc —| miR-146a tumor suppressor pathway:

Yet another miRNA that appears to be suppressed in response to high levels of c-myc is miR-146[123]. Interestingly, bioinformatics analysis of its promoter revealed a number of p53REs, suggesting that it could be a direct transcriptional target of p53 (Boominathan, unpublished). Down regulation of miR-146 has been shown to play a critical role in the progression of papillary thyroid carcinoma [233]. Furthermore, polymorphisms in miR-146 gene results in decreased mature miR-146 transcript in thyroid cancer [234]. Loss of miR-146a expression has also been shown in prostate cancer; and reintroduction of miR-146a into prostate cancer cells results in reduced proliferation, invasion, and metastasis [235]. Interestingly, Breast cancer metastasis suppressor-1, a suppressor of NFKB & miR-lOb expression, has recently been shown to increase the expression of miR-146 [236]; and reintroduction of miR-146 into breast cancer cells results in down regulation of EGFR expression and inhibition of invasion, migration, and metastasis [236] [Fig.6]. Further, c-Myc's transcriptional target, miR-17-92 cluster is predicted to suppress the expression of Breast cancer metastasis suppressor-1 [Target scan], suggesting that it could inhibit the up regulation of miR-146 [Fig.5]. Interestingly, miR-146 is predicted to target β-catenin (Target scan), which has been shown to promote the generation of stem cells in CML [133]. Together, these data suggest that p53/TAp73/p63, by suppressing the expression of c-Myc, it could increase the expression of Breast cancer metastasis suppressor-1, and thereby increase the expression of miR-146 to prevent invasion, migration, metastasis and CSCs proliferation.

12. The p53/TA-p73/p63 → miR-192, 215, 145 -| ZEB2/SIPl—\ E-cadherin; The p53/TA-p73/p63 → miR-145/34/let-7 —| c-myc → miR-9-3 —| E-cadherin; The p53/TA-p73/p63 → miR-145/34/let-7—i c-myc — ▸ miR-221/222 —| TIMP3/PTEN/CDKNlb, c tumor suppressor pathways:

As previously discussed, miR-145, and miR-192/215 are transcriptional targets of the tumor suppressor p53/TA-p73/p63. Remarkably, analysis of miR-145 and miR-192/215 targets suggests that they may suppress the expression of ZEB2/SIP1 [SMAD1 interacting protein 1] [Target scan]. Together, these data suggest that p53, p63, and p73, by increasing the expression of miRs-192, -215, & -145, they could suppress the expression of ZEB2 [Fig.4] [53-54]. Remarkably, ZEB2 also appears to be a common target of a number of other p53-induced miRs, such as miR-30a-e, miR-200b/c, miR-183, miR-92a/b, miR-132, suggesting a conserved mechanism mediating the p53/p63/p73-dependent inhibition of EMT, invasion, and metastasis [53]. ZEB2 has been shown to function as a repressor of the tumor suppressor E-cadherin, suggesting that p53, TA-p73, and TA-p63, by suppressing the expression of ZEB2, they could increase the expression of E-cadherin [Fig.4] [54]. This in turn could inhibit EMT, invasion, and metastasis [237]. Of interest, activation of EMT has been shown to promote generation of cells with stem cell properties (e.g. expression of stem cell markers) [238], suggesting that p53-miR-dependent up regulation of E-cadherin (or, inhibition of EMT) could inhibit the generation of CSCs.

Further, it has recently been shown that miR-9-a-3 is a transcriptional target of c-Myc/MYCN [239]. Interestingly, increased expression of miR-9-a-3 has been shown to suppress the expression of E-cadherin and its downstream targets, such as β-catenin and VEGF [239-240]. This data suggests that activation of the p53/TA-p73/p63-miR-145/34/let-7 pathway may down regulate both c-myc and its downstream target miRNA, miR-9-a-3, and thereby up regulate the expression of E-cadherin [Fig.5]. This in turn will result in down regulation of β-catenin and VEGF and inhibition of invasion and metastasis. Interestingly, N-myc downstream-regulated gene 1, a transcriptional target of p53 and a metastasis suppressor, appears to be targeted by miR-9 (Target scan), suggesting that p73/p63/p53, by decreasing the expression of c-myc/miR-9-a-3, it could increase its expression, and thereby inhibit metastasis [241].

A recent study suggests that c-myc increases the expression of miR-22 1, and miR-222 [242]. Another study suggests that these miRNAs are transactivated by c-met/c-Jun [243]. Furthermore, increased expression of miR-22 1/miR-222 suppresses the expression of the tumor/metastasis suppressor proteins such as PTEN and TIMP3 [Fig.5] [243]. Interestingly, increased expression of miR-22 1/222 has been inversely correlated with the expression of TIMP3 and PTEN in human non-small cell lung carcinoma and hepatocellular carcinoma. Yet another important study suggests that miR-22 1/222 suppresses the expression of CDK inhibitors, such as CDKN1B and CDKN1C [244] [Fig.5]. These data together suggest that p53-miRs (miR-145, miR-34 & miR-let-7)-dependent suppression of c-myc/c-met/c-Jun expression may result in decreased expression of miR-22 1/222. This in turn will result in up regulation of PTEN, CDKN1B, CDKN1C, and TIMP3 expression, and inhibition of tumorigenesis, migration, and invasion. Taken together, the data discussed in this patent application strongly suggest that p53, TA-p73, and p63 could function as negative regulators of the EMT, migration, invasion, metastasis, and CSCs proliferation.

13. Therapeutics

The match between c-myc-suppressed tumor suppressor miRNAs [173] and p53-induced miRNAs [118] suggests a link between p53-activated and c-myc-suppressed miRNAs-dependent tumor suppressor pathways. p53/TA-p73/p63, by suppressing the expression of c-myc through *TRIM32/PTEN/FBXW7lmiR-145/34/let-7,* it could up regulate the expression of tumor suppressor miRNAs, such as *miR-15/16a, miR-29, miR-34, miR-26, let-7a/d/g, miR-30b/c/d/e, miR-146a, miR-150 and miR-22,* and a number of tumor suppressor genes [Fig.5 & 6]. Evidently, inactivation of c-myc has been shown to induce senescence by inducing INK4a and INK4B expression [245-246]. This could perhaps be due to the down regulation of the c-myc's target genes BMI-1 and HMGA2 (an indirect target) [Fig.3]. It appears that p53/TA-p73/p63-mediated repression of c-myc [and its repressed miRNA targets] is disadvantageous for AML, glioblastoma, acute lymphoblastic leukemia, adenocarcinoma of the lung, and B-cell lymphoma [173]. In support of this data, it has recently been shown that: a) knockdown of p73 promotes dissemination of c-myc-induced B-cell lymphomas [247]; b) inactivation of both p53 and its target gene PTEN results in activation of c-myc in glioblastoma. This in turn results in increased self-renewal of neural stem cells/tumor-initiating cells [248]; and c) deletion of PTEN in HSCs increases the expression of β-catenin and c-myc. This in turn results in increased number of leukemic stem cells, which aid the progression of acute T-cell lymphoblastic leukemia [249]. The fact that deletion of PTEN in T-cells/ hematopoietic stem cells/bronchioalveolar epithelium

