TRANSCRIPTIONAL REGULATION IN CANCERS AND METABOLIC DISEASES

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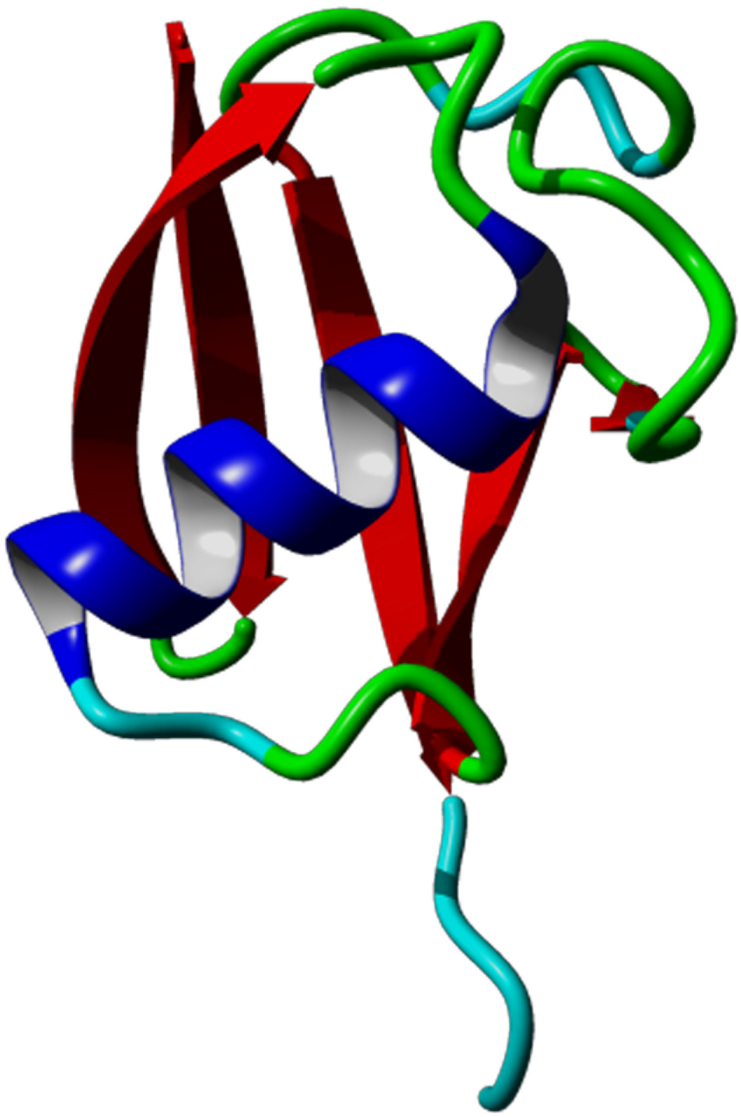

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TRANSCRIPTIONAL REGULATION IN CANCERS AND METABOLIC DISEASES

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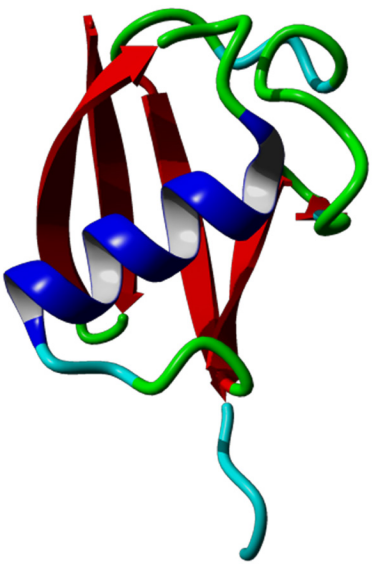

Figure modified from Yoo HM, Park JH, Jeon YJ, Chung CH. Ubiquitin-fold modifier 1 acts as a positive regulator of breast cancer. Front Endocrinol (Lausanne). 2015 Mar 20;6:36. doi: 10.3389/fendo.2015.00036.

The transcription factor (TF) mediated regulation of gene expression is a process fundamental to all biological and physiological processes. Genetic changes and epigenetic modifications of TFs affect target gene expression during the formation of malignant cells. Extensive work has been done on the critical TFs in various disease models. Despite the success of numerous TF-targeted therapies, there remain significant hurdles understanding the mechanisms, transcriptional targets and networks of physiologic pathways that govern TF action. This effort is now beginning to produce exciting new avenues of research.

A clinically relevant topic for genetic change of TF is the mutant isoforms of p53, the most famous tumor suppressor. The p53 mutations either results in loss of function, or acting as dominant negative for wild-type protein, or 'gain of function' specifically promoting cancer survival. The gain of function is achieved by shifting p53 binding partner proteins, or changed genomic binding landscape leading to a cancer-promoting transcriptome. Another example of genetic change of TF causing malignancy is the AML-ETO fusion protein in the human t(8;21)-leukemia. The fusion protein is an active TF, and more interestingly, new studies link the disease causing role of AML-ETO to the unique transcriptome in the hematopoietic stem cells. Nuclear receptors (NR) are a group of ligand-dependent TFs governing the expression of genes involved in a broad range of reproductive, developmental and metabolic programs. Genetic changes and epigenetic modifications of NRs lead to cancers and metabolic diseases. Androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR) are well studied NRs in prostate, breast and endometrial cancers. The development in

sequencing technology and computational genomics enable us to investigate the transcription programs of these master TFs in an unprecedented level.

This Research Topic aims to present the most up-to-date progress in the field of transcription regulation in cancers and metabolic diseases.

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EDITORIAL
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Editorial: Transcriptional Regulation in Cancers and Metabolic Diseases

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Keywords: p53, nuclear receptor, cell cycle, cancer stem cell, epigenetics, microRNA, exosome, transcriptional regulation

I am privileged to edit this Research Topic, *Transcriptional Regulation in Cancers and Metabolic Diseases* under the guidance of Dr. Carol Prives. We hereby thank Drs. Antonino Belfiore and Claire Perks for giving us the opportunity of editing the Research Topic. Given the large number of recent publications on transcriptional regulation, this Research Topic is timely needed. It covers many research hotspots, including p53's gain-of-function mutation, p63's role in epithelial cells, mitosis- or senescence-related transcription, and cancer-specific exosome. Overall, this *Research Topic* reviews and updates the current trends in transcriptional regulation.

Two *Mini Reviews* summarize the roles of heat shock proteins in transcriptional regulation (1, 2). In the first *Mini Review*, Alexandrova and Marchenko focused on a heat shock protein HSF1 in mutant p53 (mutp53)-mediated oncogenic activation. They highlighted a novel connection between HSF1 and mutp53 and hypothesized that pharmaceutically disrupting HSF1-mutp53 cooperation might be beneficial to cancer patients. In the second *Mini Review*, Khurana and Bhattacharyya outlined mechanisms through which another heat shock protein HSP90 activates gene expression. That is, HSP90 co-activates transcription factors, interacts with chromatin remodeling factors, and evicts histones from certain gene promoters.

Three *Reviews* explain transcriptional regulation of fate-determining transcription factors or cofactors (3–5). In the first *Review*, Yoh and Prywes illustrated the regulatory network that impinges upon the key epithelial transcription factor p63. In the second *Review*, Yoo and colleagues focused on a ubiquitin-fold modifier, UFM1 in breast cancer. In the third *Review*, Sun and colleagues categorized lysine acetyltransferases in normal and abnormal development of blood cells. Notably, Sun et al. reported current drug developments in lysine acetyltransferase inhibitors.

Two *Original research articles* evaluate transcriptional regulation in uveal melanoma and aging, respectively (6, 7). In the first *Original research article,* Huffman, Carstens, and Martinez profiled the expression levels of 48 human NRs across a panel of cell lines from uveal melanoma, cutaneous melanoma and melanocytes. In addition, the NR-to-NR and NR-to-genome expression correlation analyses identified RXRγ as a potential driver for melanoma-specific signaling, and ERRα as the uveal-melanoma-specific NR. In the second *Original research article*, Ma and colleagues analyzed microarray data of differentially expressed genes after the knockdown of the cellular senescence-inhibited gene (CSIG). CSIG, originally identified by this research group, is critical in regulating cell senescence, cell cycle progression, stress response, and tumor metastasis. In this article, they discovered novel CSIG targets that correlate with senescence. Interestingly, they inferred that CSIG regulates the stability of certain target-gene transcripts.

Nath and colleagues presented an interesting *Review* on mitotic proteins in cancer development (8). As the authors pointed out, deregulated transcriptional regulation of mitotic genes are common in cancers, yet mutations are rarely observed for mitotic genes. Cell cycle-related transcription program is interesting and warrants further investigation.

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One *Review* and one *Opinion article* appraised the possible use of exosome in early cancer diagnosis (9, 10). In the *Review* article, Qin and colleagues summarized the recent progress on the identification of miRNAs from tumor samples. They grouped different miRNAs based on their location as the cytosolic, body-fluid or exosomal miRNAs. The authors proposed exosomal miRNAs as diagnostic biomarkers for lung tumors. In addition, they described the current methods for the isolation and detection of these RNAs in the tumor samples. Moreover, the authors hypothesized how miRNAs have been transported/released into body fluids from the tumor cells. In accompanying *Opinion article*, Oltra supported cancer-associated exosome in cancer diagnosis and prognosis.

In the end of this Research Topic, Davis and colleagues presented a very interesting *Review* on Nanotetrac in treating cancers (11, 12). In many cancers, Thyroid hormones, T_3 and T_4 have pro-angiogenic effects, which might be mediated by $\alpha_V\beta_3$ integrin. Nanotetrac is a nanoparticulate preparation of a T_4 substitute, tetrac. Nanotetrac blocks T_4-triggered $\alpha_V\beta_3$-mediated transcriptional regulation. Therefore, Nanotetrac might be useful in treating cancers.

By compiling all these excellent manuscripts into one Research Topic, we hope that our readers will find this Research Topic enlightening. We owe our thanks to the staff of Frontiers Endocrinology Office, for their work in the completion of this Research Topic. We are particularly thankful to Davor Vidic, Shaun Evans, Caroline Drage and Byron Bitanihirwe for help in communicating with authors during initiation and completion of the Research Topic. All authors in this Research Topic have provided their broad perspectives on transcriptional regulation, whose insightful thoughts will both benefit the field and be appreciated by fellow researchers. We are indebted to our reviewers/review editors for their contribution to this work.

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MINI REVIEW
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Mutant p53 – heat shock response oncogenic cooperation: a new mechanism of cancer cell survival

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The main tumor suppressor function of p53 as a "guardian of the genome" is to respond to cellular stress by transcriptional activation of apoptosis, growth arrest, or senescence in damaged cells. Not surprisingly, mutations in the p53 gene are the most frequent genetic alteration in human cancers. Importantly, mutant p53 (mutp53) proteins not only lose their wild-type tumor suppressor activity but also can actively promote tumor development. Two main mechanisms accounting for mutp53 proto-oncogenic activity are inhibition of the wild-type p53 in a dominant-negative fashion and gain of additional oncogenic activities known as gain-of-function (GOF). Here, we discuss a novel mechanism of mutp53 GOF, which relies on its oncogenic cooperation with the heat shock machinery. This coordinated adaptive mechanism renders cancer cells more resistant to proteotoxic stress and provides both, a strong survival advantage to cancer cells and a promising means for therapeutic intervention.

Keywords: mutant p53, GOF, heat shock response, HSF1, Her2, Neu, EGFR

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Oncogenic Functions of Mutant p53

Mutations in the p53 tumor suppressor gene are the most frequent tumor-associated genetic alterations throughout the entire spectrum of human cancers (1). In contrast to other tumor suppressors that are commonly inactivated by frameshift and nonsense mutations resulting in loss-of-function, the majority of p53 alterations are missense mutations clustered in six "hot-spots" of the DNA-binding domain of p53 (2). Numerous mouse models, *in vitro* and clinical studies have demonstrated that in addition to simple loss of the tumor suppressor function of p53, many mutant p53 (mutp53) proteins gain neomorphic oncogenic activities, termed as gain-of-function (GOF) (2, 3). These GOF activities contribute to malignant transformation by enhancing cells proliferation, invasion, metastatic ability, and chemoresistance (2, 3). The concept of mutp53 GOF is strongly supported by human clinical studies on Li–Fraumeni syndrome (LFS) patients carrying germline p53 mutations and by GOF mouse models (4–7). Thus, several studies have found that the median age of cancer onset in LFS patients with mutp53 missense mutations is 9–20 years earlier than in LFS patients with loss of p53 expression (8, 9). Moreover, clinical evaluation of 1,794 breast cancer patients revealed that *somatic* p53 mutations are also associated with a shorter overall survival, independently of stage, grade, and hormone receptors status (10), similarly to other cancer types harboring mutp53 (1). This is fully confirmed by mouse mutp53 knock-in models, manifesting GOF by increased

Abbreviations: EGFR, epidermal growth factor receptor; GOF, gain-of-function; HSE, heat shock elements; HSF1, heat shock factor 1; HSP, heat shock protein; LFS, Li–Fraumeni syndrome; mutp53, mutant p53; NF1, neurofibromatosis type 1; PDGFR, platelet-derived growth factor receptor.

metastases, broader tumor spectrum, more invasive tumor fronts, more malignant histology, and higher tumor bulk compared to p53-null tumors (4–7).

A broad spectrum of GOF activities has been described for mutp53 [reviewed in Ref. (2, 3)]. Among the most prominent ones is the ability of mutp53 to promote cells proliferation, invasion, and motility by stimulating signal transduction pathways downstream of growth factor receptors, such as TGFβ receptor (11), EGFR (4, 12, 13), MET (14), PDGFRβ (15), as well as ErbB2/Her2 (4, 13) discussed below. Also, a number of *in vitro* and *in vivo* studies have described a critical role of mutp53 in tumor initiation via enhanced generation and expansion of cell populations with stem cell/cancer stem cell properties (4, 5, 16, 17). In addition, recent reports indicate that mutp53 promotes the inflammatory response and inflammation-associated cancers by stimulating NF-κB activation (18, 19). Finally, a novel intriguing mutp53 GOF activity has been described in tumor-associated fibroblasts (20, 21), suggesting that mutp53 can play oncogenic roles not only in cancer cells but also in the tumor stromal cells.

Mechanistically, although the majority of mutp53 missense mutations map to its DNA-binding domain, mutp53 GOF activities are still largely attributed to transcriptional regulation of specific target genes, which differ from typical wild-type p53 targets [reviewed in Ref. (2)]. While mutp53-specific DNA consensus sites have not been identified, it appears that mutp53 is a potent modulator of other transcription factors and co-factors, via regulation of their DNA binding and transcriptional properties, such as p63 (11, 12, 22, 23), p73 (22), SP1 (24), SREBP (25), and others [reviewed in Ref. (2, 3)] including the master heat shock regulator HSF1 (13), discussed below.

A critical feature of most mutp53-harboring tumors is significantly increased mutp53 protein stability, manifested by massive mutp53 accumulation in tumors, but not in normal tissues (6, 26). We and others showed that cancer-specific accumulation of mutp53 is crucial for many aspects of tumorigenesis and is the key determinant of mutp53 GOF *in vitro* and *in vivo* (7, 11, 27, 28). Thus, acute downregulation of stabilized mutp53 by RNA interference (RNAi) strongly inhibits malignant phenotypes (11, 27, 28). For example, we found that stable and Tet-inducible knockdown of endogenous mutp53 in breast (MDA231) and colon (SW480) cancer cells by p53 RNAi dramatically inhibits growth of these human cancer cells *in vitro* and in xenografts and their invasive properties (28). These data are consistent with other reports showing that mutp53 downregulation by RNAi suppresses invasion (11, 28), restores normal mammary architecture in 3D culture of breast cancer cell lines (25), and inhibits metastasis *in vivo* (11, 15). Thus, cancer cells appear to be addicted to high levels of mutp53 for their survival and oncogenic properties.

Addiction of cancer cells to stabilized mutp53 underscores the translational significance of mutp53 as a promising therapeutic target. However, targeting mutp53 by conventional modalities is a very challenging task, since mutp53 is neither an enzyme nor a cell surface protein. Therefore, one promising alternative for abolishing mutp53 GOF in mutp53-harboring cancers could be its depletion/destabilization. We recently proposed that this could be achieved by exploiting mutp53 interdependence with the heat shock response machinery (13, 28, 29). Thus, we showed that mutp53 has a novel GOF activity as an essential regulator of protein homeostasis in cancer. Specifically, it augments the pro-survival heat shock response machinery via activating the master transcriptional regulator heat shock factor 1 (HSF1), which in a positive feed-forward loop further stabilizes mutp53 itself, along with other tumor-promoting clients (13). This novel oncogenic GOF activity of mutp53 may represent a unique adaptive mechanism for superior survival of mutp53-harboring cancer cells in the hostile tumor environment.