increases the expression of c-myc suggests that they share a double negative feedback loop (c-myc-miR-17-92-PTEN; PTEN-c-myc) [250-251]. Together, these data suggest that tumors that harbor mutation in p53/PTEN(the second most frequently mutated gene next to the p53 tumor suppressor)/FBXW7 gene will have increased levels of c-myc, which in turn will activate oncogenic miRNAs and thereby suppress a number of tumor suppressor miRNAs/genes [Fig.4-6].

It has recently been shown that c-myc promotes k-ras/c-Raf-driven metastasis in a mouse model of non-small cell lung carcinoma, and inactivation of both c-myc and k-ras inhibits tumorigenesis of lung cancer/lymphomas. Remarkably, both c-myc and k-ras expression are suppressed by the tumor suppressor miRNA, let-7, suggesting that reintroduction of let-7 or its mimics may be helpful for patients suffering from lung adenocarcinoma and lymphoma.

In support of the data discussed in this patent application, c-myc-suppressed miRNAs—such as let-7, miR-34a-c, miR-15/16, & miR-29—are down regulated in lung cancer, while c-myc-activated miRNAs—such as miR-17-92 & miR-22 1/222—are up regulated [252], suggesting that the c-myc-orchestrated activation/suppression of the miRNAs may play a key role in tumor suppression.

Further, the *p63-AMLl/Rurix-l-ARF* tumor suppressor pathway may suppress the transcriptional activity of c-myc, as ARF has been shown to suppress its transcriptional activity [253]. Likewise, the *E2F-l-p73/miR-15/16-JunB-INK4a/ARF* tumor suppressor pathway may suppress the transcriptional activity of c-myc, as ARF is a transcriptional target of E2F-1. Remarkably, p73 loss of heterozygosity has been observed (LOH) in 60% of human non-small cell lung carcinomas that harbor mutation either in pl4ARF or p53 gene, suggesting the importance of the E2F-l-TA-p73-ARF pathway in inhibiting the development of non-small cell lung carcinomas [1; 254]. Furthermore, JunB, a putative target of p73/p63, has been shown to increase the expression of the lung cancer suppressor DMTF (deleted in 40% of human non-small cell lung cancer) [255]. Bioinformatics analysis of its promoter revealed a number of p53/p63-REs, suggesting that it could be a transcriptional target of p73/p53/p63. Interestingly, increased expression of DMTF has been shown to increase the expression of ARF [256-257], suggesting that the *p73/p63-JunB/DMTF-ARF* tumor suppressor pathway may suppress the expression of c-myc. Considering TAp73, p73, p63, E2F1 and DMTF heterozygous/null mice are prone to lung adenocarcinoma suggests that they may co-operate with each other in tumor suppression. Together, these findings suggest that TA-p73 and p63: a) may suppress the expression of c-myc, and thereby increase the expression of c-myc-repressed tumor suppressor miRNAs/genes; and b) are no longer the specter of the tumor/metastasis suppressors, but they are indeed tumor/metastasis suppressors[258].

. In addition, p53/TAp73/p63 could increase the expression of a number of tumor suppressor miRNAs directly. Therefore, the data discussed in this patent application posit that reintroduction of p53/TA-p63/p73-dependent miRNAs, such as *miR-145, miR-23, let-7, miR-15/16, miR-26, miR-29, miR-30, and miR-34,* in *human cancers over expressing c-myc will up regulate the tumor suppressor miRNAs/genes and thereby inhibit tumor progression, invasion, metastasis, and CSCs proliferation* [259]. Considering "cancer pathway-specific therapy" will be the mode of treatment in the future for better cancer management, the tumor suppressor pathways described in this patent application may aid cancer therapy.

Further, considering suppressing c-myc expression in a number of human cancers will increase the expression of tumor suppressor miRNAs and tumor suppressor genes, one can conduct a genetic screen to identify compounds or small molecules that simultaneously suppress the expression of c-myc and induce the expression of tumor suppressor genes/miRNAs. To conduct the genetic screen, c-myc promoter will be fused to the renilla reporter plus TAp63/p73 p53/INK4a/b/c/d/CDKN 1a/b/c/ARF/RKIP/CDH 1/PTEN/Ago 1to4/FB W7/RBs/CycG2/DEC2/D OK2/AML 1/2/BRCA l/p38a/TSC 1/MEK4/PPP2R2A/TSP 1/BRMS 1/E2F 1/2/TIMP3/CTGF/SM AD2/RRM2B/MXIl/DMTF/miR-15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23/22 promoter will be fused to the firefly reporter. These gene fragments will be cloned into an expression vector containing resistance genes for selection. This vector will be used to generate a stable cell line that expresses c-myc(oncogenic promoter) promoter linked to renilla[R] luciferase gene plus TAp63/p73/p53/INK4a/b/c/d/CDKNla/b/c/ARF/RKIP/CDHl/PTEN/Agol-4/FB W7/RBs/CycG2/DEC2/DOK2/AML 1/2/BRCA l/p38a/TSC 1/MEK4/PPP2R2A/TSP 1/BRM Sl/E2Fl/2/TIMP3/CTGF/SMAD2/RRM2B/MXIl/DMTF/miR-15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23(tumor suppressor) promoter linked to firefly[F] reporter gene (Fig. 7). This stable cell line will be used to screen for compounds. Compounds that simultaneously suppress c-myc & induce tumorsuppressor TAp63/p73/p53/INK4a/b/c/d/CDKNl a/b/c/ARF/RKIP/CDHl/PTEN/Ago lto4/FBW7/RBs/CycG2/DEC2/DOK2/AML 1/2/BRCA l/p3 8a/TSCl/MEK4/PPP2R2A/TSPl/BRMSl/E2Fl/2/TIMP3/CTGF/SMAD2/RRM2B/MXIl/DMT F/15/16/let-7/miR-34/miR-200b/c/miR-145/miR-26/miR-29/miR-30/miR-23/miR-203/miR-22 promoter activities (or, any other tumor suppressor miRNA promoter stated in this patent application) will be selected—using F(firefly)/F+2R(renilla) ratio— for further evaluation. Compounds, such as Ascochlorin, Dihydroartemisinin, and 5-Fluro uracil and curcumin, have been shown to suppress c-myc [260-261]. Interestingly, curcumin has also been shown to activate miR-15/16/22) [262] expression, suggesting that curcumin can be used as a positive control to check whether it simultaneously suppress the expression of c-myc and induce the expression of tumor suppressor miRNAs.