Mutant p53 and the Heat Shock Response

Inherent to malignant transformation is the constant proteotoxic stress due to aneuploidy, accumulation of reactive oxygen species (ROS), hypoxia, acidosis, and accumulation of mutated, conformationally aberrant proteins (30–32). To overcome these potentially deadly conditions for their survival, cancer cells heavily depend on molecular chaperones, heat shock proteins (HSPs), whose induction in cancer constitutes the powerful adaptive pro-survival mechanism known as the heat shock response (32). Under proteotoxic stress, induction of HSPs restores protein homeostasis by repairing and proper folding of damaged and mutated proteins with aberrant conformation. HSPs induction in cancer cells is triggered by the transcription factor HSF1 that binds to unique DNA sequence motifs known as heat shock elements (HSEs) in the promoters of HSPs, inducing their transcription (33). In unstressed cells, HSF1 is sequestered by HSP90 predominantly in the cytoplasm (30). However, proteotoxic stress induces HSF1 phosphorylation, liberation from the HSP90 inhibitory complex, trimerization, and translocation to the nucleus to activate HSPs expression (34). It has been shown that phosphorylation of HSF1 at serine 326 (pSer326) is pivotal to render HSF1 transcriptionally competent (30, 34). Also, HSF1 stabilization and activation may be induced by genetic changes such as loss of the neurofibromatosis type 1 (NF1) tumor suppressor gene (35).

The essential role of HSF1 in malignant transformation and progression is well documented in literature. Specifically, HSF1 induces a diverse array of HSP-mediated pro-survival mechanisms, including stabilization of oncogenic clients, altered glucose metabolism and signal transduction, and upregulation of protein translation (4, 32, 35, 36). Interestingly, a recent study by the Linquist group has shown that HSF1 has both distinct and overlapping activities in the maintenance of normal protein homeostasis vs. tumorigenesis (36). In cancer HSF1 orchestrates, a wide range of fundamental cellular processes that are not related to heat shock response but are critical for malignant transformation and maintenance, including cell-cycle control, ribosomal biogenesis, protein translation, and inhibition of apoptosis (36). Importantly, cancer-specific HSF1-bound genes ("HSF1 cancer signature") were found enriched in the biopsies of human breast, colon, and lung tumors and strongly correlated with poor patient outcomes underscoring the critical role of HSF1 in tumorigenesis (36). In agreement, another clinical study found HSF1 upregulation in 80% of breast cancers, which was also associated with high histologic grade and increased

mortality (37). Finally, the pivotal role of HSF1 in tumorigenesis is demonstrated in various animal cancer models. For example, genetic deficiency of HSF1 dramatically reduces mammary tumor formation in the Her2/Neu mouse model (38), tumorigenesis in the DMBA-induced skin carcinogenesis and in the mutp53 mouse models (31).

The connection between mutp53 and heat shock response has been known for nearly two decades (39). Thus, mutp53 and HSP90 (one of the most explored HSF1 transcription targets) were shown to physically interact, which was linked to cancer-specific mutp53 stabilization (28, 29, 39, 40). We and others subsequently demonstrated that pharmacological or RNAi-mediated inhibition of HSP90 leads to ubiquitination and proteasomal degradation of mutp53, mediated by the E3 ubiquitin ligases MDM2 and CHIP (28, 29, 40, 41). Moreover, it is likely that other E3 ligases might be involved as well. Thus, a recent report suggests that arsenic trioxide, a drug used to treat acute promyelocytic leukemia, cooperates with HSP90 inhibitors and promotes mutp53 degradation in tumor cells by the Pirh2 ubiquitin ligase (42). It would be interesting to see whether Pirh2 is inhibited by HSPs during malignant transformation similarly to MDM2 and CHIP (29, 40), leading to mutp53 aberrant stabilization, and fueling of its oncogenic properties.

An important evidence for mutp53-HSP90 oncogenic cooperation comes from the studies showing that HSP90 inhibition shows preferential cytotoxicity in mutp53 – rather than in wild-type p53 or p53null – cancer cells and destabilizes mutp53 (28, 29). *In vivo* studies also provide compelling evidence for mutp53-heat shock response oncogenic cooperation. Thus, HSF1 genetic deficiency prolonged median overall survival of mutp53 mice (R172H) in a dose-dependent manner, from 427 to 470 to >622 days in $HSF1^{+/+}$ vs. $HSF1^{+/-}$ vs. $HSF1^{-/-}$ animals, respectively (31). This strongly suggests that either (i) HSF1/HSPs directly maintain mutp53 levels/activity or (ii) the oncogenicity of mutp53 critically depends on HSF1 and/or HSF1-mediated transcriptional program, or both. Although mutp53 levels were not examined in this study (31), it is tempting to speculate that they were reduced in HSF1-deficient tumors as a result of insufficient transcriptional upregulation of HSP90, leading to restrained malignant transformation. In support of this idea, we previously showed that shRNA-mediated knockdown of HSF1 in mutp53 cancer cells induces rapid destabilization of mutp53 and reduces its half-life, along with reduction of HSP90 levels (29). Further studies are needed to confirm this idea *in vivo* and to test the other possible mechanisms of mutp53-HSF1 oncogenic cooperation. Besides that, it would be interesting to examine whether other HSF1 transcriptional chaperone targets besides HSP90, e.g., HSP70 and HSP27, also can stabilize mutp53 in cancer cells and via what mechanisms. Interestingly, a novel mechanism of mutp53 degradation via chaperone-mediated autophagy was recently reported. It has been shown that heat shock cognate protein 70 (HSC70), a constitutive cytosolic protein (*not* regulated by HSF1), can target mutp53 to lysosomal degradation, but only in non-proliferating tumor cells under the condition of proteasomal and macroautophagy inhibition (43). However, the importance of this mechanism in proliferating cancer cells remains to be elucidated.

Mutant p53 Induces HSF1 Transcriptional Activity via Her2 and EGFR

Although mechanisms regulating the heat shock response in tumor cells are not fully understood, it is well established that heat shock response strongly depends on the transcriptional activity of HSF1. Stress-activated transcriptionally competent form of HSF1 is a homo-trimer, which can be post-translationally modified resulting in HSF1 nuclear translocation and binding to HSP promoters (27, 30). In response to proteotoxic stress, HSF1 becomes phosphorylated at serine 326, which is essential for HSF1 transcriptional activity (27, 31).

A number of studies suggest that the Her2/EGFR2/Neu signaling pathway is an important activator of HSF1, at least in breast cancer. Thus, Her2 overexpression in MCF7 cells leads to increased HSF1 levels and its trimerization (44). Also, HSF1 is necessary and sufficient for Her2-induced transformation of normal breast epithelial cells MCF10A *in vitro* (45). Finally, HSF1 genetic knockout significantly reduces mammary tumorigenesis in the ErbB2 transgenic mouse model (38). Clinically, the presence of high levels of nuclear HSF1 in Her2-positive mammary tumors correlates with poor patient prognosis (37). Moreover, recent studies demonstrate existence of a linear signaling pathway between Her2 and HSF1 in Her2-positive breast cancer, both *in vitro* (13, 46, 47) and *in vivo* (4, 47). Thus, Schulz et al. showed that Her2 overexpression constitutively activates HSF1, resulting in stabilization of HSP90 clients, such as MIF, AKT, mutp53, and HSF1 itself (47). Moreover, pharmacological inhibition of Her2 strongly suppresses HSF1 activation *in vitro* and in the Her2 mouse transgenic model, which correlates with reduced mammary tumor progression (13, 47). Mechanistically, Her2 signals via the phosphoinositide-3 kinase (PI3K)–AKT axis to induce pSer326 phosphorylation of HSF1 and its transcriptional activity (47).

Intriguingly, the Lindquist group found that activation of HSF1 can be triggered not only by environmental and proteotoxic insults but also by genetic alterations. Thus, loss of the tumor suppressor gene neurofibromatosis type 1 (NF1) increases HSF1 levels and induces HSF1 phosphorylation at Ser326, which depends on dysregulated RAS/MAPK signaling, suggesting a key role of RAS/MAPK pathway in the transcriptional activation of HSF1 (35). Consistently, Stanhill et al. showed that RAS activation induces HSF1-mediated upregulation of HSP70 (48). These findings are strongly supported by clinical data. Thus, elevated expression of HSF1 and pSer326-HSF1 was found in surgical specimens of malignant peripheral nerve sheath tumors driven by loss of NF1 (35). Given the importance of HSF1 in cancer cell physiology (36), it will be interesting to see whether loss of other tumor suppressor genes, especially those leading to MAPK/PI3K pathway activation, could trigger transcriptional activity of HSF1 in different tumor types.

How exactly does mutp53 cooperate with HSF1 in cancer? On one hand, as mentioned above, HSF1 genetic ablation profoundly alleviates tumorigenesis driven by mutp53 *in vivo* (31), which could be due to insufficient levels of HSP90 to support mutp53 accumulation in tumors (29, 39, 40). On the other hand, our recent findings demonstrate that mutp53 is also an important

determinant of HSF1 activity. Thus, overexpression of various mutp53 alleles in cancer cell lines leads to upregulation of HSF1, with concurrent increase in heat shock response and prototypical HSP clients (13). Furthermore, we found that this mutp53-HSF1 positive feed-forward loop depends on growth factor receptor signaling, specifically EGFR and Her2 pathways, for HSF1 transcriptional activity (4, 13).

Since mutp53 is known to exert its GOF activities via other growth factor receptor signaling pathways besides Her2 and EGFR (see above), these pathways could also be involved in mutp53-HSF1 oncogenic cooperation. Preliminary indirect evidence of this possibility comes from the finding by the Linquist lab that HSF1 enables cellular transformation of mouse embryo fibroblasts via PDGFRβ signaling (31), which was shown to be positively regulated by mutp53 as well (15). However, it remains to be determined whether other growth factor receptor signaling pathways – besides EGFR and Her2 – can modulate HSF1 activity in a mutp53-dependent manner.

Recently, we found another, more direct mode of the mutp53-HSF1 circuit regulation in cancer cells. Thus, upon proteotoxic stress and nuclear translocation, phospho-activated pSer326-HSF1 interacts (directly or indirectly) with mutp53. Moreover, mutp53 facilitates HSF1 recruitment to specific DNA sites (HSEs) in target gene promoters and augments a broad pro-survival HSF1-induced transcriptional program, including expression of HSPs (13).

Finally, as mentioned above, HSF1 transcriptional activity can be stimulated by genetic alterations in cancer (35). Thus, the well-known ability of mutp53 to induce genomic instability (49) could in theory be another mechanism contributing to HSF1 activation in cancer, which remains to be tested.

Based on the described findings, we propose a novel mechanism of mutp53 GOF, via regulation of the heat shock response, as depicted in **Figure 1**. We propose that mutp53 through enhanced recycling (12) and/or stability of EGFR and Her2/ErbB2 – augments MAPK and PI3K signaling, leading to phospho-activation of HSF1. Concurrently, mutp53 directly interacts with activated HSF1 and facilitates its binding to specific promoter elements and stimulates transcription of HSPs. In turn, HSPs further stabilize their oncogenic clients, including EGFR, Her2, and mutp53 itself (via suppression of ubiquitin ligases MDM2, CHIP, etc.), reinforcing tumorigenesis (**Figure 1**). Importantly, this mechanism has been confirmed by *in vivo* studies. First, clinical data show a strong enrichment (83%) of the Her2-positive breast cancer in LFS women with p53 germline mutations, compared to 20–25% Her2 positivity in patients with sporadic breast cancer (50). This suggests that the presence of mutp53 germline mutations strongly predisposes Li–Fraumeni women specifically to the initiation of Her2-driven breast cancer. Second, we found a strong correlation between mutp53 and nuclear pSer326-HSF1 levels only in strongly Her2-positive (3+), but not in Her2-negative/ER/PR-positive human breast cancer specimens (13). Finally, we demonstrated that the Her2/ErbB2 signaling is much more amplified in mutp53 (R172H) compared to p53null tumor cells in the MMTV-ErbB2 mouse breast cancer model, leading to a more aggressive disease (4).

Many physiological consequences can result from the described mutp53-HSF1 liaison in cancer cells. First, the resulting increased expression of HSPs endows cancer cells with a superior resistance to proteotoxic stress caused by harsh tumor environment (13). Second, it leads to enhanced stabilization of numerous tumor-promoting HSP oncogenic clients, including Her2, EGFR, and mutp53 themselves, which cell-autonomously further amplify this feed-forward circuit and oncogenesis. Third, HSPs have been shown to inhibit oncogene-induced senescence pathways in cancer cells (51). Thus, by enhancing HSP expression at early stages of tumorigenesis, mutp53 may facilitate disabling of oncogene-induced senescence and therefore empower tumor progression. Fourth, we showed that mutp53-HSF1-mediated amplification of Her2 pathway can promote expansion of mammary stem cells and induce cancer cell proliferation *in vivo* (4), which can enhance tumor initiation and progression. Finally, by augmenting the broad HSF1-dependent transcriptional program, mutp53 may promote global cancer-related changes, including cell-cycle progression, altered signaling pathways, metabolism, adhesion, protein translation, etc. (36).

In sum, it is evident that the functional oncogenic interaction between mutp53 and HSF1 can initiate a wide range of tumorigenic processes in the complex landscape of mutp53-harboring cancers.

Summary

While mutations in the p53 gene are prevailing in many types of cancer, specific therapeutic modalities tailored to mutp53-harboring cancers have not been developed in clinic. Three potential mutp53-targeted therapeutic strategies have been recently proposed: (i) restoration of wild-type p53 activity in mutp53 proteins (3), (ii) inhibition of mutp53-regulated downstream targets and pathways, e.g., proteins involved in integrin recycling (12), the mevalonate pathway (25), PDGFRβ signaling (15), etc. [reviewed in Ref. (3)], and (iii) mutp53 degradation (28, 29, 42). Overall, understanding the mechanisms underlying mutp53 oncogenic activity will no doubt have a profound translational impact. However, more in-depth studies are needed to establish whether these approaches will be clinically feasible and whether pharmacological targeting of relevant pathways will achieve preferential response in the patients with mutp53-harboring tumors.

The herein described novel oncogenic GOF role of mutp53 in the regulation of heat shock response – via enhanced receptor tyrosine kinase (Her2, EGFR) signaling and augmented HSF1 transcriptional activity – opens up novel therapeutic opportunities. We anticipate that the mutp53-HSF1 liaison, due to potentiating Her2 and/or EGFR pathways, sensitizes cancer cells to ErbB2 and EGFR targeted therapies. Thus, inhibition of Her2, EGFR, or their downstream effectors can intercept the sensitive mutp53-HSF1-Her2/EGFR circuitry and therefore be a potent approach for the treatment of Her2 (or EGFR) and mutp53 double-positive cancers. Convergence of the mutp53-HSF1 liaison on the Her2/EGFR pathways provides a strong rationale to test the targeted therapies that are currently on the market (e.g., Her2-targeted trastuzamab, pertuzumab, T-DM1, and lapatinib) specifically in mutp53-harboring cancers. The most promising cancer types expected to specifically respond to these therapies are Her2/mutp53 double-positive breast cancer [constituting 72% of all sporadic Her2-positive breast cancers (52)],

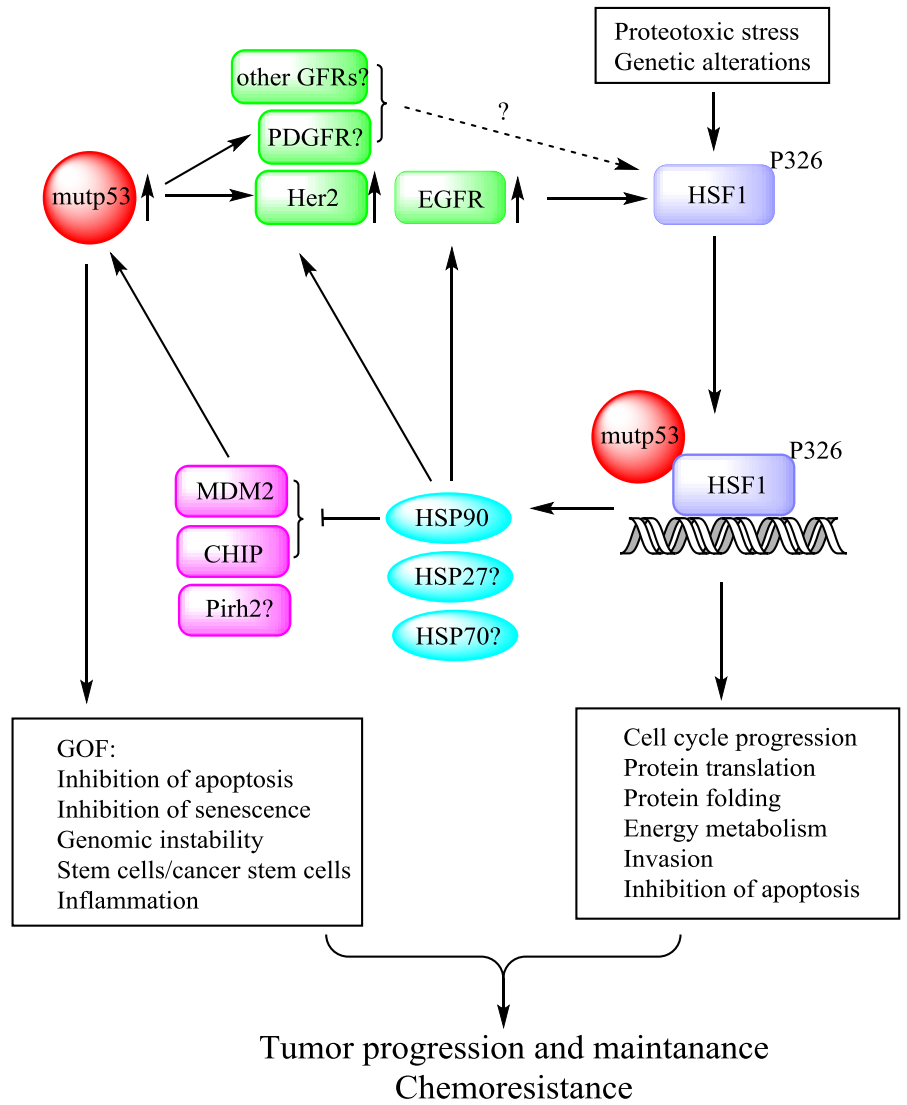

FIGURE 1 | Mutp53 potentiates HSF1 activation in an EGFR/Her2-dependent manner. Mutp53, by enhancing EGFR and Her2 signaling, potentiates HSF1 activity via a feed-forward loop and thereby up-regulates HSP90 clients including Her2, EGFR, and mutp53 itself. Thus, mutp53-HSF1 oncogenic liaison endows cancer cells with superior survival and chemoresistance, hence supporting cancer progression and maintenance.

pancreatic and non-small-cell lung cancer [both of which have high prevalence of mutp53 (1) and are commonly treated with EGFR inhibitor Erlotinib], and possibly esophageal cancer [43% mutp53-positive (1), 23% Her2-positive]. Although it remains to be determined whether the Her2-positive subtype of esophageal cancers is enriched in mutp53, similarly to breast cancer. Importantly, the data described here also predict that the patients with tumor types that commonly overexpress both, Her2/EGFR and mutp53, will need to be stratified according to their mutp53 status for the most efficient treatment outcomes.