Malone & Hannon have recently suggested that small RNAs may be considered as guardians of the genome [263]. Remarkably, it appears that most of the known miRNA processing components, including Drosha, DGCR8, Dicer, Ago-l(miRNA)/-3(piRNA)/-4(rasiRNA)(lp34-35), TARBP2, and p68/p72, are regulated by p53/p73/p63 and its target miRNAs [113]. In particular, p53-miRs appear to target the miRNA processing enzyme Dicer in a context dependent manner [113]; and p63/p73 may increase the transcription of dicer and thereby inhibit metastasis [113]. Interestingly, deletion of dicer has been shown to elicit a DNA damage response, increase the tumor suppressor p53-ARF pathway, reduce blood pressure, promote senescence, and inhibit stem cell renewal/proliferation [113]. This data suggests that: (i) p53/p63/p73, by regulating dicer in a cell context dependent manner, it could inhibit tumorigenesis, metastasis, and stem cell (possibly CSCs) proliferation/self-renewal [113]; (ii) compounds that increase dicer 1 expression may inhibit metastatic progression and promote insulin sensitivity; and (iii) compounds that decrease dicerl expression may reduce blood pressure. In addition, genome sequence analysis suggests that nearly half of the 326 miRNA

promoters contain p53-REs [264]. Therefore, p53, p63, and p73, by regulating both the miRNAs expression and their processing components, they could function as regulators of the *miRNA/siRNA/piRNA* (silences transposons in the *germ Yme)/rasiRNA* (suppresses DNA damage response in the germ line) biogen'esis [265-266]. Together, the data discussed in this patent application suggests for the first time thaf'the guardians of the genome" p53, TA-p73, and TA-p63 are: (i) in control of the production of small RNAs; and (ii) not only in control of the expression of a number of protein-coding tumor suppressor genes, but also non-coding tumor suppressor small RNAs [267]. In conclusion, using the dual promoter vector—Promoter: 1 Oncogenic promoter eg., c-myc; and Promoter 2: tumor suppressor gene/miRNAs promoter—, one can identify compounds that simultaneously suppress the expression of c-myc (or, any other oncogene or protein that suppresses the expression of a tumor suppressor gene(s)) and induces the expression of tumor suppressor genes/miRNAs.

Footnotes

1. Target scan: http://www.targetscan.org/
2. Diana: http://diana.cslab.ece.ntua.gr/microT/
3. Mami: http://mami.med.harvard.edu/

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DESCRIPTION OF THE DRAWINGS

Fig.l. **p53/TA-p73/p63 functions as a tumor/metastasis suppressor.** The tumor suppressor p53/p73/p63 increases the expression of HDM2, which in turn promotes the degradation of metastasis initiators, SNAI1 and SNAI2. SNAI1/SNAI2 suppresses the expression of the metastasis/invasion/migration suppressors, such as RKIP, E-Cadherin, TIMP3, PTEN, and ΔN-p63. SNAI1 also promotes immune suppression, while p53/p63/p73 opposes it. The metastasis suppressor RKIP inhibits the activation of Ras-Raf-MEK-HMGA2-SNAI1 signaling cascade by inhibiting the expression of c-Raf. Additionally, it inhibits the expression of c-myc and its target gene Lin-28, and thereby increases the expression of the tumor suppressor miRNA, let-7(a putative transcriptional target of p53/p63/p73). This in turn inhibits the expression of lin-28, c-myc, Ras and HMGA2. Down regulation of Ras-MEK signaling cascade may inhibit the expression of HDM2. This in turn will result in increased stability and activity of the tumor suppressor p53/p73/p63. Dicer 1, a putative transcriptional target of p63/p73, suppresses invasion and metastasis. Dotted arrow, an indirect target.

Fig.2. **How TA-p73/p63/p53 induces the expression of Iet-7.** The tumor suppressor TA-p73/p63/p53 increases the expression of let-7, which in turn suppresses the expression of genes involved in cell cycle, cell proliferation, replication, oncogenic kinases, and transcription factors. Let-7-dependent down regulation of these proteins may result in up regulation of tumor suppressor genes (let-7; p53/TA-p73/p63; INK4a/b/ARF; CDH1; PTEN; CDKN1 a/b/c; c-myc-suppressed tumor suppressor miRNAs/genes). Dotted arrow denotes a putative target.

Fig.3. **How p53/TA-p73/p63 increases the expression of c-myc-suppressed miRNAs (let-7, miR-29, miR-15/16, miR-26, miR-34, miR-30 and miR-146).** Increased expression of let-7

suppresses the expression of key oncogenes (k-ras; HMGA2; EGFR) and stem cell factors (Lin-28; Log2/6; 4-12) that promote tumorigenesis and cancer stem cell proliferation. By negatively regulating HMGA2, let-7 increases the expression of INK4a/ARF. One of the c-myc-suppressed miRNAs, miR-29 suppresses DNMTs that are known to hypermethylate tumor suppressor gene/miRNA's promoters, including TA-p73, miR-148 and miR-34. miR-148 appears to target HIF-2a, a positive regulator of stem cell factors Oct-4, Sox-2, Klf-4, Nanog, c-mys, and Twist. The role of other c-myc suppressed miRNAs (miR-15/16, miR-26, miR-34, miR-30, and miR-146) in the inhibition of tumorigenesis is described in the text. Both c-myc and let-7 oppose each other's expression and share a double negative feedback loop. Dotted arrow = a putative target.

Fig.4. **How TA-p73, TA-p63, and p53 inhibit EMT, invasion and metastasis.** p53/TA-p73/p63 negatively regulates the metastasis initiators (ZEB 1 and ZEB2) and the EMT through its target miRs (miR-145, miR-192, miR-29, miR-215, and miR-23). Down regulation of ZEB1 and ZEB2 results in up regulation of the metastasis suppressors E-Cadherin, TA-p73, and ΓNK4B. c-Myc increases the expression of genes—such as Skp-2 [RhoA-mDIA/ROCK], HIF-2a [Oct-4-Sox-2-Klf4-Nanog; Twist] and lin-28 [let-7-log2/6; log4-12]~that promote metastasis and CSCs proliferation. c-Myc-dependent up regulation of Skp-2/BMI-l down regulates CDK inhibitors. HIF-1a increases the expression of Twist and thereby activates the metastasis cascade miR-lOB-HB-lOD-RhoC. Together, p53/TA-p73/p63 suppresses c-myc, HIFs, and ZEB 1/2 expression through its target miRs and thereby inhibits EMT, CSCs, invasion, and metastasis.