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Hsp90, the concertmaster: tuning transcription

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In the last decade, Hsp90 has emerged as a major regulator of cancer cell growth and proliferation. In cancer cells, it assists in giving maturation to oncogenic proteins including several kinases and transcription factors (TF). Recent studies have shown that apart from its chaperone activity, it also imparts regulation of transcription machinery and thereby alters the cellular physiology. Hsp90 and its co-chaperones modulate transcription at least at three different levels. In the first place, they alter the steady-state levels of certain TFs in response to various physiological cues. Second, they modulate the activity of certain epigenetic modifiers, such as histone deacetylases or DNA methyl transferases, and thereby respond to the change in the environment. Third, they participate in the eviction of histones from the promoter region of certain genes and thereby turn on gene expression. In this review, we discuss the role of Hsp90 in all the three aforementioned mechanisms of transcriptional control, taking examples from various model organisms with a special emphasis on cancer progression.

Keywords: Hsp90, transcription, chromatin modifiers, transcription factors, cancer

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Introduction

Considerable progress has been achieved in understanding the cellular role of the major eukaryotic cytoplasmic chaperone, Hsp90. It aids in the folding and stability of numerous classes of proteins (collectively known as clients), under normal as well as stressful conditions. In normal cell, Hsp90 comprises about 2% of the total cellular proteins. However, in stressful condition, its level is increased significantly (up to 10%) with concomitant increase in its activity. Cancer cells experience a variety of stressful conditions like hypoxia, nutrient deprivation, acidosis, high interstitial pressure (1), and consequently, Hsp90 levels are found to be up-regulated in melanoma (2), breast cancer (3), gastric and pancreatic carcinoma (4, 5), ovarian and endometrial carcinoma (6, 7), etc. The increased level of Hsp90 causes chaperoning of the potentially dangerous oncogenic clients that are otherwise metastable. Thereby, Hsp90 impairs the apoptotic signaling in cancer cells. One such candidate is mutant p53, whose stability and intracellular concentration are aided by Hsp90 (8). Experimental findings establish that Hsp90 inhibition by geldanamycin (GA) in rat embryo fibroblast cell lines A1–5 increases the proteolytic turnover of mutant p53 and enhances its nuclear translocation, although it is unable to restore the wild-type transcriptional activity of target genes.

Although Hsp90 is a cytoplasmic chaperone, a small fraction of Hsp90 (about 3% of the total cellular pool) is present in the nucleus. In recent days, the focus has been shifted in understanding the function of Hsp90 in the nucleus. Two decades back, it was first observed that during heat-shock treatment, Hsp90 is specifically localized in the salivary gland of *Drosophila melanogaster* 93D chromosomal locus as well as at the telomere region of *Chironomus thummi* (9). Intriguingly, the fact that its localization to those regions of chromatin was hindered in the presence of transcription inhibitor suggests a role of Hsp90 in transcription during heat-stressed condition. It also assists in the

degradation of unfolded or un-required proteins and thereby plays a significant role in maintaining the protein homeostasis in cell. Hsp90 acts as a master regulator of gene expression as it controls the trafficking of steroid hormone receptors to nucleus in a hormone-dependent manner. Recent study shows that Hsp90 and its co-chaperone FKBP51 also promotes hormone-independent nuclear localization of androgen receptor in prostate cancer cells (10) and thereby plays a critical role in progression of prostate cancer. It is observed that in hormone refractory or androgen-independent (AI) prostate cancer cells, a large pool of androgen receptor is translocated into the nucleus even in the absence of androgen and thus leads to the transcriptional activation of target genes resulting in tumor growth (11, 12). The specific inhibitor of Hsp90, 17-allylamino-17-demethoxygeldanamycin (17-AAG), prevents the nuclear localization of androgen receptor in AI tumor at much lower doses than that required to inhibit androgen induced nuclear import of androgen receptors (AR) (13).

In this review, we shall focus on various transcription factors (TF), which interact with Hsp90. Also, we will discuss about the latest understanding on how Hsp90 is involved in regulating chromatin structure and thereby controls gene expression. Although the cellular role of Hsp90 in transcriptional regulation by modulating chromatin dynamics is apparent, its relevance in cancer progression is yet to be appreciated.

Major Transcription Factors Belong to the Hsp90 Network Society

The role of Hsp90 in transcriptional regulation is foremost attributed to a wide variety of TFs that serve as its clients. One of the ways by which Hsp90 aids in cell survival upon stressed conditions is by regulating the expression profiles of many genes. However, Hsp90 does not do so by binding to DNA as it lacks DNA binding ability. Nevertheless, it chaperones different proteins that act as either activator (like SP1, STAT5) or repressor (for example, Bcl-6) (14, 15) to govern gross transcriptional program (16). TFs serve as tools to regulate different downstream biological processes. Therefore, by providing its services to TFs, Hsp90 is able to regulate multiple pathways simultaneously and hence, plays a vital role in facilitating the progression of many diseases, infections, and cancer (17, 18). When it comes to get hold of processes relevant to cancer, Hsp90 has its branches penetrating into all the six hallmarks of cancer (19). Among the TFs, which serve as Hsp90 clients, NF-κB, STATs, p53, and Bcl-6 (20–26) top the scores owing to the importance of the processes governed by them, which favor malignant transformation. To orchestrate the transcriptional response in a pathway, two or more TFs, which are Hsp90 clients, work together and allow the progression of a pathway dance to their tune. In this light, Hsf-1, which serves as a client of Hsp90 under normal conditions and drives transcriptional programs that are cancer specific, indulges in a positive feedback loop with mutp53 (another Hsp90 client) and endow cancer cells more resistant to proteotoxic stress. The direct interaction between these two proteins in a feed forward loop reinforces tumorigenesis by stabilizing the transcription of *HSPs* that further stabilize EGF, ErbB2, mutp53, and other oncogenes (27). In another scenario, the broad array of clientele of Hsp90 gives it the advantage to regulate the expression of a single protein in different conditions via different TFs. The parallel effect of the TFs upon cellular machinery is witnessed when Hsf-1 and Hif-1 (hypoxia-inducible factor), the clients of Hsp90, regulate the expression of the same protein FoxM1 under different conditions. On one hand, FoxM1 (a key TF for cell cycle progression and a critical molecule for tumor development and progression) is shown to be induced by hypoxia via direct binding of Hif-1 to its promoter sequence, which causes its up-regulation. Induction of FoxM1 leads to promotion of tumor cells proliferation by diminishing nuclear levels of p21 protein and increasing cyclin B1 and cyclin D1 expression (28). On the other hand, FoxM1 is also regulated by Hsf-1 under heat-shock stress conditions and the induction of FoxM1 by Hsf-1 is required for cell cycle progression through regulating the expression of downstream Cdc20, Cdc2, and Cdc25B proteins (29). The importance of Hsp90 in tumor progression is further portrayed by the following study, which reveals that inhibition of Hsp90 leads to the suppression of Lmp1 expression (a major oncogene encoded by Epstein–Barr virus) that plays a crucial role in development of lymphomas. The effect was due to compromised JAK/STAT and NF-κB signaling pathways owing to the repression of STATs and NF-κB TFs upon Hsp90 inhibition (30).

Hsp90 has long been known to regulate transcription when it comes to steroid hormone signaling and was studied extensively (31). The relevance of steroid hormone receptors in cancer is very well reflected by estrogen and progesterone receptors in breast cancer, and by AR in prostate cancer (32, 33). However, Hsp90 does not fail to add one more layer of regulatory step in stabilizing AR by involving breast carcinoma amplified sequence 2 (Bcas2). Bcas2 is a transcriptional cofactor of estrogen receptor (ER), which is involved in breast cancer malignant progression and also overexpresses in prostate cancer. A recent study reports that Bcas2 interacts with Hsp90 to bring about AR stabilization in a p53-independent manner (34). Hsp90 not only stabilizes its clients but also helps them to localize in the right compartment in the cell where their function is required. This aspect was explicitly shown in a study with a *bona fide* client TF, AF9, which is vital for hematopoiesis. It is also called master regulator of *HOX* gene expression. It is observed that it depends on Hsp90 for proper subcellular localization (35). Nevertheless, another study exemplifies the role of Hsp90 in deciding the fate of cell death whether it would be necrosis or apoptosis. The inhibition of Hsp90 dictates the inhibition of Atf3 (a TF that regulates gene expression in response to oncogenic stresses) expression, which regulates the switch from necrosis to apoptosis (36). In these ways, the parameters of *HSP90* regulation are extended to the normal cellular processes as well.

The versatile nature of Hsp90 does not restrict it to stay "in-house" rather reflects its ability to tune the transcriptional program being "outdoor." This particular molecular chaperone now is reported to be secreted out in extracellular "reactive" stroma by tumor cells and also under other stressed conditions. This secreted form of Hsp90, addressed as eHsp90, sustains cancer cell motility, invasion, and metastatic spread (37). The extent of secretion of eHsp90 is more in aggressive tumors as it is reported in prostate cancer (38). A recent study suggests eHsp90 as a potent initiator of stromal inflammatory response, which is executed by transcriptional modulation of NF-κB and STAT3, the master regulators of inflammatory pathway (39). Thus, Hsp90 creates a

hub of regulatory network where not only the client TFs lead to required alteration in the progression of pathway but also cross-talks among client proteins dictate the downstream effectors for better response to stress stimuli. Thus, Hsp90 regulates the activity of several key transcriptions factors involved in cancer progression via two different mechanisms: in the first place, by regulating the cellular abundance of these factors and second, by regulating their intracellular transports (**Figure 1**, 1 and 2).

Communication between Hsp90 and Chromatin Remodeling Factors

So far, we have discussed the role of Hsp90 in transcriptional regulation by directly modulating the activity of TFs. Now, we will discuss how Hsp90 alters the epigenetic marks on chromatin and thereby modulates transcription of several genes that might include proto-oncogenes. Abnormal methylation marks on DNA, altered histone modifications, or RNA-mediated silencing could potentially result in inappropriate gene expression. Any of these epigenetic abnormalities might cause development of cancer. There are increasing amounts of evidence that suggest mutual cross-talks between Hsp90 and several chromatin modifiers (**Figure 1**, 4). Hsp90α is shown to interact and enhance the activity of (H3–K4) histone methyltransferase (HMTase) SMYD3 whose over-expression is essential for the growth of colorectal-, liver-, and breast cancers (40, 41). Hsp90 induces a conformational change of SMYD3 upon binding to its N-terminal domain, which is essential for the regulation of its cognate HMTase activity (42). It is also reported that the tetratricopeptide repeat (TPR) present at the C-terminal domain of SMYD3 is involved in the physical interaction with MEEVD regions of Hsp90. This interaction is proved to be essential for the chromatin localization and enhancement of HMTase activity of SMYD3 (43). It has been speculated that disruption of the interaction between Hsp90α and SMYD3 might be responsible for inactivation of WNT gene transcription (44). Recent findings show that increased ATPase activity of Hsp90 by Aha1 results in enhanced expression of WNT target genes in colon cancer in a p53-dependent manner (45). It has also been observed that functional inactivation of Hsp90 or post-translational modification of Hsp90 leads to the dysfunction of several chromatin remodelers, which eventually cause alteration of chromatin state associated with many oncogenes and tumor suppressor genes. The "maintenance" methyltransferase DNMT1 is stabilized by Hsp90. Elevated level of DNMT1 is observed in MCF-7 breast cancer cells (46). DNMT1 along with HDAC1 and (H3–K9) HMTase remain associated with the ER-α promoter, causing hypermethylation of 5′ C_pG islands and thereby causes silencing of ER-α expression in breast cancer cells (47, 48). Studies with HDAC1 inhibitors reveal that post-translational modification (hyperacetylation) of Hsp90 destabilizes its interaction with DNMT1 and promotes ubiquitin-dependent degradation of DNMT1 (49).

In lower eukaryotes like *Saccharomyces cerevisiae*, genome-wide two-hybrid interaction study revealed that Hsp90 may influence global gene expression through interactions with histone deacetylases. Strong association between $Hsp90^{E33A}$ and Sir2 (Type III histone deacetylase) as well as Sap30 (a component of Rpd3L histone deacetylase complex) has been observed (50).

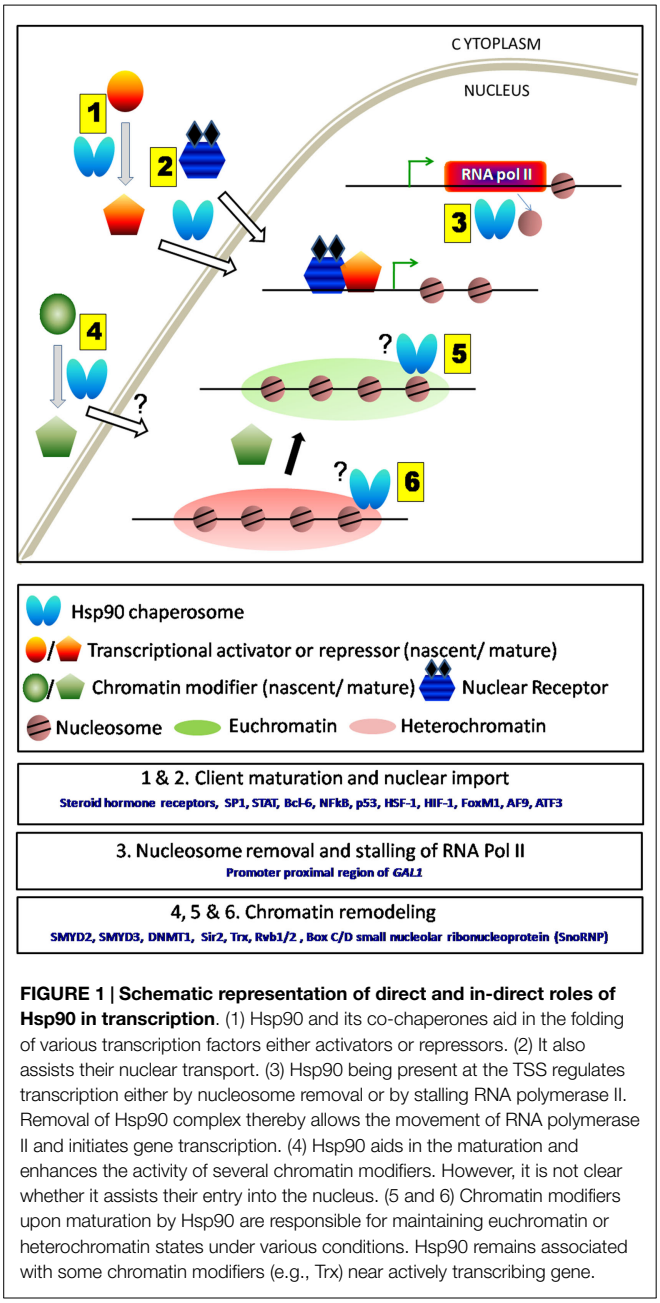

FIGURE 1 | Schematic representation of direct and in-direct roles of Hsp90 in transcription. (1) Hsp90 and its co-chaperones aid in the folding of various transcription factors either activators or repressors. (2) It also assists their nuclear transport. (3) Hsp90 being present at the TSS regulates transcription either by nucleosome removal or by stalling RNA polymerase II. Removal of Hsp90 complex thereby allows the movement of RNA polymerase II and initiates gene transcription. (4) Hsp90 aids in the maturation and enhances the activity of several chromatin modifiers. However, it is not clear whether it assists their entry into the nucleus. (5 and 6) Chromatin modifiers upon maturation by Hsp90 are responsible for maintaining euchromatin or heterochromatin states under various conditions. Hsp90 remains associated with some chromatin modifiers (e.g., Trx) near actively transcribing gene.