Fig.5. **The p53/TAp73/p63-dependent degradation of c-myc results in down regulation of oncogenic miRNAs and activation of tumor/metastasis suppressor genes.** p53/TAp73/p63 suppresses c-myc through its protein-coding (PTEN,TRIM32 & FBXW7) and non-coding (miR-145, let-7 & miR-34) target genes. c-Myc increases the expression of both its protein-coding (Skp-2) and non-coding (miR- 17-92, miR-22 1/222 & miR-9) targets to suppress the expression of tumor suppressor genes. * denotes a putative target.

Fig.6. **The p53/TA-p73/p63-dependent tumor suppressor miRNAs network.** An integrated view of how p53/TA-p73/p63-dependent tumor suppressor miRNAs' network activates tumor suppressor genes and thereby inhibits EMT, CSCs, migration, invasion, and metastasis. Dotted arrow denotes a putative target.

Fig 7. A dual promoter containing expression vector. Myc-LR(renilla); TS-G(Tumor suppressor gene/miR(miRNA)-LF(firefly); SV-40 sarcoma virus promoter; PA-poly adenylation tail; Neo-Neomycin gene.

2. CLAIMS

I claim:

1. A stable cell line that expresses both c-myc and tumor suppressor TAp63/p73/p53/INK4a/b/c/d/CDKNla/b/c/ARF/RKIP/CDHl/PTEN/Ago-lto4/FBW7/RBs/CycG2/DEC2/DOK2/AMLl/2/BRCAl/p38a/TSCl/MEK4/PPP2R2A/TSPl/BRMSl/E2Fl/2/TIMP3/CTGF/SMAD2/RRM2B/MXIl/DMTF/CHD5/miR-15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23/miR-22/miR-203/miR-200/miR-134/miR-miR-192/miR-215 promoters will be generated. Any combination of c-myc promoter plus tumor suppressor promoter will be chosen (for e.g., c-myc+p53, c-myc+INK4a, c-myc+miR-145, c-myc+miR-15/16 and so on) to generate stable cell lines.

2. c-Myc promoter will be linked to renilla luciferase; and TAp63/p73/p53/rNK4a/b/c/d/CDKN 1a/b/c/ARF/RKIP/CDH 1/PTEN/Ago 1to4/FB W7/RBs/Cyc G2/DEC2/DOK2/AML1/2/BRCA l/p38a/TSC 1/MEK4/PPP2R2A/TSP 1/BRMS 1/E2F 1/2/TIMP3 /CTGF/SMAD2/RRM2B/MXIl/DMTF/CHD5/miR-15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23/miR-22/miR-203/miR-200/miR-134/miR-192/miR-215 promoter will be linked to firefly luciferase. These two gene fragments will be cloned into a single mammalian expression vector containing resistance markers (eg., G418, purinomycin etc.).

3. The expression vector, as stated in 2, will be used to generate stable cell lines/clones (any mammalian cell lines).

4. The stable cell lines, as stated in 3, will be used to screen for compounds that simultaneously suppress c-myc promoter and induce tumor suppressor TAp63/p73/p53/INK4a/b/c/d/CDKN 1a/b/c/ARF/RKIP/CDH 1/PTEN/Ago 1-4/FBW7/RBs/CycG2/DEC2/DOK2/AML 1/2/BRCA 1/p38a/TSC 1/MEK4/PPP2R2A/TSP 1/BRMS 1/E2F 1/2/TIMP3/CTGF/SMAD2/RRM2B/MXI l/DMTF/CHD5)/miR(microRNA)- 15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23/miR-22/miR-203/miR-200/miR-134/miR-miR-192/miR-215 promoter will be selected for further evaluation. Compounds that induce tumor suppressor genes/miRNAs alone will also be selected for further evaluation.

5. RNPC1 promoter will be linked to renilla luciferase; and p63/p53/miR-15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23/miR-22/miR-203/miR-200/miR-134/miR-192/miR-215 promoter will be linked to firefly luciferase. These two gene fragments will be cloned into a single mammalian expression vector containing resistance markers (eg., G418, purinomycin etc.).

6. The stable cell line, as stated in 5, will be used to screen for compounds that simultaneously suppress RNPC1 and induce tumor suppressor p63/p53/miR-15/16/let-7/miR-34/miR-145/miR-26/miR-29/miR-30/miR-23/miR-22/miR-203/miR-200/miR- 134/miR- 192/miR-2 15 promoter activities will be selected for further evaluation.

7. A stable cell line—any mammalian cell line—that expresses Dicer l/let-7/Pax5/CDC6/ARK-1/2/MYCN promoter linked to renilla/firefly luciferase gene will be generated.

8. The stable cell line, as stated in 7, will be used to screen for compounds that induce or suppress Dicer l/Pax5/let-7/CDC6/ARK-l/2/MYCN promoter activity will be selected for further evaluation.

9. The components of the biological pathways mentioned below will be used for diagnosis, prognosis, and treatment of a number of disease conditions, including cancer.

a. p53/TA-p73/p63-miR-145-c-myc-lin-28/miR-17-92-let-7 (e.g., lung cancer)

b. p53/TA-p73/p63-miR-145/let7/miR-34-c-myc- miR- 17-92

c. TA-p73/p63-miR-145/let7/miR-34-c-myc- miR- 17-92/
CDKN 1c/CDKN 1a/CycG2/DEC2/AML l/DOK2/p 105/CD82/Dicer/DMTF/CHD5/BCMS 1

d. p53/TA-p73/p63-TRIM32- c-myc-let-7

e. p53/TA-p73/p63/miR-145/miR-34/let-7/FBXW7- c-myc

f.p53/TA-p73/p63-miR- 145/miR-34/let-7/FBXW7-c-myc-let-7/miR- 15/16/miR-34/miR-26/miR-23/miR-29/miR-30/miR- 146/miR-22/miR- 150

g. p53/TA-p73/p63-miRs-192, 215, 145, 200-ZEB2/SIPl-E-cadherin

h. p53/TA-p73/p63-miR-145/34/let-7-c-myc miR-9-3-E-cadherin

i. p53/TA-p73/p63-miR-145/34/let47-c-myc- miR-221/222-TIMP3/PTEN/CDKNlb, c

j. p63-AMLl/Runx-l-ARF

k. E2F- 1-p73/miR- 15/ 16-JunB-INK4a/ARF

1. p73/p63-JunB/DMTF-ARF

m. E2F- 1-TA-p73/p63/p53-Snail-RKIP-c-myc-lin-28-let-7a/g-HMGA2-ras(Ha/N/K)

n. p53/p73/p63-JunB/AP-2/KAI 1-KiSS

o. TA-p73/p53/p63-JunB-miR-203-Snail 1/Slug/E-cadherin/PTEN

p. TA-p73/p53/p63-JunB-miR-203-BMI- INK4a/ARF

q. p53/p73/p63-let-7-c-myc/Skp-2/CKS 1B/CDK 1-p 130/RhoA-CDKN 1-A/-B/p57Kip2/INK4-a,-b-,c,-d

r. p53/TA-p73/p63-miR-200-ZEBl-TA-p73/E-cadherin/INK4B/CDKNlA

s. p53/p73/p63-miR-34/130-SRC-l-ETS-2-c-myc-Twistl— E-cadherin

t.p53/p73/p63-c-myc-miR-26-EZH2-INK4a/ARF/p 130/CDKN1B/C-DNMT3b/Klf-4/HOXA9/ HMGA2/Jagged-l/HIF-2a/AIB

u. p53/p73-c-myc-miR-30-WWPl-p63/let-7/CDKNlB/C/pl30/E-cadherin

v. p53/TA-p73/p63-let-7/miR-145-c-myc-miR-23-HIF-2a/ZEBl/CDHl /rNK4b/Skp2

w. p53/TA-p73/p63-let-7/miR-145-c-myc- miR-146 -β-catenin

x.E2F-l/2-TA-p73/p63-p57kip2/LZTSl/TSCl/PTENl/RBs/14-3-3o/AML2/INK4-miR 145/143/let-7/101/29/34(eg. lung cancer; and bladder cancer)

y. p53/p73/p63-c-myc-miR-26- HDM2, HMGA2, and Skp2-p53, INK4a, ARF, pi30, and CDKN1B/C.

10. BMI1/WWP1 promoter ,will be linked to renilla luciferase; and miR-15/16/30/CDKNlA/INK4A/PTEN/TA-p63 promoter will be linked to firefly lucifease. These gene fragments will be cloned into a single mammalian expression vector containing resistance markers (e.g., G418, purinomycin etc.). This expression vector will be used to generate stable cell lines/clones (any mammalian cell lines). These stable cell lines will be used to screen for compounds that simultaneously suppress BMI-1/WWP1 and induce miR-15/16/CDKNlA/INK4A/PTEN/TA-p63 promoter activities will be selected for evaluation (for e.g., WWPl+p63; BMIl+INK4a; BMI1+PTEN promoters and so on).

.3. **DATE AND SIGNATURE** (to be given at the end of last page of specification)