Recent studies have established that Hsp90 is required for the stability and functional activity of Sir2. In Hsp90 loss of function mutant, the endogenous level of Sir2 reduces considerably and it results in de-repression of silencing at telomeres and at the mating loci *HMLα* and *HMRa* (**Figure 1**, 6). The temperature-sensitive mutant of Hsp90 behaves similarly as Δ*sir2* mutant resulting in sterile yeast (51). On the other hand, Hsp90 over-expression, which is a natural outcome of heat-stressed condition, drives downregulation of *SIR2* at the transcription level (52). Such reduced abundance of *SIR2* transcript is maintained through several generations before it gradually returns to its normal level. Hence, the level and activity of the chromatin modifier Sir2 are modulated by two independent pathways both controlled by

Hsp90. In addition to the regulation of histone deacetylase activity, Hsp90 chaperosome also amends the activity of other types of chromatin modifiers. Two co-chaperones of Hsp90; Tah1 (human ortholog RPAP3) and Pih1 (also known as NOP17 and Pih1D1) are found to interact with Rvb1/2 (53), which are the essential components of INO80 (42, 54); SWR-C chromatin remodeling complex (55–57); and histone acetyl transferase TIP60 complex (58). In *Drosophila melanogaster*, it has been reported that Hsp90 interacts with Trithorax G, which is an important chromatin modifier complex that controls *Drosophila* development. Inhibition of Hsp90 function by radicicol causes depletion of intracellular Trx. As a result, the recruitment of Trx at the specific chromatin locus is reduced thereby leading to the down regulation of Trx target genes (59).

The third arm of epigenetic control, namely the small interfering RNA-mediated post-transcriptional gene silencing is also influenced by Hsp90 chaperone complex. It has been demonstrated that Hsp90/Hsc70 chaperone complex is required for the loading of small RNA duplexes onto the Argonaute proteins (60). Its involvement in the assembly and maintenance of box C/D small nucleolar ribonucleoprotein (SnoRNP) complexes is also observed. Hsp90 along with Tah1 and Pih1 interact with Rvb1/2 to form R2TP complex, which participates in assembly of snoRNPs (61). Interestingly, while Hsp90 controls the activity of chromatin modifiers, its own activity is often regulated by non-histone methyl transferases. Such regulation provides another layer of regulation where Hsp90 is a central molecule. Recent report witnesses that SMYD2-mediated methylation of Hsp90β induces its dimerization and chaperone complex formation, which accelerates the proliferation of cancer cell (62).

Hsp90 collaborates with histone deacetylases to influence the stability of oncogenic TFs and tumor suppressors. The Hsp90–HDAC6 complex is critical for the stability of mutant p53 (63). Recent reports establish that the regulation of tumor suppressor TAp73 stability is mediated by Hsp90–HDAC1 combo protein complex. HDAC1 knockdown induces hyperacetylation of Hsp90, which disrupts the interaction between TAp73 and Hsp90 and promotes proteasomal degradation of TAp73 (64). Thus, Hsp90 influences the activity of several epigenetic modifiers as well as the micro-RNAs. Independent studies have revealed the link between cancer progression and the improper functioning of such epigenetic writers, speculating a general role of Hsp90 in cancer progression through the modulation of chromatin dynamics. However, any such direct connection between Hsp90, chromatin modification, and clinical progression of cancer is yet to be established.

The Function of Hsp90 at Promoter Proximal Regions

The transcription machinery including RNA polymerase, transcription activators, and other factors need to be recruited at the promoter adjacent region at the onset of transcription and once transcription is over they must be dislodged from the DNA. Hsp90 actively participates at all the above steps of transcription. Genome wide ChIP-seq analysis reveals that Hsp90 is recruited at the transcription start site (TSS) of about one-third of *Drosophila* genome suggesting a general role of Hsp90 in transcription initiation (65). Interestingly, Hsp90 targeted promoters include TFs like c-myc, p53; genes involved in stress response and developmental signaling such as WNT, JNK, etc.; as well as several environmental responsive genes like Hsp70, Hsp68, and Hsp22. It is observed that Hsp90 together with negative elongation factor (NELF) represses the expression of its target genes by forming stalled RNA polymerase II at the target locus. Hsp90 inhibitory condition causes robust up-regulation of Hsp90 target genes by converting stalled RNA polymerase to the elongated form. However, Hsp90 may not have a general role in transcription as it is evident from another study where Hsp90 and Trx are co-localized only at the TSS of the actively transcribed region *Abd-B* in *Drosophila* SF4 cells (59) (**Figure 1**, 5) but neither it is found to be associated with Trx at the TSS of silent genes (*Dfd* or *Ubx*) nor at the TSS of house-keeping genes. There are reports, which show that Hsp90 also enhances transcriptional activation in cancer cells by binding to the DNA–protein complex. It is observed that Hsp90 interacts strongly to the hTERT promoters in telomerase positive oral cancer cell lines compared to the normal human oral keratinocytes (NHOKs) cell lines and thereby causes enhanced promoter activity of telomerase gene in cancer cells (66). Hsp90 inhibition by GA specifically destabilizes the interaction between Hsp90 and hTERT promoter causing loss of hTERT mRNA expression.

It turns out that the role of Hsp90 in transcriptional regulation begins much earlier than the recruitment of TFs or RNA pol II. It is observed that Hsp90 is involved in the steps prior to the transcription initiation, which involves precise removal of nucleosomes (**Figure 1**, 3). The transcriptional induction of *GAL1* is found to be delayed in Δ*hsc82* strain background due to the retention of nucleosomes at the *GAL1* promoter (67). However, the precise mechanism of how Hsp90 aids in the eviction of nucleosomes is not clear.

Consistent with the function of Hsp90 in the removal of histone proteins from several promoters, Hsp90 also removes other proteins from the promoter proximal regions. Hsp90 controls the exit of steroid hormone receptors from nuclear locus. Hsp90 and its co-chaperone p23 also play pivotal roles during the dislodging of steroid hormone receptor complexes from hormone response elements (HRE) in a hormone-dependent manner (68). First, over-expression of p23 causes significant (35-fold) reduction of GR activity *in vitro*. Similarly, Hsp90 over-expression results in modest (twofold) reduction. Second, ChIP assay shows increased recruitment of Hsp90, p23, and gluococorticoid receptor at GRE upon addition of dexamethasone. Finally, forced localization of Hsp90/p23 to HRE precludes GR-induced transcriptional activation.

In summary, Hsp90 has multifaceted cellular functions in transcription regulations. It could evict nucleosomes from the promoter and thereby makes space for loading of RNA pol II and other TFs; it could alter the heterochromatin to euchromatin states by modulating chromatin modifiers; it could give functional maturation to the TFs and regulate their nuclear entry; and finally, it could remove the TFs from the promoter proximal regions upon the completion of transcription.

Future Perspective

In the light of the recent findings, it is becoming clear that besides the well known chaperone function Hsp90 plays significant roles at many stages of transcriptional control. However, it is not clear whether Hsp90 has a generalized role during transcription or its involvement is confined to certain specific promoters. In the later case, it would be extremely important to decipher the molecular mechanism behind such promoter specificity. It will also be interesting to unravel whether human Hsp90 also targets promoters of tumor suppressors/oncogenes. The interplay between Hsp90 and chromatin modifiers during carcinogenesis needs to be investigated. Studies focusing on whether and how human Hsp90 modulates post-transcriptional gene regulation via non-coding micro-RNAs in cancer cells demand special attention. The classical chaperone function of cytosolic Hsp90 and several newly emerged moonlighting functions of Hsp90 at the nucleus prompt us to propose that the nuclear Hsp90 could be structurally different (due to certain post-translational modification: PTM) from the cytosolic form. Identification of different PTM of Hsp90 might give us valuable handle in separating the cytosolic versus the nuclear functions of Hsp90. This field is still at its infancy and more experimentations are needed to understand the yet to be discovered newer nuclear functions of Hsp90.

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Pathway regulation of p63, a director of epithelial cell fate

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The p53-related gene p63 is required for epithelial cell establishment and its expression is often altered in tumor cells. Great strides have been made in understanding the pathways and mechanisms that regulate p63 levels, such as the Wnt, Hedgehog, Notch, and EGFR pathways. We discuss here the multiple signaling pathways that control p63 expression as well as transcription factors and post-transcriptional mechanisms that regulate p63 levels. While a unified picture has not emerged, it is clear that the fine-tuning of p63 has evolved to carefully control epithelial cell differentiation and fate.

Keywords: p63, epithelial cells, notch signalling, Wnt proteins, Hedgehog pathways, EGFR, epithelial–mesenchymal transition

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Introduction

At first glance, the tumor suppressor p53 and its family member p63 seem quite similar in function and exhibit a high degree of evolutionary conservation. In particular, the DNA-binding domains are about 60% identical at the amino acid level; however, the adjacent domains and C-termini diverge drastically (1). While it was first thought that p63 and p53 could regulate similar sets of genes, it has become clear that these potent transcription factors possess some partially redundant functions, and some that are entirely unique (2–4).

p63 is also unlike its family member p53 in that it is rarely mutated in human cancers. Instead, mutations in p63 lead to disorders with ectodermal dysplasia such as ankyloblepharon-ectodermal dysplasia-clefting (AEC)/Hay–Wells syndrome, which can include symptoms like cleft lip/palate and skin erosions (5, 6). Other p63 syndromes can include split hand/foot malformation and alopecia, but cancer predisposition is generally not seen (7–9).

Due to differential promoter usage and splicing, there are at least six common isoforms. There are two classes that arise from different promoters, one with the N-terminal transactivation domain (TA), and the other set lacking the N-terminal transactivation domain (ΔN). While the ΔN form can be dominant negative to the TA isoforms (2), the ΔNp63α isoform has been shown to contain an alternate transcriptional activation domain, suggesting it can also directly activate target genes (3, 10). Alternative splicing of the 3′ end of the TA and ΔNp63 mRNAs produces the α, β, and γ isoforms, although only the α isoforms contain the sterile-α motif (SAM) domain and the transcription-inhibitory (TI) domain. Mutations in these domains can disrupt binding to the target Apobec-1-binding protein-1 (ABBP1), and deletion of both domains led to increased $p21^{Waf1/Cip1}$ signaling, indicating that these domains can modulate target gene specificity (11, 12).

As to the specific functions of these isoforms, mouse models have been instrumental in providing us with clues. Two groups reported that $p63^{-/-}$ mice were found to have severe limb and epithelial defects, including partial or missing epithelial stratification, and truncated forelimbs (13, 14). More recently, both the whole animal- and epidermal-specific deletion of ΔNp63α in mice led to skin erosions and impaired terminal differentiation of keratinocytes, demonstrating the importance of this isoform in the epithelial stratification process (15–17). It is possible that deregulation

of p63 targets linked to cell–matrix adhesion and epithelial morphogenesis causes these skin abnormalities (18–20).

Furthermore, loss of epithelial cells in ΔNp63-null mice suggested that this isoform is essential for the establishment of epidermal progenitor cells (13). Pellegrini et al. (21) suggested that p63 is found in the stem cells of the proliferative compartment, but not in the transit amplifying keratinocytes that have exited the compartment. When it comes to the caudal endoderm, Pignon et al. (22) revealed that the p63-expressing cells are capable of differentiating into prostate, bladder, and colorectal epithelia. Another report found p63 to be essential for the proliferative ability and differentiation of the epidermis; however, in a thymic model, p63 was only required for clonogenicity but not for lineage commitment or differentiation (23, 24). Intriguingly, depletion of ΔNp63 or its target DGCR8, an miRNA processing factor, allowed keratinocytes to enter a multipotent stem cell state, suggesting that ΔNp63 is needed to maintain the keratinocyte differentiation state (25). Finally, an AEC-like mutation in p63 led to reduced proliferative and clonogenic potential in epithelial cells (26). Together these studies make a compelling case for p63 in the maintenance and regulation of epithelial stem cells.

Meanwhile, TAp63 ablation demonstrated that this isoform monitors the integrity of the germline after cellular stresses (27, 28). In particular, γ-irradiation was shown to induce tetramerization of TAp63α from inactive dimers, leading to greatly increased target binding ability (29), and inducing cell cycle arrest or an apoptotic response.

Yet, p63 levels are sometimes altered in tumors. Many groups have reported increased expression in cancers, especially in head and neck squamous cell carcinomas (HNSCC) (30, 31). Indeed, amplification or overexpression of p63 has frequently been observed in lung cancers, and more rarely in HNSCC (32–34). However, p63 expression is lost in more invasive prostate and breast cancers, and this loss is associated with worse prognosis in some cases (35, 36). It has been theorized that the tissue context, as well as the balance between TA and ΔN isoforms, could partially explain this dichotomy.

So how does p63 impact cancer formation? The last decade has seen a preponderance of direct targets unearthed, including adhesion-related β4 integrin, the tissue integrity factor Perp, the Notch ligands Jagged1 and Jagged2, keratins 5 and 14, and EGF receptor (18, 19, 37–41). Cancer-related targets like N-cadherin, Id3, MMP13, and Wnt-4 can be activated by p63; however, p63 can also induce Sharp1 and Cyclin G2 expression, which have been shown to be suppressors of breast cancer metastasis (42–45). Additionally, phosphorylated ΔNp63α was found to associate with components of the splicing machinery, as well as transcription factors SREBP1 and E2F1, in regulation of metabolic and cell cycle-related processes (46).

p63 is also known to regulate a diverse set of microRNAs. A prominent target is miR-205, a repressor of epithelial–mesenchymal transition (EMT) and metastasis in bladder and pancreatic cancers (36, 47, 48). In contrast to the role of miR-205, members of the miR-17 family (miR-17, miR-20b, and miR-106a) are regulated by p63 and Myc, and were found to target Rb, p21, and JNK2, suggesting that they are oncomirs (49–51). Additionally, p63 can repress the prominent cell cycle regulators miR-34a and miR-34c, thereby affecting cellular progression in a p53-independent manner (52).

A data mining approach also identified p63 and the p53-related p73 gene as key regulators of microRNAs differentially expressed in ovarian carcinomas, including miR-200a, miR-200b, and miR-429 (53). Similarly, mir-193a was repressed by both p63 and p73, although its induction leads to p73 inhibition (54). For more on p63 regulation of microRNAs, see the review by Candi et al. (55).

Taken together, p63, like p53 and p73, can regulate a host of processes, some of which are known regulators for or against tumor growth. As suggested by the opposite expression of p63 in different tumor types, the context of the cell type appears to be critical to which p63 targets have the dominant effects in each cell. Whether targets are differentially expressed or have different activities in different cell types needs to be investigated further.

As ΔNp63 is required for the formation of stratified epithelial layers and is the primary isoform expressed in the basal layer of epithelial tissues, it is subject to multiple modes of tissue-specific regulation (13, 14). As described below, a number of signaling pathways and transcription factors have been identified that affect p63 expression in epithelial cells (**Figure 1**).

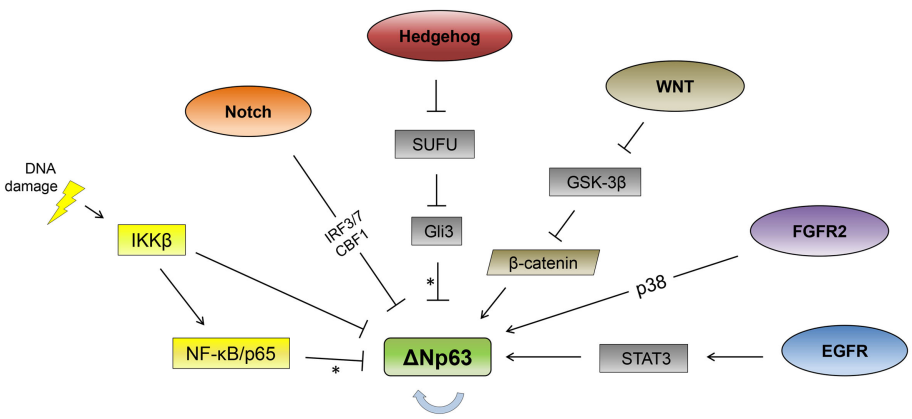

FIGURE 1 | Signaling pathway regulation of ΔNp63. Signaling pathways reported to regulate ΔNp63 levels are indicated. The thick, blue arrow indicates autoregulation. *Note that the Hedgehog and NF-κB pathways repress ΔNp63 levels while simultaneously activating expression from the TAp63 promoter. See text for feedback regulation where p63 regulates multiple components of these pathways (not shown here).

Notch Signaling

One prominent pathway is Notch, which can control epidermal differentiation as well as other developmental pathways (56, 57). Notch activation was found to suppress p63 expression in keratinocytes, ectodermal progenitor cells, and mammary epithelial cells (58–60). The repression in keratinocytes was dependent on the IRF3 and IRF7 transcription factors (59). In mouse mammary epithelial cells, the Notch-mediated repression of p63 functions through the CBF1/RBP-Jk transcription factor (60). In addition to these cases, there has been a report of Notch *activation* of p63 in fibroblasts (61), suggesting differing cell-specific modes of regulation.

The Notch-to-p63 pathway is subject to feedback regulation by ΔNp63, as it can activate Notch pathway gene expression (58, 60, 62). This loop could delineate the boundary between basal and luminal mammary cells as well as allow for ectodermal specification during development (58, 60).