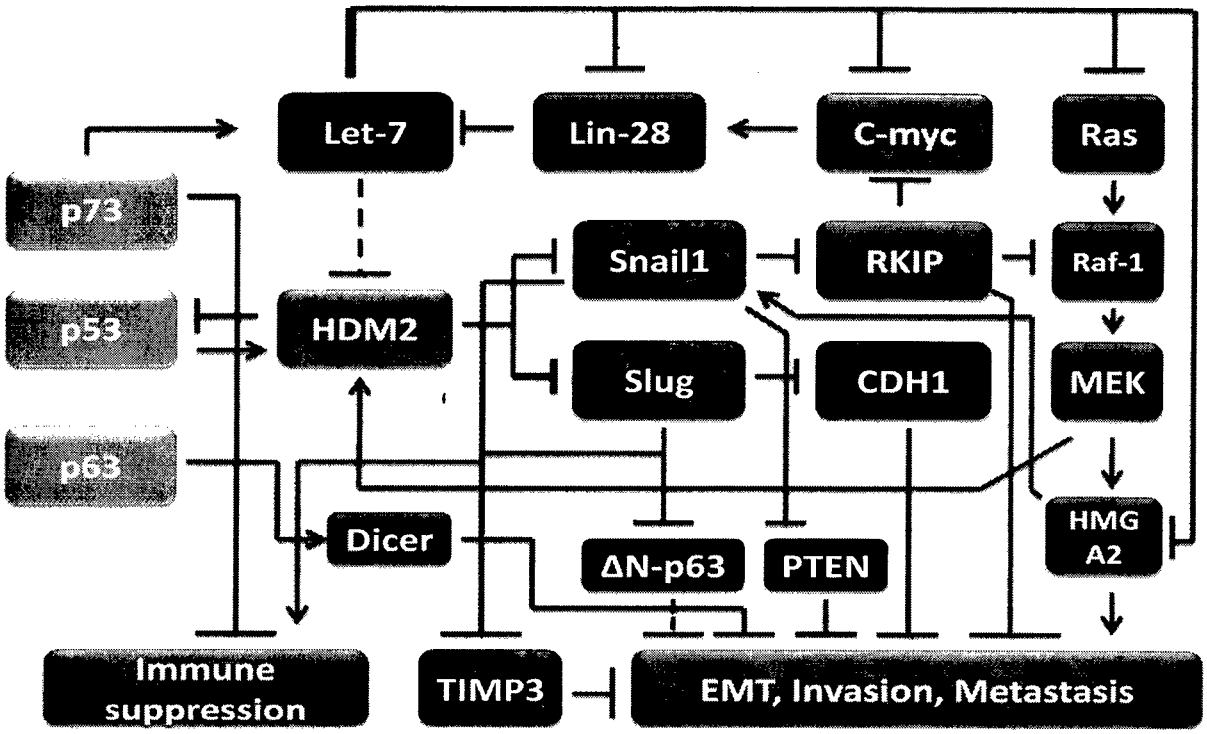

Figure1

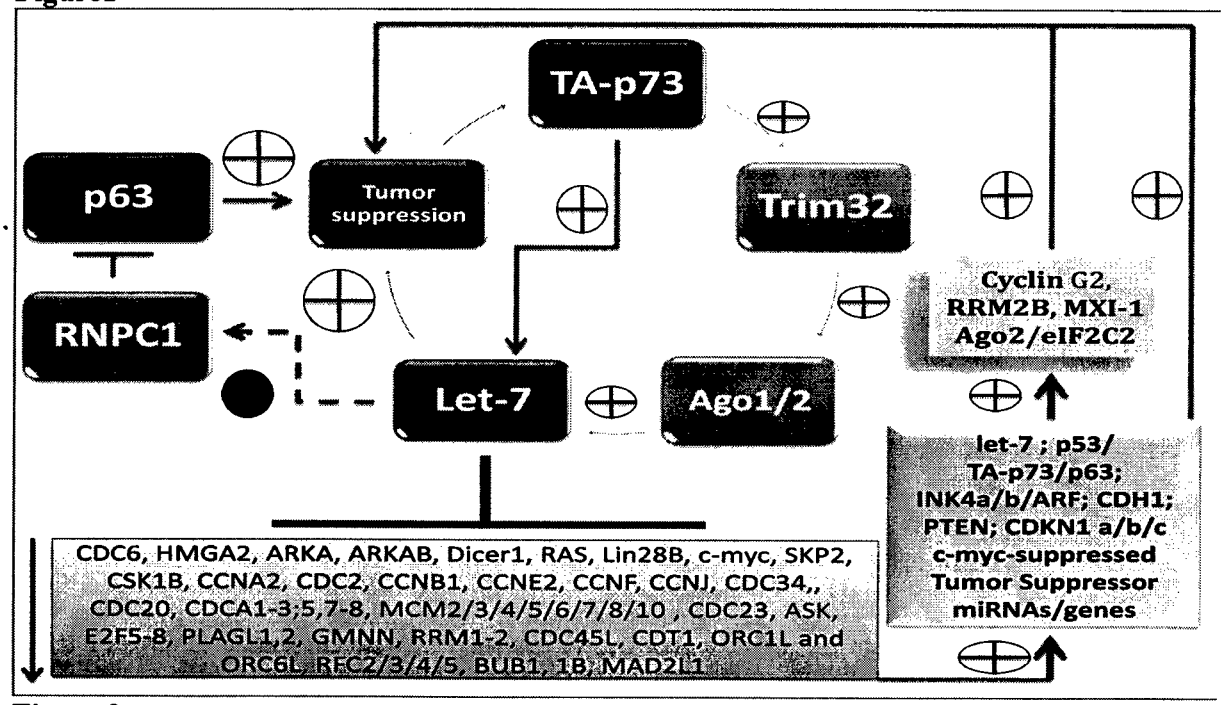

Figure 2

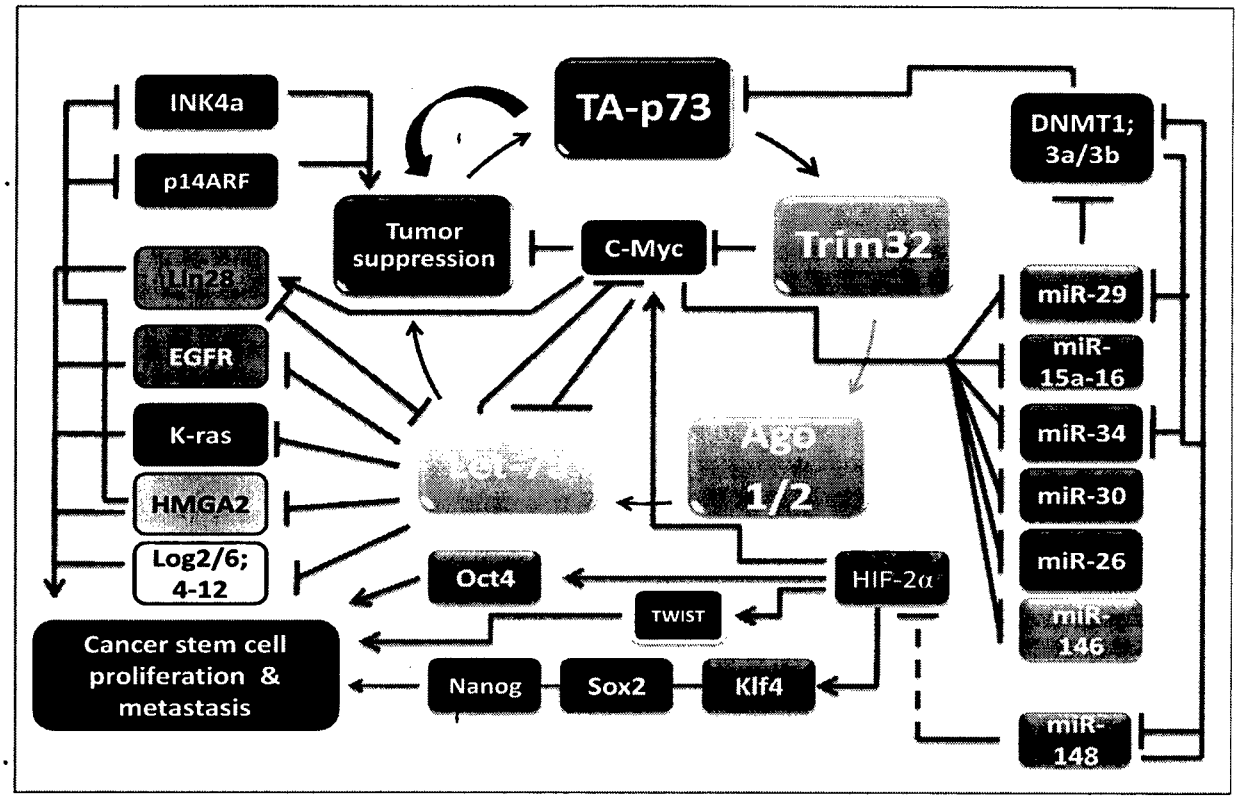

Figure 3

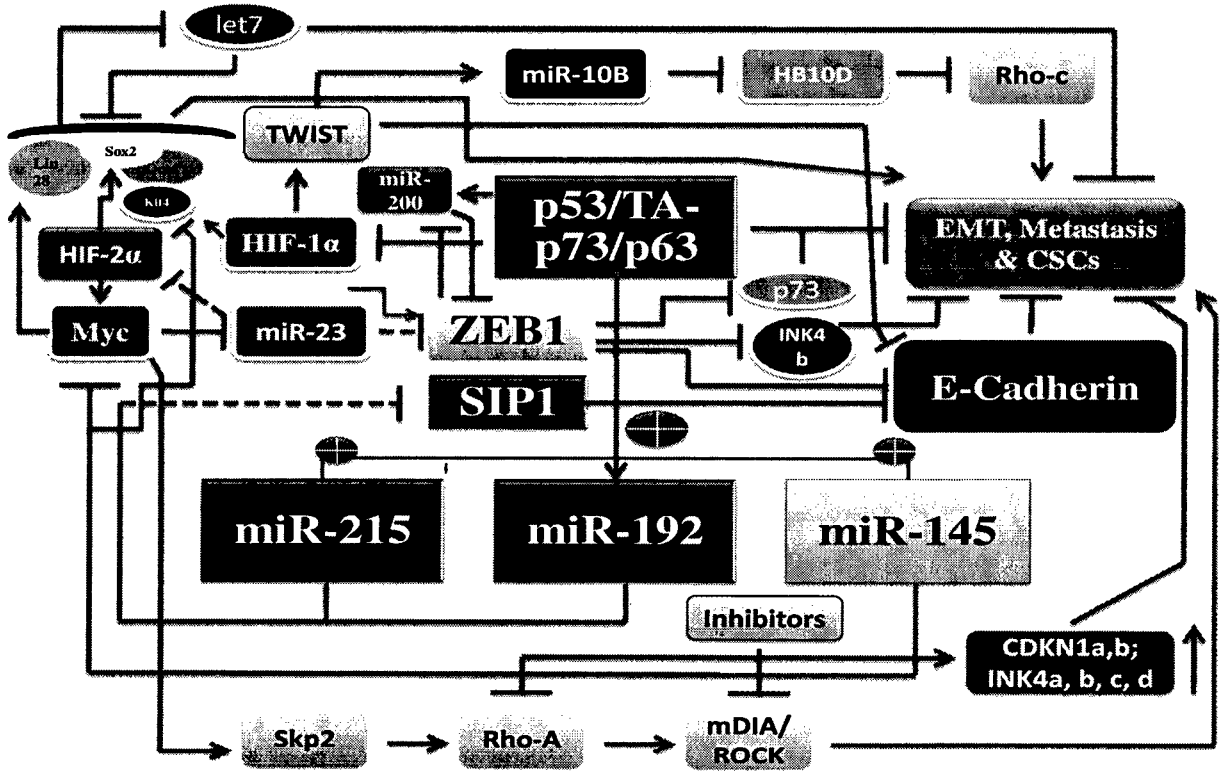

Figure 4

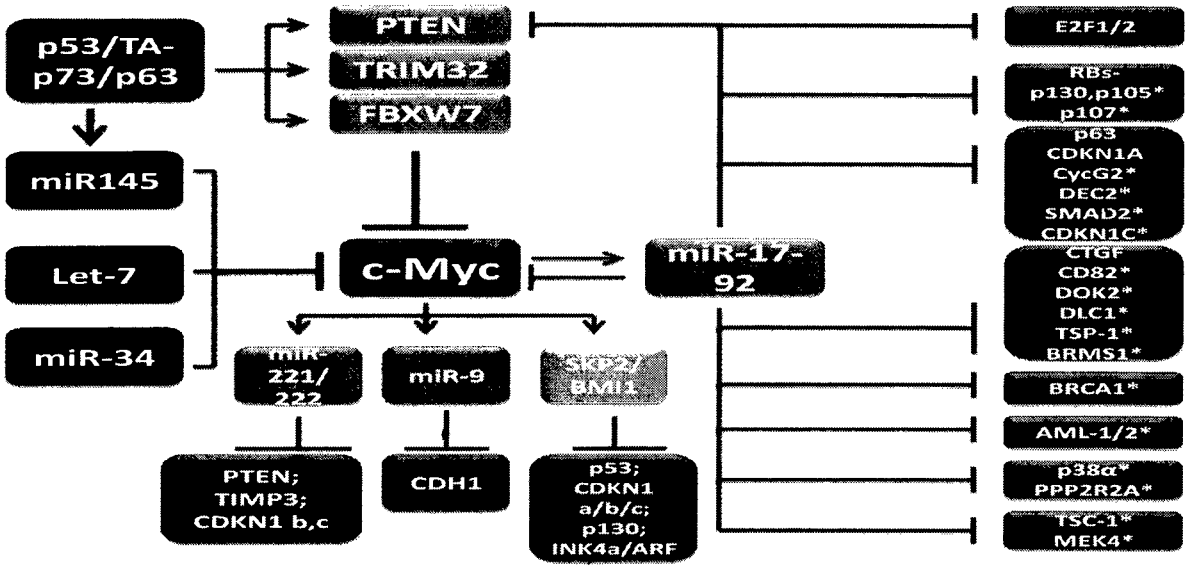

Figure 5

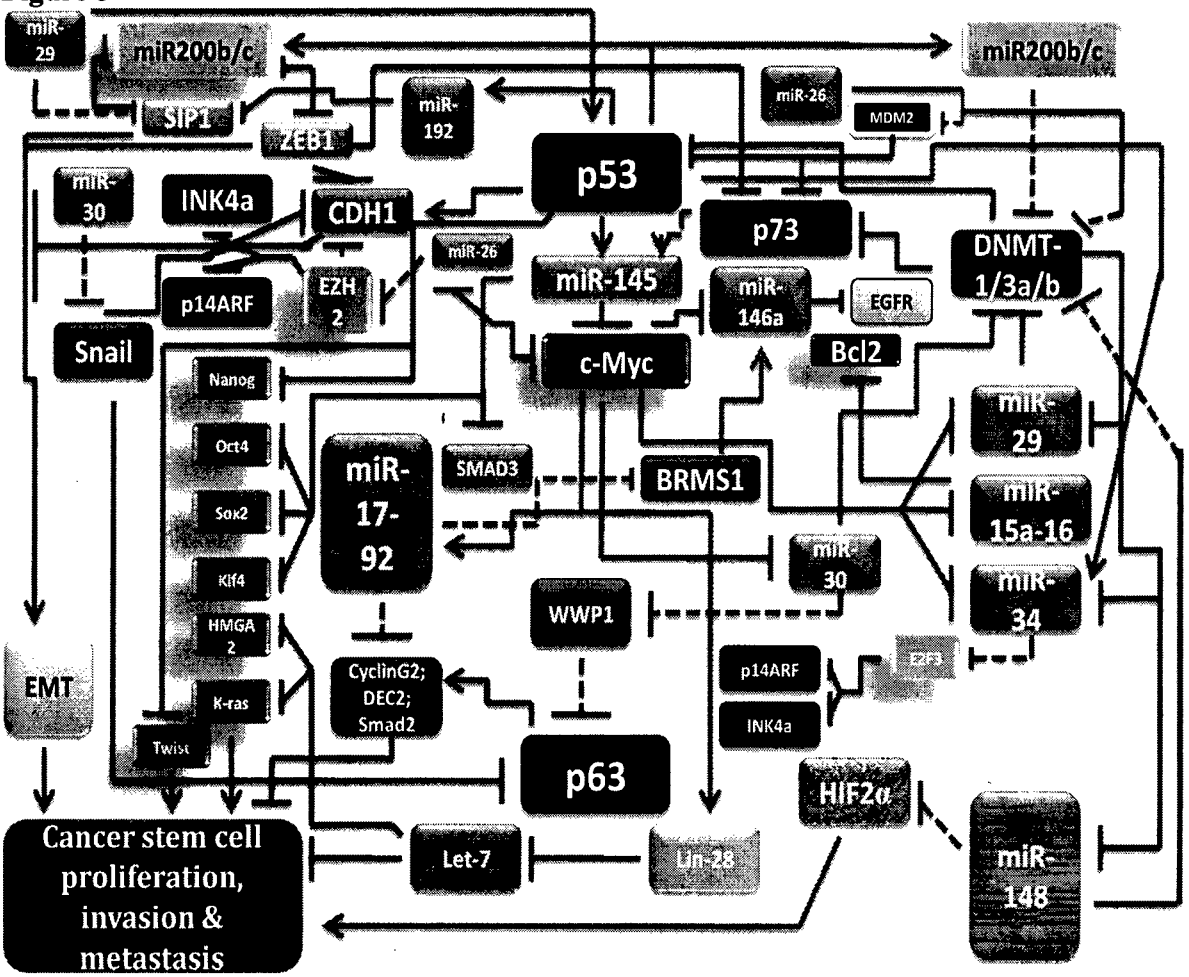

Figure 6

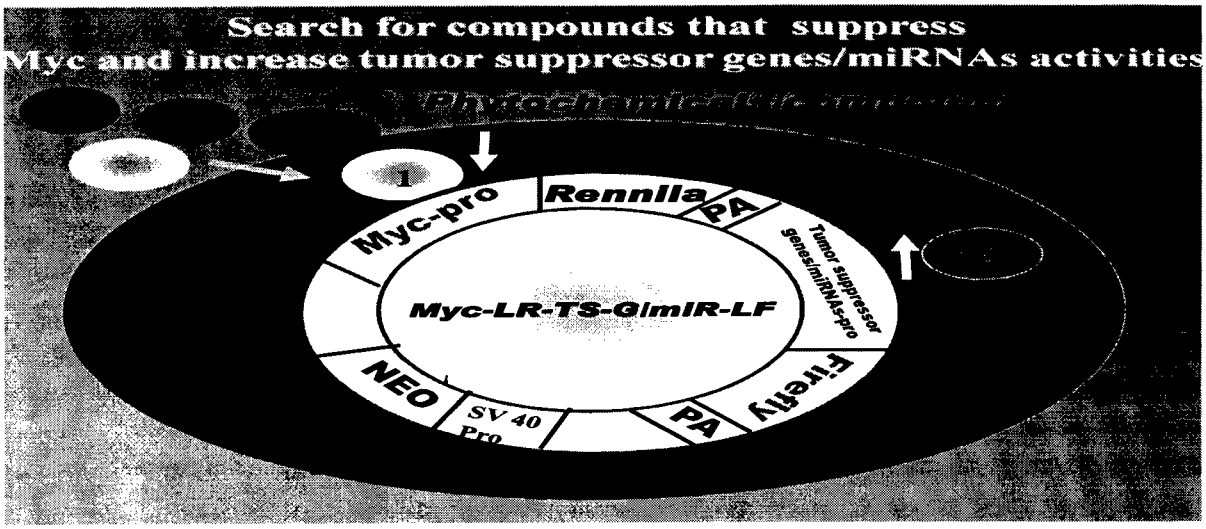
Search for compounds that suppress
Myc and increase tumor suppressor genes/miRNAs activities
1
Myc-pro
Renilla
RA
Tumor suppressor genes/miRNAs-pro
Myc-LR-TS-GlmIR-LF
Firefly
PA
SV 40 Pro
NEO

Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2011/000684

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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: C12N5/-;C12N15/-;A61K48/-

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

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

CNPAT,WPI, EPODOC, CNKI, GOOGLE SCHOLAR:p53,p73,p63,c-myc,tumor, suppress+,RNPCl, promoter,BMIl,WWPl, miR,miR-145 ,PTEN,luciferase, vector, cell, line, mammalian, let-7, dicerl ,INK4α

C. DOCUMENT S CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SACHDEVA, M. et al. p53 represses c-Myc through inductionof the tumor suppressor miR-145.PNAS. 3 March 2009 (03.03.2009), vol. 106, No. 9, pages 3207-3212. see page 3209 right column paragrph 4, page 3208 right column paragrph 1, Figures 3A,3B,4E and 4F.	*1-4,9*
X	SITU, Limin et al. RNPCl , an RNA-binding protein and a target of the p53 family, is required for maintaining the stability of the basal and stress-induced p21 transcript. Genes Dev. 18 October 2006(18.10.2006) ,vol.20, pages 2961-2972. see page 2970 left column paragrph 2.	5,6
X	LEE,Y.S. et al. The tumor suppressor microRNA let-7 represses the ITMGA2 oncogene, Genes Dev. 16 April 2007 (16.04.2007), vol. 21, pages 1025-1030. see page 1027 left column paragrph 3, Figures 2A and 2B.	7,8

☒ Further documents are listed in the continuation of Box C. ☒ 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 February 2012 (07.02.2012)	**15 Mar. 2012 (15.03.2012)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer XINQWeiling Telephone No. (86-10)62414331

Form PCT/ISA /210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2011/000684

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FAN,C. et al. PTEN inhibits BMIl function independently of its phosphatase activity.Molecular Cancer. 10 November 2009 (10.11.2009), vol. 8, pages 98-111. see page 9 of 14, left column paragrph 3,Fig 6, page 2 of 14,right column paragrph 2.	10
A	WO 2008/088858 A2 (THE JOHNS HOPKINS UNIVERSITY) 24 July 2008 (24.07.2008). see the whole document.	1-10
A	EP 2202309 A1 (KYOTO UNIVERSITY) 30 June 2010 (30.06.2010).see the whole document.	1-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2011/000684

Continuation of: A. CLASSIFICATION OF SUBJECT MATTER OF SECOND SHEET

C12N5/00 (2006.01)i

C12N5/09 (2010.01)i

C12N15/79 (2006.01)i

A61K48/00 (2006.01)i

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2011/000684

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
WO 2008/088858 A2	24.07.2008	EP2111408 A2	28.10.2009
		JP2010516249A	20.05.2010
		US2010298407A1	25.11.2010
		WO2008088858A3	18.12.2008
EP 2202309 A1	30.06.2010	US2009246875A1	01.10.2009
		JP2010158171A	22.07.2010