As with p63 mutations, alterations in interferon regulatory factor 6 (IRF6) are associated with craniofacial abnormalities like cleft lip and/or palate (63, 64). Both IRF6 and p63 are required for normal palate development, so the finding that ΔNp63 induces IRF6 expression is logical, but surprisingly, IRF6 in turn causes proteasomal degradation of ΔNp63 (65, 66). Notch has also been found to activate IRF6 expression in keratinocytes (67). Together, these results suggest a Notch/p63/IRF6 axis regulates genes involved in epithelial development. Importantly, Notch, p63, and IRF6 genes were found mutated in about 30% of HNSCC cases, suggesting that this developmental pathway can be hijacked to promote tumor growth (68).

Hedgehog Signaling

Hedgehog is another essential pathway for development (69, 70), and it is reported to regulate p63 expression. Hedgehog activation is seen in various cancers including lung, prostate, and breast (71, 72). Hedgehog ligands including Indian Hedgehog (IHH) can lead to activation of the Gli3 transcription factor, while absence of these ligands leads to a repressive form of the Gli3 transcription factor, termed $Gli3^R$ (73, 74). This balance of Gli3 forms can control p63 isoform formation, as IHH induction of Gli3 actually upregulates TAp63 expression while reducing ΔNp63 promoter usage (75). Again, there is a regulatory loop here since TAp63 expression can increase IHH expression. Similarly, both TA and ΔNp63 β and γ isoforms can activate Sonic Hedgehog (SHH) expression and recently ΔNp63 was found to induce expression of Gli2 and the Hedgehog receptor Ptch1, affecting mammary stem cell renewal (76, 77). In addition, it was posited that some of the developmental defects observed in the $p63^{-/-}$ mice may occur due to subsequent repression of SHH and other Hedgehog pathway genes (76). Other connections between the p63 and Hedgehog pathways include ΔNp63 activation of Gli2 and Gli3 as well as suppressor of fused (SUFU) (78–80). As SUFU is an inhibitor of the Gli proteins, these contrasting effects show the complexity of this signaling system. Nevertheless, together these results suggest a strong connection between the Hedgehog and p63 signaling pathways that could control normal epithelial differentiation or cancer progression.

Wnt Signaling

Strikingly, mutations in the WNT genes also cause similar craniofacial abnormalities as p63 and IRF6 mutations (81, 82). Moreover, mutations in the Pbx genes in mice resulted in a similar phenotype and perturbed Wnt signaling (82). Further analysis demonstrated a Pbx-Wnt9b/Wnt3-p63-IRF6 signaling axis controlling development of the midfacial ectoderm (82). Chromatin immunoprecipitation and reporter genes suggested that p63 is directly regulated by the Wnt pathway through binding of Lef1/Tcf with β-catenin to a region between the TA and ΔNp63 promoters (82), although another report identified a β-catenin responsive site within the proximal ΔNp63 promoter (83). Recently, the Hedgehog pathway was also shown to be connected to craniofacial defects (84). Compound mutations in the Hedgehog pathway genes Hedgehog acyltransferase (Hhat) and Patched 1 (Ptch1) led to a cleft lip-like phenotype and these acted through reduced Wnt-p63-IRF6 signaling.

Analysis of keratinocyte differentiation has led to a different characterization of the p63, Wnt, and Notch signaling pathways. Knockdown of p63 caused reduced Wnt and Notch signaling (50, 51), suggesting that they lie downstream of p63 in contrast to the models of craniofacial development. This could be reconciled as part of a feedback regulation pathway as described above for Notch and p63. Additionally, the activation of Wnt and Notch by p63 may be dependent upon the availability of other transcription factors. For instance, the depletion of p63 led to reduced Myc gene expression via lowered Wnt/β-catenin and Notch signaling, and this is consistent with the requirement of both p63 and Myc for keratinocyte proliferation (50, 51). p63 was also found to regulate the expression of Myc and β-catenin in esophageal squamous cell carcinomas, suggesting the general functioning of a p63/β-catenin/Myc pathway in tumorigenesis (85). Finally, ΔNp63 was shown to upregulate the Wnt receptor Fzd7, leading to enhanced mammary stem cell formation and clonogenic potential (86).

FGFR2/EGFR Pathways

Mutations in the FRGR2 gene (also known as KGFR) can also lead to craniofacial disorders such as cleft lip and Crouzon's syndrome (87, 88). The splice variant FGFR2-2b is an epithelial-specific receptor for ligands like FGF1 and FGF7 (KGF), and is required for embryogenesis and adult tissue homeostasis (89). FGFR signaling and ΔNp63 can influence each other, as ΔNp63 activates expression of FGFR2 in thymic epithelial cells (90) and KGF-induced ΔNp63 expression in limbal epithelial cells (91). KGF's effects on ΔNp63 require p38 MAPK, suggesting a novel pathway for regulation of p63 (91). Furthermore, mutations in p63 that cause AEC syndrome led to impaired FGFR2 gene expression and increased splicing of the mesenchymal FGFR2-2c isoform (11, 26). Together, the combination of FGFR2 activation of ΔNp63 and ΔNp63 induction of specific isoforms of FGFR2 are likely to lead to increased proliferation of specific epithelial cell types. This could enhance proliferation of progenitor cells, but might block progression of specific epithelial cancers.

Interestingly, FGFR2 can induce expression of the epithelial-specific transcription factor Elf5, and deletion of Elf5 causes altered expression of ΔNp63 in the luminal compartment of

mouse mammary tissue (92–94). This suggests a pathway for cell type-specific expression of ΔNp63 mediated by Elf5.

The tyrosine kinase receptor EGFR has also been found to induce ΔNp63 expression. In one case, this was through phosphatidylinositol-3-kinase (PI3K) signaling in keratinocytes (42), while in two types of carcinomas EGFR activation of ΔNp63 was found to be mediated by STAT3 (95, 96). STAT3 was also required for ΔNp63 expression in limbal keratinocytes (97). The inhibition of the STAT3 growth-stimulatory pathway allowed the concomitant differentiation of the limbal keratinocytes, further suggesting the importance of ΔNp63 regulation in these and likely other epithelial cells. The PI3K and STAT3 pathways may be connected through mTOR signaling, as Ma et al. (62) found that PI3K activation of mTOR led to mTOR-dependent activation of the STAT3-p63-Jagged pathway. This highlights the interconnectedness of these signaling pathways, and the role of STAT3 as a key regulator of p63. However, a clear mechanism for how STAT3 directly regulates p63 remains to be determined.

Regulation of ΔNp63 during the Epithelial to Mesenchymal Transition

Epithelial cells can undergo an EMT during development and during carcinogenesis, progressing to a more invasive and metastatic phenotype. This differentiation is thought to allow the cancerous cells greater motility and increased metastatic potential [see reviews by Thiery (98) and Kang and Massagué (99)]. The expression of ΔNp63 is repressed during this transition (100, 101). Transcription factors that can induce EMT include Snail, Slug (also known as Snail2), and Zeb1, and all of these can repress ΔNp63 in epithelial cells (100, 102–104). This inhibition, however, may be due to a feedback loop, as ΔNp63 expression can inhibit EMT by activation of miR-205, which suppresses Zeb1 and Zeb2 expression (36, 48).

Other transcription factors involved in control of EMT are Ovol1 and Ovol2 (85, 105). These factors can repress Zeb1 expression; however, it was also found that ΔNp63 expression increased in Ovol1- and Ovol2-deficient cells, and that Ovol2 could bind to several sites within the ΔNp63 promoter (85). Ovol2 may be upstream of ΔNp63 in an EMT-inducing pathway; alternatively, there may be feedback of Ovol2 to ΔNp63 (as there is with Zeb1 and ΔNp63) with ΔNp63 being an activator of Ovol2. In general, it remains to be characterized how ΔNp63 is regulated during EMT in different epithelial cell types.

Transcription Factor Control

While we have mentioned a number of transcription factors as regulators of ΔNp63, a clear picture has yet to emerge on which factors are critical direct regulators of ΔNp63 and through which sequence elements they act near the ΔNp63 gene.

It is possible that multiple pathways regulate p63 through the C/EBP family of transcription factors, as they have been repeatedly found to regulate p63. C/EBPδ was found to bind to multiple regions of the ΔNp63 gene in human keratinocytes (106, 107). Antonini et al. (108, 109) assayed all conserved regions throughout the p63 gene and identified two, termed as C38 and C40, in the second intron of the ΔNp63 gene that affect expression in mouse keratinocytes. The C40 region was needed for expression in keratinocytes, while C38 provided repression during calcium-dependent differentiation. They found that C/EBPα and β bound to the C38 and C40 regions, and that overexpression of these factors repressed reporter gene expression. In addition, siRNA depletion of C/EBPα and β slightly increased p63 mRNA levels in differentiating cells, suggesting that C/EBPα and β are direct repressors of p63 expression. Furthermore, these investigators found AP-2 to be an activator of the C40 region and the POU domain protein Pou3f1 to be a repressor (108, 109). In contrast to the repression by C/EBPα and β described above, another group described a C/EBP site within the proximal human ΔNp63 promoter, which was required for expression in A431 epidermal carcinoma cells (100). C/EBPα was also found to positively activate a site within the mouse ΔNp63 promoter in mouse keratinocytes (110). Finally, after chemical stress, the cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) was found to bind to and inhibit C/EBPα, partially accounting for its inhibition of ΔNp63 expression (110, 111). These contrasting effects of C/EBP may reflect different family members, DNA-binding sites or cell types used, suggesting that further studies are needed to better understanding of the roles these factors play in regulating p63.

Other transcription factors have also been found to regulate the p63 gene. An OCT4 binding site within the TAp63 promoter activates its expression, suggesting its involvement in stem cell regulation (112). Another pluripotency factor, Sox2, bound to p63 protein and localized with it to common gene loci in chromatin immunoprecipitation experiments. This binding occurred in squamous cell carcinoma cells, but not in embryonic stem cells, suggesting that p63 may co-opt pluripotency factors for differentiated cell-specific expression (105).

p63 Autoregulation and Interaction with p53

p63 positively activates its own expression through binding to the C38 and C40 intronic enhancers as well as to its own proximal promoter (108, 109, 113). Overexpression of the ΔNp63γ isoform increased expression of ΔNp63α in HeLa cells, and of a promoter reporter gene in keratinocytes (108, 113). Overexpression of ΔNp63 was also found to increase expression of endogenous ΔNp63 in a nasopharyngeal carcinoma cell line where activation was dependent upon the STAT3 transcription factor (95). Whether binding of p63 to its promoter is direct or through another transcription factor, the evidence consistently shows that it positively feeds back to augment its own expression.

Initially, p63 expression was found to be suppressed by stresses, such as UV irradiation, that stimulate p53 expression (114–116). Binding of p53 to the ΔNp63 proximal promoter was detected in a mammary epithelial cell line, suggesting direct regulation by p53 of ΔNp63 expression (116). Mutant p53 proteins could also bind to the p63 protein in tumor cell lines and inhibit its activity (117), while in carcinoma cells it was shown that mutant p53 together with SMADs could sequester p63, resulting in inhibition of p63 and increased metastatic potential (45). While these results suggest that wild-type and mutant p53 can repress p63 expression and

function, more work is needed to demonstrate the significance of this effect in human cancers, and exactly how this could contribute to tumorigenesis.

Post-Transcriptional Regulation

p63 levels are also regulated by miRNA, ubiquitin-dependent proteasomal degradation, and protein phosphorylation. Notably, miR-203 can repress p63 expression in supra-basal epithelial cells, contributing to definition of the border between progenitor and differentiated epithelial cells (118, 119). In addition, miR-203 expression was activated during luminal mammary epithelial differentiation and ectopic expression of miR-203 stimulated EMT (120). These results suggest that miR-203 is an essential part of the epithelial differentiation pathway.

Other miRNAs have also been found to regulate p63 expression. miR-92 targets ΔNp63α and β in the HaCaT keratinocyte cell line and in myeloid cells, respectively, and miR-302 suppressed p63 expression in germ cells (121, 122). The apotosis stimulating protein of p53 (ASPP) family of p53 coactivators has similarities with protein phosphatases (123). A related family member iASPP (also known as PPP1R13L) is an inhibitor of apoptosis and can also bind to p63 (124). The expression of iASPP in the basal layer of skin cells is strikingly similar to that of p63, and knockdown of iASPP promoted epithelial differentiation (125). However, rather than regulating p63 by protein–protein interaction, Chikh et al. (125) found that iASPP inhibits the expression of two miRNAs, miR-574-3p and miR-720, which inhibit p63 expression. There is an auto-regulatory loop as p63 is needed for expression of these miRNAs and binds to the promoter of the iASPP gene. These experiments point to a critical role of iASPP and repression of its target miRNAs in maintenance of p63 expression and the epithelial phenotype.

p63 protein stability is also regulated by the ubiquitin–proteasome system, adding another layer of regulation (126). One example is p53-induced RING-H2 (Pirh2), an E3 ubiquitin ligase, which can directly bind to p63 and cause its poly ubiquitination and degradation in keratinocytes (127, 128). Pirh2 was also a transcriptional target of ΔNp63, establishing an auto-regulatory loop, and was required for epithelial differentiation. Another E3 ubiquitin ligase, Ring1B, part of the polycomb repressive complex 1 (PRC1), was found to target p63 (129). Ring1b is overexpressed in breast and pancreatic cancer cells (129, 130), suggesting a possible mechanism for p63 suppression in these tumors.

While p53 is stabilized by DNA damaging agents, such as UV irradiation, ΔNp63 is degraded (115, 131, 132). Two mechanisms related to the NF-κB pathway have been found to mediate this degradation. In the first, IKKβ binds to ΔNp63 and phosphorylates it to induce ubiquitination and degradation (133). A second mechanism is direct binding of the p65 subunit of NF-κB to ΔNp63 in cisplatin-treated cells, leading to proteasomal degradation of ΔNp63 (134). The reduction of ΔNp63 augmented activation of p53 target genes and may contribute to cell death in UV-damaged cells. NF-κB repression of p63 may also have a role in epithelial cell differentiation, as overexpression of the NF-κB factor p65 in epithelial cells led to p63 downregulation and increased EMT (103). ΔNp63 also bound to target genes with p65, suggesting that these two factors coordinately regulate a gene program promoting cell survival (135).

NF-κB can also activate the TAp63 promoter, suggesting that a shift to the TAp63 form could also underlie the DNA damage response (136). Again, there is an auto-regulatory loop where TAp63 activates p65 expression as well as stabilizes p65 protein by direct binding (137, 138).

An alternative mechanism for ΔNp63 degradation as part of the DNA damage response is phosphorylation on threonine 397 by the protein kinase HIPK2 (139). HIPK2 has previously been identified as a DNA damage-induced kinase targeting p53 (140), such that it provides a mechanism to coordinate p63 levels with p53 and other aspects of the DNA damage response. For more regulators of p63 protein stability, see the review by Li and Xiao (126).

Conclusion

p63 has been termed as a master regulator of epithelial cells, and it is often suppressed in order for these cells to differentiate (21, 141, 142). We now understand more about how p63 is regulated, uncovering a large array of signaling pathways (**Figure 1**) and feedback regulation that controls expression of components of the signaling pathway as well as p63. Besides the processes of differentiation and development, p63 is also regulated during the DNA damage response, suggesting that it can mediate the more immediate fate of cells. The regulation of ΔNp63 expression, the predominant form in epithelial cells, includes transcriptional and post-transcriptional components. The relative importance of each pathway is still unclear and their usage will likely vary in different cell types and developmental stages. While there are multiple reports of some pathways and mechanisms, common regulatory sequence element(s) for control of the p63 gene across systems have yet to be established.

It will also be important to understand how modulation of p63 levels affects cancer formation. The combination of heterozygous p63 and p53 genotypes in mice yielded conflicting results, giving either greater or reduced tumor burdens (143, 144). Additionally, while ΔNp63 is often highly expressed or amplified in squamous carcinomas, other tumors such as esophageal adenocarcinomas and hepatocellular carcinomas generally lack expression (145–147). Naturally, these cancers arise from diverse tissues, but it is confounding that p63 can have oncogenic effects in some cases and tumor suppressive ones in others. As EMT is part of metastatic progression of some carcinomas, it is interesting that repression of p63 was seen during this differentiation process; is this regulation critical for progression of tumor cells to a more aggressive state? Further, which of the pathways described here, if any, are altered in cancer cells and modulate p63 levels in a critical manner?

Other open questions concern p63 promoter usage and splicing – what factors determine the balance of usage of the TA and ΔN promoters, and what governs the presence of different 3′ splicing isoforms? How does the balance of these 3′ isoforms lead to differences in development or oncogenesis? Finally, can the signaling pathways that control p63 levels be controlled to provide a therapeutic benefit in specific cancers? We can hope that the following years will bring a greater understanding of this master regulator of epithelial biology.

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Ubiquitin-fold modifier 1 acts as a positive regulator of breast cancer

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Estrogen receptor-α (ERα) is a steroid hormone-sensitive transcription factor that plays a critical role in development of breast cancer. The binding of estrogen to ERα triggers the recruitment of transcriptional co-activators as well as chromatin remodeling factors to estrogen-responsive elements (*ERE*) of ERα target genes. This process is tightly associated with post-translational modifications (PTMs) of ERα and its co-activators for promotion of transcriptional activation, which leads to proliferation of a large subset of breast tumor cells. These PTMs include phosphorylation, acetylation, methylation, and conjugation by ubiquitin and ubiquitin-like proteins. Ubiquitin-fold modifier 1 (UFM1), one of ubiquitin-like proteins, has recently been shown to be ligated to activating signal co-integrator 1 (ASC1), which acts as a transcriptional co-activator of nuclear receptors. Here, we discuss the mechanistic connection between ASC1 modification by UFM1 and ERα transactivation, and highlight how the interplay of these processes is involved in development of breast cancer. We also discuss potential use of UFM1-conjugating system as therapeutic targets against not only breast cancer but also other nuclear receptor-mediated cancers.

Keywords: ASC1, breast cancer, ERα, post-translational modification, UFM1

INTRODUCTION

Protein modifications by ubiquitin and ubiquitin-like proteins, including SUMO and ISG15, have emerged as critical regulatory processes, such as in the control of cell cycle, stress response, signaling transduction, and immune response. Moreover, deregulation of the modification systems often gives rise to numerous human diseases, such as cancers, neurodegenerative diseases, and immune diseases (1–4).

Ubiquitin-fold modifier 1 (UFM1) is the most recently identified ubiquitin-like protein (5). Like ubiquitination, protein modification by UFM1 (ufmylation) utilizes a cascade three-enzyme system: UBA5 as an UFM1-activating E1 enzyme, UFC1 as an UFM1-conjugating E2 enzyme, and UFL1 as an UFM1 E3 ligase. This ufmylation process can be reversed by UFM1-specific proteases (UFSPs) (6). All of the proteins involved in reversible protein modification by UFM1 are conserved in metazoa and plants, but not in yeast, implicating its specific roles in multicellular organisms.

Not only estrogen receptor α (ERα) itself but also its co-regulators, including SRC1 and p300, are known to undergo a wide variety of post-translational modifications (PTMs), such as phosphorylation, acetylation, methylation, ubiquitination, and sumoylation. Moreover, these PTMs have been identified as critical events that regulate the expression of ERα and its transcriptional co-regulators and their stability, subcellular localization, and sensitivity to hormonal response (7–12). Although the components of estrogen signaling pathways are suitable and efficient targets for breast cancer therapies, the role of their PTMs in initiation and progression of breast carcinogenesis remains largely elusive.

Activating signal co-integrator 1 (ASC1), originally identified as thyroid hormone receptor interactor 4 (TRIP4), is one of ERα transcriptional co-activators (13). It also serves as a co-activator of other nuclear receptors, such as thyroid hormone receptor (TR) and retinoic acid receptor α (RARα) (14–16). However, it remained unknown whether ASC1 also undergoes PTMs and how the PTMs of ASC1 influence its co-activator function toward nuclear receptors.

In this review, we will provide an overview on current and emerging roles of the UFM1 system, with a focus on ASC1 ufmylation in regulation of breast cancer development. A thorough understanding of ASC1 ufmylation would promote not only the identification of new markers for prognosis of breast cancer but also the development of novel therapeutic strategies.

PROPERTIES OF UFM1

UFM1 consists of 85 amino acids with a predicted molecular mass of 9.1 kDa. Its gene is located in human chromosome 13q13.3. UFM1 is expressed in human cells as a precursor with a C-terminal Ser-Cys dipeptide extension, which needs to be processed by UFSPs

Abbreviations: AR, androgen receptor; ASC1, activating signal co-integrator 1; Cdk5rap3, CDK5 regulatory subunit-associated protein 3; DDRGK1, DDRGK domain-containing protein 1, ERα, estrogen receptor-α; ISG15, interferon-stimulated gene 15; LZAP, LXXLL/leucine zipper-containing alternative reading frame (ARF)-binding protein; Maxer, multiple α–helix protein located at ER; NCAM, neuronal cell adhesion molecule; NLBP, novel LZAP-binding protein; RARα, retinoic acid receptor α; RCAD, regulator of C53/LZAP and DDRGK1; SRC1, steroid receptor coactivator 1; SUMO, small ubiquitin-related modifier; TRIP4, thyroid hormone receptor interactor 4; UBA, ubiquitin-like modifier activating enzyme; UBE1DC1, ubiquitin activating enzyme E1-domain containing 1; UFBP1, UFM1-binding protein 1 containing a PCI domain; UFC1, ubiquitin-fold modifier-conjugating enzyme 1; UFL1, UFM1-specific ligase 1; UFM1, ubiquitin-fold modifier 1; UFSP, UFM1-specific protease; Urm1, ubiquitin-related modifier 1.

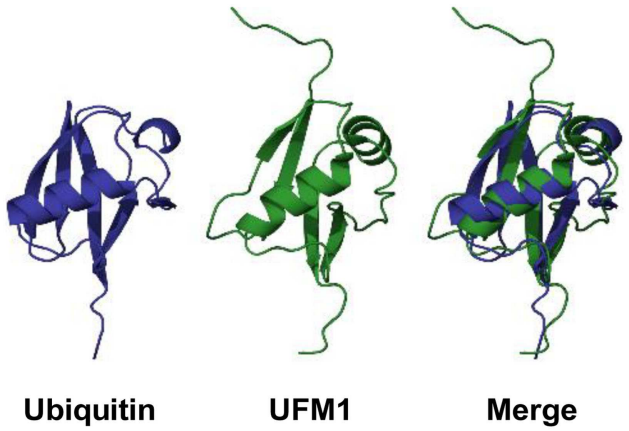

FIGURE 1 | 3D structures of ubiquitin and UFM1 in human. PDB IDs for ubiquitin and UFM1 are 1UBQ and 1WXS, respectively.

prior to conjugation to target proteins (5). Matured UFM1 has a single glycine residue at the C-terminus, which also is required for conjugation to its target proteins, unlike ubiquitin and most other ubiquitin-like proteins, such as SUMO and NEDD8, which have a conserved C-terminal di-glycine. UFM1 is localized in both the nucleus and the cytoplasm (5).

Although UFM1 has a limited amino acid sequence identity (~16%) with ubiquitin, it displays a striking similarity in its tertiary structure to ubiquitin (**Figure 1**). UFM1 adopts an ubiquitin-like α + β fold with ordered β−β−α−β−β−α−β secondary structure along the sequence (17). A special feature in UFM1 structure is the absence of the cluster of the acidic residues in the α1 surface, which is displayed by ubiquitin (17). Therefore, it has been suggested that UFM1 employs the uncharged surface for binding to its putative partners.

ENZYMES FOR UFM1 MODIFICATION

UBA5

UBA5 (also known as UBE1DC1), an UFM1-activating E1 enzyme, consists of 404 amino acids with a predicted molecular mass of 44.7 kDa (18). Its gene is located in human chromosome 3q22.1. UBA5 is expressed in human as two distinct isoforms (amino acid sequences: 1–404 and 57–404) due to alternative splicing of its primary transcript (19, 20). The role of the additional N-terminal region (1–56) is unknown, as it is not required for UFM1 activation (19). Typically, E1 enzymes consist of the first and second catalytic cysteine half-domains (FCCH and SCCH, respectively), the adenylation domain, and the C-terminal ubiquitin-fold domain (19). UBA5 lacks the FCCH and SCCH domains, but instead simply comprises an adenylation domain, in which the catalytic cysteine (Cys250) is located, and an ubiquitin-fold domain (19, 20). Therefore, UBA5 is much smaller than other E1 enzymes, which comprise >1,000 amino acid residues. UBA5 is predominantly localized in the cytoplasm (20).

At the expense of ATP, UBA5 activates UFM1 (i.e., generates adenylated UFM1 and inorganic pyrophosphate). UFM1 is then conjugated to Cys250 of UBA5 via a thioester bond with the release of AMP (5). It has been reported that UBA5 can also activate SUMO2 under both *in vitro* and *in vivo* conditions (20). However, the loss of mouse *UBA5* has no effect on the conjugation of ubiquitin-like proteins to cellular proteins, except that of UFM1 (21). In addition, overexpression of UBA5 promotes the modification of ASC1 by UFM1, but not by any other ubiquitin-like proteins (22), indicating that UBA5 is a specific E1 enzyme for UFM1.

Significantly, UBA5-deficient mice die *in utero* due to severe anemia associated with defective differentiation of both megakaryocytes and erythrocytes, although UBA5 is dispensable for the production of erythropoietin (21). Moreover, transgenic expression of UBA5 in the erythroid lineage rescues the UBA5-deficient embryos from anemia and prolongs their survival, revealing that the UFM1-conjugating system has an essential role in erythroid differentiation. However, it is necessary to clarify whether UBA5 has other functions distinct from protein conjugation in the control of erythrocyte biogenesis in mice, as UBA4, the E1 enzyme of the Urm1 system, is known to function in tRNA uracyl thiolation in yeast, independent of protein modification by Urm1 (18).

UFC1

UFC1 (also known as HSPC155) is an UFM1-conjugating E2 enzyme consisting of 167 amino acids with a predicted molecular mass of 19.4 kDa. Its gene is located in human chromosome 1q23.3. UFC1 is mainly localized in the nucleus and partly in the cytoplasm, but excluded from the nucleoli (http://www.proteinatlas.org). UFC1 shows low sequence homology (within a range of 13–17%) with other E2 enzymes (23). However, UFC1 has the catalytic core domain conserved in all E2-like enzymes, except that it contains an additional N-terminal helix. The active site Cys116 is located in a flexible loop that is highly solvent accessible. Upon binding of UFC1 to the ubiquitin-fold domain of UBA5, UFM1 is transferred to the cysteine residue of UFC1 by a transesterification reaction.

The neuronal cell adhesion molecule (NCAM) plays important roles in the control of cell migration, synaptogenesis, and axonal outgrowth (24). Recently, NCAM140, an isoform of NCAM, was shown to interact with UFC1 upon analysis by protein macroarray and ELISA (24). NCAM140 and UFC1 co-localize in the surface of B35 neuroblastoma cells and overexpression of UFM1 increases NCAM140 endocytosis. Therefore, UFM1 has been suggested to play a role in trafficking of cell surface molecules, although it remains unknown whether NCAM140 or other cell surface proteins are modified by UFM1.

UFL1

UFL1 (also known as Maxer, NLBP, and RCAD) is an UFM1 E3 ligase consisting of 794 amino acids with a predicted molecular mass of 89.5 kDa. Its gene is located in human chromosome 6q16.1. UFL1 has a transmembrane domain and localizes predominantly in ER membrane. It also has a nuclear localization signal (NLS) sequence, which is functional only when the transmembrane domain is deleted (25, 26).

UFL1 does not have any domain conserved for ubiquitin E3 ligases, such as HECT, RING finger, and U-box. However, its N-terminal region (amino acid sequence: 1–202) is highly conserved across species, and sufficient for the transfer of UFM1 from UFC1 to C20orf116, the first target substrate identified for ufmylation, under both *in vitro* and *in vivo* conditions (26). Since the

N-terminal region lacks the active site cysteine residue, which is typically found in HECT type E3 ligase for transthiolation reaction, UFL1 may play a role as a scaffold protein that recruits E2 enzyme and target proteins similarly to RING type ubiquitin E3 ligase.

UFBP1

UFM1-binding protein 1 (UFBP1: also known as Dashurin and DDRGK1) consists of 314 amino acids with a predicted molecular mass of 35.6 kDa. Its gene is located in human chromosome 20q13. UFBP1 contains a transmembrane helix (amino acid sequence: 4–21), a NLS sequence (64–68), a PCI [proteasome, COP9, and initiation factor domain (228–272)], and a DDRGK sequence (253–267) (27). UFBP1 also has an N-terminal signal sequence (1–26) for its localization in ER. However, deletion of the N-terminal signal sequence leads to nuclear localization of UFBP1.

UFBP1 was originally identified as C20orf116, which is the first target protein identified for ufmylation (26). It interacts not only with UFM1 but also with UFL1 and target proteins for ufmylation, such as ASC1 and LZAP. Depletion of UFBP1 abrogates ufmylation of the target proteins, indicating that UFBP1 serves as a cofactor as well as a substrate for ufmylation. Interestingly, ASC1 ufmylation could also be prevented by substitution of the UFM1 acceptor site Lys267 in UFBP1 with Arg. Moreover, this Lys-to-Arg mutation markedly reduces the interaction of UFBP1 with UFL1, although not with ASC1. Thus, it appears that UFBP1 may first act as a substrate of UFL1 through their weak binding and the ufmylated UFBP1, then binds to the ligase with high affinity, which might be required for the activation of UFL1. **Figure 2** summarizes the overall process of protein ufmylation.

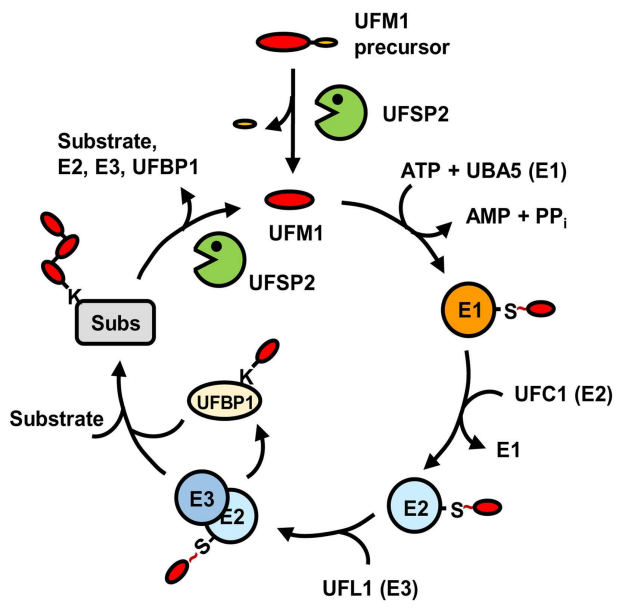

FIGURE 2 | Pathway for protein modification by UFM1. Matured UFM1 generated from its precursor by UFSP2 is activated by UBA5 (E1), transferred to UFC1 (E2), and then conjugated to target substrates by UFL1 (E3) with the aid of UFBP1. This ufmylation pathway can be reversed by USFP2. "S ~" indicates the thioester bond.

UFM1-SPECIFIC PROTEASES

UFSP1

Mouse Ufsp1 consists of 217 amino acids with a predicted molecular mass of 23.4 kDa. Like most deubiquitinating enzymes (DUBs) and ubiquitin-like protein-specific proteases (ULPs), mouse Ufsp1 belongs to the family of cysteine proteases. However, it shows no sequence homology to previously known proteases (28). This novel cysteine protease has a papain-like fold with a unique active site that is composed of a Cys box and a conserved "Asp-Pro-His" box, instead of the canonical Cys-His-Asp catalytic triad. This novel active site configuration appears to form a new subfamily of the cysteine protease superfamily (28).

Human USFP1 consists of 142 amino acids with a predicted molecular mass of 15.0 kDa. Its gene is located in human chromosome 7q22.1. However, unlike the catalytically active mouse Ufsp1, human UFSP1 is expected to be non-functional, since it is shorter on the N-terminus and thereby lacks the conserved cysteine active site (**Figure 3**).

UFSP2

Human USFP2 consists of 469 amino acids with a predicted molecular mass of 53.16 kDa. Its gene is located in chromosome 4q35.1. The crystal structure of mouse UFSP2 shows that the protease is composed of two domains (29). The C-terminal catalytic domain is similar to UFSP1 with the active site composed of a Cys box and a conserved Asp-Pro-His box. The novel N-terminal domain shows a unique structure and plays a role in the recognition of UFBP1. UFSP2 resides in both the nucleus and the cytoplasm. However, overexpressed N-terminal domain co-localizes with UFBP1 in ER, where UFBP1 predominantly localizes, suggesting that the N-terminal domain of UFSP2 plays an important role in the recruitment of UFBP1 to ER for reversal of ufmylation process.

A mutation within the human *UFSP2* gene has been identified in a family with an autosomal dominant form of hip dysplasia, called Beukes familial hip dysplasia (29). This mutation predicts the replacement of the highly conserved Tyr290 by His in the encoded protein. Interestingly, the substitution of Tyr282 in mouse UFSP2, which is equivalent to Tyr290 in human UFSP2, abolishes the *in vitro* UFM1-processing activity (22). Thus, it appears that impairment of reversible modification of unknown protein(s) by UFM1 is associated with an autosomal dominant form of hip dysplasia.

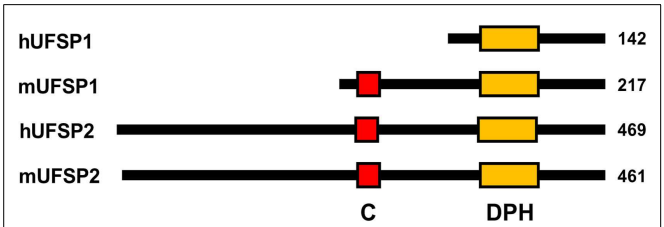

FIGURE 3 | Schematic diagram for primary structures of UFBPs. "C" and "DPH" denote the active site Cys-box (red) and Asp-Pro-His box (orange), respectively. Note that hUFSP1 lacks the Cys box.

ROLE OF ASC1 UFMYLATION IN BREAST CANCER DEVELOPMENT

IDENTIFICATION OF ASC1 AS A TARGET FOR UFMYLATION

Although UFBP1 (C20orf116) was identified as the first target protein for ufmylation (26), its biological function remained unknown. Recently, however, numerous target proteins for ufmylation have been identified by stable expression of Flag-His-UFM1 in NIH3T3 and double affinity purification using Ni^{2+}-nitrilotriacetic acid-conjugated agarose and anti-Flag antibody-conjugated resins, followed by mass spectrometry (22). The identified ufmylated proteins include ASC1, a transcriptional coactivator of ERα, and LZAP (also known as CDK5RAP3 and C53) that has tumor suppressive functions, including activation of p53, induction of apoptosis, and suppression of NF-κB signaling (30–32).

All of Lys324, Lys325, Lys334, and Lys367 in ASC1 serve as the acceptor sites for UFM1 (22). Of the six lysine residues in UFM1, only Lys69 is involved in poly-UFM1 chain formation on ASC1 via isopeptide bond linkage. However, it is possible that other lysine residues may also participate in poly-UFM1 chain formation on other target proteins. Knockdown of any of UBA5 (E1), UFC1 (E2), UFL1 (E3), and UFBP1 abrogates poly-UFM1 chain formation on ASC1, indicating that UFBP1 serves as an essential cofactor for ufmylation process.

REQUIREMENT OF ESTROGEN FOR ASC1 UFMYLATION

Endogenous ASC1 can be ufmylated upon treatment of ERα-negative cells with estrogen, but not without it (22). In the absence of estrogen, UFSP2 remains bound to the N-terminal zinc-finger domain of ASC1, and rapidly removes UFM1 molecules that are conjugated to ASC1. In the presence of the hormone, ERα forms a dimeric complex, translocates to the nucleus, and displaces UFSP2 for its binding to the zinc-finger domain of ASC1, thus allowing ASC1 ufmylation. On the other hand, no ufmylation of ASC1 can be observed in ERα-negative cells, such as MDA-MB-453, regardless of the presence of estrogen. In addition, 4-hydroxy-tamoxifen, an ERα antagonist, abrogates ASC1 ufmylation by preventing the interaction of ASC1 with ERα, indicating the requirement of estrogen binding to ERα for ASC1 ufmylation.

ASC1 acts as a general transcriptional co-activator of nuclear hormone receptors upon binding to not only ERα but also other nuclear receptors, such as androgen receptor (AR) (15). Accordingly, dihydrotestosterone (DHT), an AR agonist, induces ASC1 ufmylation in LNCap (AR-positive) cells, but not in PC3 (AR-negative) cells (22). Thus, ligand-dependent ASC1 ufmylation appears specific to cognate nuclear hormone receptors and it is likely that ligands for other nuclear receptors (e.g., all-trans-retinoic acid for RARα) can induce ASC1 ufmylation.

REQUIREMENT OF ASC1 UFMYLATION FOR ERα TRANSACTIVATION

The zinc-finger domain of ASC1 serves as a binding site for nuclear hormone receptors, transcriptional co-activators (e.g., SRC1 and p300), and basic transcriptional machinery (e.g., TFIIA and TBP) (14). Thus, ASC1 plays an important role as a platform that recruits the necessary components for nuclear receptor-mediated transcription. However, it remained unclear how the zinc-finger domain, a short region in ASC1 (amino acid sequence: 125-237), can simultaneously interact with such a group of the proteins. Remarkably, poly-UFM1 chain conjugated to ASC1 plays a crucial role as a scaffold protein that recruits SRC1, p300, ERα, and ASC1 itself to estrogen-responsive elements (*ERE*s) located in the promoters of ERα target genes, such as *pS2*, *Cyclin D*, and *c-MYC* (22, 33, 34). Moreover, this recruitment leads to a dramatic increase in ERα transactivation. Whereas knockdown of UFSP2 markedly promotes ERα transactivation, its overexpression abolishes it. Knockdown of UBA5 or overexpression of an ufmylation-deficient ASC1 mutant, in which the four UFM1 acceptor lysine residues are replaced by arginine, also abrogates ERα transactivation, indicating that ASC1 ufmylation is crucial for ERα transactivation.

PROMOTION OF TUMOR FORMATION BY ASC1 UFMYLATION

Recently, colony-forming assay has shown that estrogen-induced ASC1 ufmylation is critically involved in anchorage-independent growth of ERα-positive MCF7 breast cancer cells (22). Xenograft analysis using ovariectomized mice has further revealed that ASC1 ufmylation is tightly associated with estrogen-dependent tumor formation *in vivo* (22). Whereas depletion of ASC1, UBA5, or both prevent colony formation and tumor growth, overexpression of ASC1 markedly increases them. Remarkably, depletion of UFSP2 most dramatically promotes the cell growth and tumor formation, and this promotion can be abrogated by simultaneous depletion of ASC1, implicating the role of UFSP2 as a tumor suppressor. In addition, tamoxifen could completely reverse the stimulatory effects of ASC1 overexpression and UFSP2 depletion on colony formation and tumor growth. These findings implicate a crucial role of ASC1 ufmylation in development of ERα-positive breast cancer by promoting ERα transactivity. **Figure 4** summarizes estrogen-induced ASC1 ufmylation pathway for ERα transactivation, which leads to development of breast cancer.

POSSIBILITY FOR DEVELOPMENT OF ANTI-BREAST CANCER DRUG

Breast cancer is one of the most prevailing cancers of woman. It is well-known that estrogen plays a critical role in the pathogenesis and development of breast cancer (35). Moreover, nearly 70% of breast cancer is ERα-positive (36). Therefore, patients with ERα-positive cancer have been treated with aromatase inhibitors, which prevent the synthesis of estrogen or with tamoxifen, which blocks the binding of estrogen to ERα (35–38). These treatments are highly effective, but many patients inevitably develop the drug-resistant invasive tumors. Therefore, new drugs against ERα-positive breast cancer are of high demand.

As to the findings that estrogen-induced ASC1 ufmylation is required for ERα transactivation and tumor formation (22), UBA5 and other components of UFM1-conjugating machinery involved in ASC1 ufmylation could be used as potential targets for development of new therapeutic drugs against ERα-positive breast cancer. Significantly, the induction of ASC1 ufmylation is not limited to estrogen, but could also occur in the presence of other ligands, such as testosterone and retinoic acid, if their cognate nuclear receptors are present in cells (22). Thus, the components of UFM1-conjugating system may also represent potent therapeutic targets in patients with other nuclear receptor-related cancers, such as prostate and leukemic cancers. Since UFSP2 knockdown leads

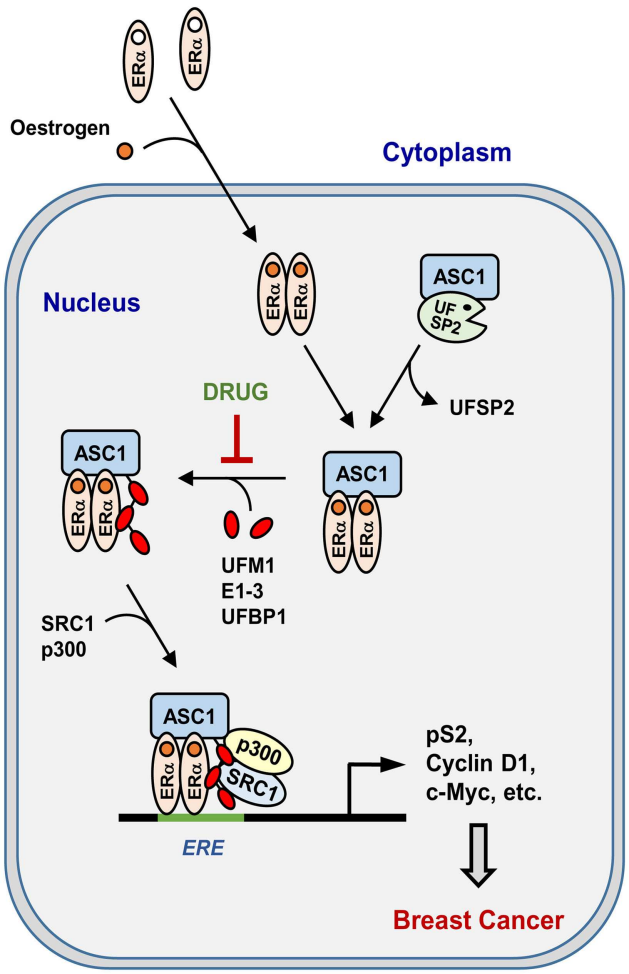

FIGURE 4 | Pathway for estrogen-induced ASC1 ufmylation for ERα transactivation. "DRUG" indicates any small molecule that can act as a therapeutic anti-breast cancer drug by blocking ASC1 ufmylation.

to the most dramatic effect on the increase in cell proliferation, anchorage-independent colony formation, and tumor formation, small molecules that increase the affinity of UFSP2 to the zinc-finger domain of ASC1, or the activity of the protease could also be used as a potential drug against the nuclear receptor-mediated cancers.

OTHER BIOLOGICAL FUNCTIONS OF THE UFM1-CONJUGATING SYSTEM

UFL1 IN TUMORIGENESIS

The human *UFL1* gene is located in chromosome 6q16.1, a region that was reported to be frequently lost in prostate and gastric cancers as well as in bile duct cancer cell lines (39–41). It has also been reported that the expression of UFL1 (also called NLBP, RCAD, and Maxer) cannot be detected in invasive hepatocellular carcinoma cells including HepG2, Hep3B, HLE, and PLC, whereas it can be detected in non-invasive Huh7 hepatocellular carcinoma cell line (39). In addition, UFL1 was shown to cooperate with LZAP in suppression of cell invasion and NF-κB signaling by mutual stabilization, suggesting that UFL1 may act as a tumor suppressor (27).

However, it has also been reported that UFL1 knockdown suppresses the proliferation of C6 glioma cells and LZAP-mediated inhibition of Cyclin D1 transcription (25). In addition, UFL1 is highly expressed in human lung adenocarcinoma and its overexpression promotes the proliferation of rat H1299 lung cancer cells through interaction with p120 catenin, suggesting that UFL1 may play a role in development of lung carcinoma (42). Thus, UFL1 seems to have two opposite functions: one in tumor suppression and the other in tumor development, perhaps depending on its target proteins for ufmylation in different types of cells and tissues. In this respect, it would be of high interest to see if UFL1-mediated ufmylation differentially influences the function of LZAP and p120 catenin in the control of tumorigenesis, although it is also possible that UFL1 may regulate their functions independently of its E3 ligase activity.

Interestingly, UFBP1 was shown to bind to I-κB, stabilize it, and thereby inhibit NF-κB signaling (43). In addition, UFBP1 knockdown leads to inhibition of cell migration and invasion. Thus, UFBP1, like UFL1, plays two opposite roles as a tumor suppressor by inhibiting NF-κB signaling and as a tumor promoter by serving as a cofactor of the UFM1-conjugating system for ASC1 in development of breast cancer. UFBP1 may regulate NF-κB pathway independently of its cofactor function in ufmylation. Further studies are required to clarify the opposite dual functions of UFBP1 and UFL1 in the control of tumorigenesis and NF-κB signaling.

THE UFM1 SYSTEM IN ER STRESS RESPONSE

The expression of UFM1 was shown to be up-regulated in type 2 diabetes and ischemic heart disease in mice, whose pathological conditions are associated with activation of ER stress response (44–47). ER stress induced by cyclopiazonic acid or thapsigargin, both of which are inhibitors of the ER Ca^{2+} ATPase pump, was also shown to increase the expression of UFM1, UFBP1, and UFL1 (48). Interestingly, this increase attenuates ER stress-induced apoptosis of mouse pancreatic β-cells. Recently, brefeldin, an inhibitor of vesicle trafficking, has been shown to increase the transcript level of UFM1, UFBP1, and UFL1, and this increase could not be observed in *Xbp1*$^{-/-}$ MEFs (45). These findings suggest that the UFM1-conjugating system plays an important role in maintaining the ER homeostasis.

CONCLUDING REMARKS

The UFM1-conjugating machinery, consisting of UBA5 (E1), UFC1 (E2), UFL1 (E3), and UFBP1, is the most recently discovered post-translational protein modification system, whose biological function is largely unknown. Intriguingly, estrogen-induced ASC1 ufmylation by this system plays a crucial role in development of breast cancer by promoting ERα-transactivation. Thus, each component of the UFM1-conjugating machinery and UFSP2 that reverses ufmylation process could be potential targets for development of drug against ERα-positive breast cancer. Since ASC1 ufmylation can be achieved by specific ligands for other nuclear receptors, such as AR and RARα, it would be of interest to examine whether ASC1 ufmylation also promotes the transcriptional activity of the nuclear receptors and thereby the receptor-mediated

cancers, such as prostate and leukemic cancers, respectively. Up to present, however, the knowledge on biological function of protein ufmylation is limited to ASC1. Significantly, ER stress induces the expression of UBA5, UFBP1, and UFL1, suggesting that proteins involved in UPR response may be potential targets for ufmylation. Thus, extensive studies are of necessity to identify more target proteins for ufmylation and explore their biological function.

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The role of histone acetyltransferases in normal and malignant hematopoiesis

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Histone, and non-histone, protein acetylation plays an important role in a variety of cellular events, including the normal and abnormal development of blood cells, by changing the epigenetic status of chromatin and regulating non-histone protein function. Histone acetyltransferases (HATs), which are the enzymes responsible for histone and non-histone protein acetylation, contain p300/CBP, MYST, and GNAT family members. HATs are not only protein modifiers and epigenetic factors but also critical regulators of cell development and carcinogenesis. Here, we will review the function of HATs such as p300/CBP, Tip60, MOZ/MORF, and GCN5/PCAF in normal hematopoiesis and the pathogenesis of hematological malignancies. The inhibitors that have been developed to target HATs will also be reviewed here. Understanding the roles of HATs in normal/malignant hematopoiesis will provide the potential therapeutic targets for the hematological malignancies.

Keywords: histone acetyltransferases, hematopoiesis, transcriptional regulation, hematopoietic stem cells, hematological malignancies

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Introduction

Histone acetyltransferases (HATs) acetylate histone proteins by transferring acetyl group from acetyl-CoA to specific lysine residues (1, 2). The acetylation of histones by HATs results in a dispersed structure of chromatin, which becomes accessible by transcriptional factors. Besides histones, a variety of non-histone substrates also have been shown to be acetylated by HATs, thus the HATs are now generally categorized as lysine acetyltransferases (3). The acetylome studies have led to discovery of many new substrates of HATs, and a lot of non-histone substrates of HATs, such as AML1, AML1-ETO (AE), p53, c-MYC, NF-κB, Cohesin and Tubulin, have been found to play important roles in different cellular processes (4–10). Based on the cellular localization, HATs are classified into type A and type B HATs. The type A HATs show nuclear localization and likely catalyze the processes related to transcription (11). The type A HATs are further divided into five families according to their homology and acetylation mechanisms. The GNAT family members include PCAF, Gnc5 and ELP3. CBP and p300 form the CBP/p300 family (12). Tip60, MOZ, MORF, HBO1 and HMOF belong to the MYST family (13). The transcriptional factor related HAT family includes TAF1 and TIFIIIC90. In addition, several steroid receptor co-activators, such as p600, SRC1, CLOCK and AIB1/ACTR/SCR3 etc., are also HATs. (14, 15). Type B HATs are localized in the cytoplasm and they are shown to acetylate the newly synthesized histones. For

example, HAT1 is one of type B HAT members and functions in DNA repair and histone deposition (16).

Histone acetyltransferases play key roles in normal and malignant hematopoiesis. The acetylation of histones and non-histone proteins has been shown to regulate normal blood cell development (17–19) (**Table 1**). Analysis of chromatin factor interaction network in hematopoietic development shows multiple chromatin factor complexes, including NuA4/P300/CBP/HBO1, are required for normal hematopoiesis (20). Protein acetylation regulates hematopoietic stem cell (HSC) self-renewal, proliferation, and their differentiation into committed hematopoietic progenitors. In line with the critical functions of HATs in normal hematopoiesis, chromosomal translocations that involve HAT genes are frequently found in hematological malignancies. Recent cancer genome studies have identified HATs as common targets for mutations in these diseases. Meanwhile, the acetylation states of some oncoproteins and tumor suppressor proteins have been correlated with hematological malignancies manifestation (5, 19, 21–25) (**Table 2**). Notably, most leukemogenic fusion proteins physically interact with HATs, even though they are not directly fused with HATs, suggesting that the aberrant acetylation regulation by these fusion proteins are critically important in leukemogenesis (26). In this article, we will first briefly review a few examples of interesting findings that potentially lead to development of new therapeutic strategies for hematological malignancies, and then provide an overview of the functions of HATs in normal and malignant hematopoiesis (**Figure 1**).

TABLE 1 | The role of HATs in hematopoietic stem/progenitor cells.

Cell type	Acetyl-transferase	Target genes	Established role/function
Hematopoietic stem/progenitor cells	p300	C-Myb	Block proliferation and promote differentiation
	CBP	Gfi1b	Promote self-renewal and block differentiation
	MOZ	p16	Generate and maintain HSCs
Myeloid progenitor cells	p300/CBP	C-Myb	Block proliferation and promote differentiation
	MOZ	p16	Promote hematopoietic progenitors proliferation
	HBO1	Gata1	Promote fetal liver erythropoiesis
Lymphoid cells	p300	Foxp3/C-Myb	Regulate Foxp3(+) Treg cell function and homeostasis
	GCN5	PI3K/AKT/Syk/Btk	Regulate B cell apoptosis

Bromodomains are Promising Targets for treating Hematological malignancies

Acetylated lysine residues generated by HATs can be specifically bound by some protein domains ("readers"). Bromodomains have been identified as an important type of the readers of acetyl lysine. The human genome encodes over 60 bromodomain proteins, including HATs, HAT-associated proteins (such as GCN5L2, PCAF, and BRD9), histone methyltransferases (such as ASH1L and MLL), transcriptional co-activators (such as TRIMs and TAFs), as well as the BET family proteins (27). BRD4, a member of the BET family proteins, is shown to locate at the enhancer and/or promoter regions of many active genes. The bromodomain inhibitor JQ1 is able to disassociate BRD4 from acetylated histones, leading to downregulation of gene transcription and decreased phosphorylation of RNA polymerase II (28) (**Figure 2**). JQ1 is also able to remove BRD4 from the super-enhancers and thus repress many super-enhancer regulated oncogenes. These findings have been verified in hematological malignancies, such as diffuse large B cell lymphoma (DLBCL), and provide a basis for using JQ1, as well as other BET bromodomain inhibitors, in the treatment of hematological malignancies (29–31).

HATs Generate the Markers of Super-Enhancers in Hematological Malignancies

Super-enhancers are clusters of active enhancers bound by master transcription factors and cofactors, including the mediator complex, and can promote the high-level expression of genes that control cell identity (32, 33). Cancer cells can acquire super-enhancers to active oncogenes, which suggest that super-enhancer-associated genes could be candidate oncogenic drivers (29, 34–36). Histone H3 lysine 27 acetylation (H3K27ac) by p300/CBP is used as an active enhancer mark, which biophysically facilitates opening of chromatin and recruits the co-activators that recognize ϵ-acetyl lysine through bromodomain (37). BRD4 is most commonly associated with enhancer regions, defined by the presence of H3K27ac by p300/CBP and the absence of H3K4me3. In a genome-wide study of DLBCL, a rank ordering of the enhancer regions by H3K27ac enrichment reveals that BRD4 binds to the majority of active enhancers and that the genome-wide correlation between BRD4 occupancy and H3K27ac is extremely strong. Importantly, BRD4 is highly loaded at the super-enhancers (29). These findings suggest that the HAT-mediated H3K27ac and the BRD4 recruitment may play an important role in the formation and function of the super-enhancers.

TABLE 2 | The role of HATs in hematological malignancies.

Acetyltransferase	Disease	Non-histone substrate	Target genes	Established role/function	Inhibitors
p300/CBP	AML	C-Myb, AML1–ETO	Id1, p21, Egr1	Block differentiation and promote self-renewal	C646, EGCG, L002
p300	T cell leukemia	Notch3	Unknown	Promote Notch3-induced T cell proliferation	Garcinol
Tip60	AML, lymphoma	Unknown	C-Myc, p53	Tumor suppressor and modulate DDR signaling	Garcinol
MOZ/MORF	AML	AML1	p53, RARβ, PU.1	MOZ-related fusion proteins transduce HSPCs	Garcinol
GCN5	B cell ALL, AML	E2A-PBX1, AME	Syk, Btk	Promote cell transformation	MB-3, Garcinol
PCAF	AML, CML	AME	AML1	Promote AML1-dependent transcription	MG153, Garcinol

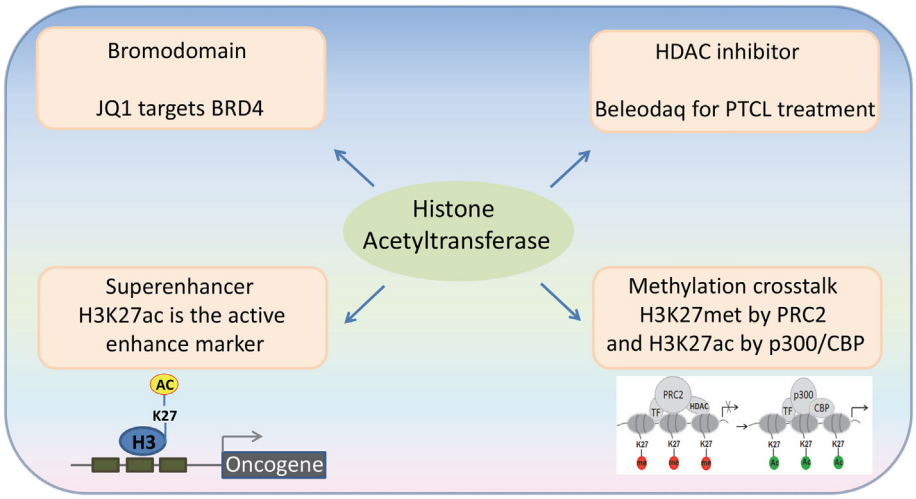

FIGURE 1 | **Recent advances in the emerging fields of histone acetylation in hematopoiesis: (1) Bromodomains are a promising target for the therapy of hematological malignancies; (2) HATs generate histone marks found in active enhancers; (3) Histone acetylation-methylation crosstalk in hematopoiesis; (4) Third generation HDAC inhibitors for the therapy of hematological malignancies.**

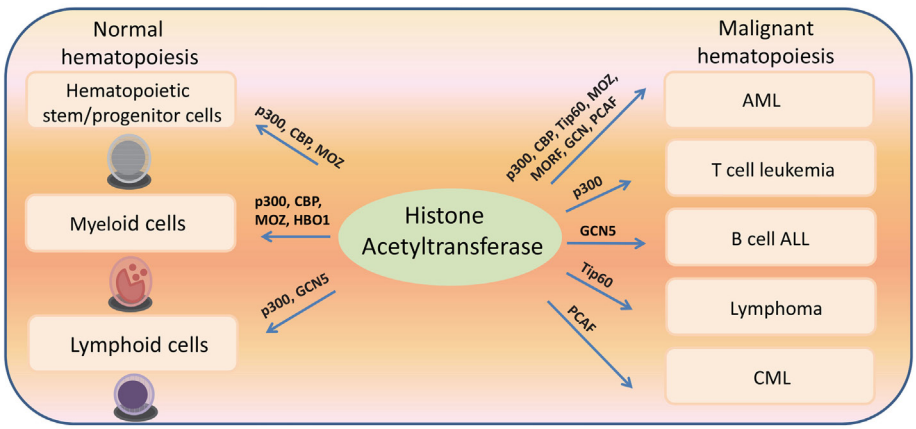

FIGURE 2 | **Histone acetyltransferases regulate both normal and malignant hematopoiesis.**

Histone Acetylation-Methylation Crosstalk in Hematological Malignancies

Polycomb group (PcG) proteins are transcriptional repressors, and their abnormal expression is frequently associated with hematological malignancies. The PcG proteins can induce transcription repression through disassociating HATs from their target genes (38). The Polycomb repressive complex 2 (PRC2) catalyzes trimethylation of H3K27, and this activity is important for transcriptional repression. Depletion of PRC2 leads to a global increase of H3K27 acetylation, which is catalyzed by p300 and CBP. In MLL-AF9-transduced HSCs, the transcriptional activation of PcG-targeted genes has been found to correlate with a methylation-to-acetylation change (**Figure 1**). These findings suggest that the acetylation–methylation crosstalk plays an important role in hematological malignancies.

The Role of Histone Acetyltransferases in Normal Hematopoiesis

The Role of HATs in Hematopoietic Stem/Progenitor Cells

p300/CBP Knockout Mouse studies

The initial studies showed that CBP, instead of p300, is pivotal for the self-renewal of HSCs. However, it has been shown that p300, but not CBP, is essential for hematopoietic differentiation (39). However, it has been recently reported that CBP regulates both self-renewal and differentiation in adult HSCs and that loss of CBP leads to an increase in apoptosis, differentiation, and quiescence in HSCs through regulating Gfi1b (40). The numbers of colony-forming cells and erythroid cells are reduced in mouse embryos expressing the truncated CBP protein (1–1084 amino acids), which suggests that CBP mutations disrupt

primitive hematopoiesis. Abnormal endothelial precursors have been found when the CBP mutant para-aortic splanchnopleural mesoderm was cultured with stromal cells, suggesting defects in the hematopoietic microenvironment (39). *CBP*$^{+/-}$ mice develop highly penetrant, multilineage defects in hematopoietic differentiation (41). These findings indicate that CBP has important functions in normal hematopoiesis. No such pathology was observed in *p300*$^{+/-}$ mice. Thus, p300 and CBP play essential but distinct roles in maintaining normal hematopoiesis (42).

MOZ is Crucial for the Generation and Development of HSPCs

MOZ contains two coactivation domains and a HAT catalytic domain. The fetal liver cells with *moz* mutation were able to help reconstitute the hematopoietic system in the lethally irradiated recipient mice. In the Moz-deficient mice, the number of hematopoietic progenitors in all lineages was reduced, and defects in HSCs were found (43). Loss of MOZ HAT activity causes abnormalities in hematopoietic stem/progenitor cell (HSPC) numbers in mice since HSPCs lacking MOZ HAT activity cannot expand. Loss of MOZ HAT activity also leads to the disruption of B cell development in mice. MOZ-mediated acetylation has been found to play an important role, controlling the balance between differentiation and proliferation in normal hematopoiesis (26, 44). MOZ controls the proliferation of HSCs at least in part by repressing the transcription of p16. The expression level of p16 is increased in HSPCs without MOZ HAT activity, which can induce the senescence of HSPCs. Loss of p16 rescues the proliferative abnormality in the hematopoietic progenitors lacking the MOZ HAT activity. These findings indicate an important role of MOZ HAT activity in the transcription of p16 and HSPC senescence (45). Together, MOZ is essential for a fundamental property of HSCs and the development of hematopoietic progenitors.

The Role of HATs in Myeloid Progenitors and Differentiation

The KIX Domains of p300/CBP are Required for Definitive Hematopoiesis

The KIX domains in p300 and CBP are responsible for interacting with other proteins, and they regulate c-Myb-mediated transcription activation and repression. Loss of the CH1 or KIX domain in p300 leads to profound abnormalities in hematopoiesis, while deletion of other portions of p300 only affects some specific lineages (46). Certain site specific point mutations in the KIX domain of p300 can disrupt the interaction between p300 and CREB/c-Myb, and mice homozygous for these mutations have many hematopoietic defects, such as anemia, thrombocytosis, megakaryocytosis, thymic hypoplasia, and B cell deficiency. However, no defects are detected in mice carrying the same point mutations in CBP. The interaction between the KIX domain of p300 and c-Myb is important for the function and development of megakaryocytes, and a synergistic genetic interaction has been found between the mutations in the KIX domain of p300 and mutations in c-Myb. CBP KIX domain mutations affect platelets, B cells, T cells, and red cells. Therefore, the KIX domains in p300 and CBP have their unique functions in normal hematopoiesis (47). Altogether, the KIX domains in p300 and CBP are essential for the normal hematopoiesis through regulating c-Myb-mediated transcription activation and repression (48).

The Hbo1-Brd1/Brpf2 Complex is Required for Fetal Liver Erythropoiesis

HBO1 is responsible for the acetylation of histone H4K5, K8, and K12. The interaction between ING4 and histone H3K4me3 augments the ability of HBO1 to acetylate histone H3 (49, 50). HBO1 and BRD1 can form a HAT complex and control erythropoiesis. Loss of Brd1 leads to severe anemia in mouse embryos due to abnormal erythropoiesis in the fetal liver. HBO1 and BRD1 are found to mostly co-localize in the erythroblast genome, and regulate critical developmental genes. Loss of Brd1 or depletion of Hbo1 significantly decreases the levels of H3K14 acetylation in erythroblasts. Loss of Brd1 leads to reduced expression of Gata1, the key erythroid developmental regulator, and the forced expression of Gata1 can partially rescue the abnormal erythropoiesis induced by loss of Brd1. Taken together, the Brd1–Hbo1 HAT complex is an important H3K14 HAT, which is essential for the transcriptional activation of key erythroid regulators (17).

The Role of HATs in Lymphoid Cells

p300 is Critical for the Function and Homeostasis of Foxp3(+) Treg Cells

Forkhead box P3 (Foxp3) is acetylated by p300 and is essential for the development of a Treg suppressor phenotype. Hyperacetylation of Foxp3 prevents its ubiquitination and proteasome mediated degradation, which leads to a significant increase in the Foxp3 protein level. Foxp3 acetylation can rapidly control Foxp3 protein levels in T cells, which provides a new mechanism for regulating the number and function of Treg cells (51). In the presence of a p300 inhibitor, Garcinol, p300 becomes disassociated from the FOXP3 protein complex, and subsequently FOXP3 is degraded through the lysosome-dependent system. A subset of four lysine residues, which together control the total acetylation of FOXP3, could also be acetylated by p300 (52, 53). The conditional deletion or pharmacologic inhibition of p300, was able to increase apoptosis induced by the T cell receptor in Foxp3(+) Treg cells, and inhibit tumor growth in immunodeficient mice. Together, p300 is critical for the function and homeostasis of Foxp3(+) Treg cells, and thus p300 inhibitors are able to impair the function of Treg cells without affecting T effector cells suggesting a new approach for cancer immunotherapy (54).

The Role of GCN5 in Lymphoid Cells

GCN5 controls the PI3K/AKT pathway activation through regulating the transcription of Btk and Syk, which are involved in PI3K/AKT pathway activation in B cells under oxidative stress. GCN5 deficiency significantly induced apoptosis in chicken DT40 cells treated with hydrogen peroxide. GCN5 is localized at the proximal 5′-upstream regions of Btk and Syk, and the expression levels of Syk and Btk were significantly decreased in GCN5-deficient chicken DT40 cells exposed to exogenous hydrogen peroxide. Moreover,

the phosphorylation level of AKT was also significantly decreased in hydrogen peroxide-treated GCN5-deficient chicken DT40 cells. Together, GCN5 regulates the transcription of Btk and Syk, and is crucial for the epigenetic regulation of the PI3K/AKT pathway activation in lymphoid cells under oxidative stress (55).

The Role of Histone Acetyltransferases in Malignant Hematopoiesis

p300/CBP

The Interaction of p300/CBP with c-Myb is Required for the Induction of Acute Myeloid Leukemia

CBP/p300 is an essential co-activator for the transforming capacity of c-Myb (56). CBP/p300 is required for the ability of c-Myb to repress several key target genes involved in myeloid differentiation and p300 is recruited to c-Myb-binding sites close to c-Myb target genes (57). The interaction of p300/CBP with c-Myb is essential for leukemic transformation by the myeloid leukemia oncogenes AE, MLL-ENL, and MLL-AF9 (58). The p300–c-Myb interaction is essential for the ability of AE, MLL-ENL, and MLL-AF9 fusion proteins to confer self-renewal properties on myeloid progenitor cells. In the absence of this interaction, these fusion oncoproteins are unable to impose a block of differentiation, leading instead to terminal differentiation. Myeloid progenitors from Plt6 mice, which have a mutation in p300, are also refractory to transformation by the AE and MLL fusion proteins. Taken together, the specific interaction between p300 and c-Myb is required to control a transcriptional program, which is essential for the acquisition of self-renewal and possibly other leukemogenic properties upon expression of fusion oncoproteins. Thus, disruption of the p300–c-Myb interaction could be a potential therapeutic strategy for acute myeloid leukemia (AML) (58).

The Acetylation of Notch 3 by p300 in T Cell Leukemia

Notch3 is acetylated at lysine 1692 and lysine 1731 by p300, which can be deacetylated by HDAC1. The acetylation of Notch3 by p300 is able to promote its ubiquitination and protein degradation through proteasome system. Consequently, the expression level of Notch3 and its transcriptional activity are decreased in the non-acetylatable Notch3 mutant transgenic mice, which leads to defects in the Notch3 downstream signaling. The non-acetylatable Notch3 mutant can enhance Notch3-induced growth of T cell leukemia proliferation, which can be blocked by a histone deacetylases (HDAC) inhibitor. In the Notch3 transgenic mouse model, HDAC inhibitor-mediated hyperacetylation of Notch3 inhibits the proliferation of T cell leukemia/lymphoma cells. Altogether, targeting Notch3 deacetylation could be a promising therapeutic strategy for T-cell leukemia (25).

The HAT Domain and Bromodomain are Required for MLL–CBP-Induced Transformation in AML

The CBP gene is fused with the MLL gene in patients with *t*(11;16) MDS; the MLL–CBP fusion contains a mostly intact CBP, suggesting involvement of CBP in leukemogenesis (59). Both the CBP HAT domain and bromodomain are required for MLL–CBP-induced transformation in AML, which is usually preceded by an MDS phase. The replacement of the MLL–CBP HAT domain with the PCAF/GCN5 HAT domain enhanced the proliferation of hematopoietic progenitor cells and led to the loss of myeloid cell surface markers in these cells. These phenotypes were not observed when the CBP bomodomain of MLL–CBP was replaced by the PCAF/TAFII250 bromodomain. The recipient mice transplanted with domain-swapped hematopoietic progenitors developed lymphoid disease or had low-frequency MDS that progressed to AML. Thus, the CBP HAT domain and bromodomain have different functions but play important roles in the pathogenesis of MLL–CBP-positive leukemias (60).

The Acetylation of the AE Fusion Protein by p300 is Required for the Induction of Acute Leukemia

We have shown that transcriptional activation by AE is crucial for leukemogenesis and that AE interacts with the transcriptional co-activator, p300. The important function of the AML1–ETO/p300 interaction is that AE can be acetylated, as acetylation of AE is essential for its self-renewal promoting effects. The acetylation of AE9a by p300 at a specific lysine residue (K43) is required for its ability to induce leukemia in mice. Pharmacological and RNA interference-mediated inhibition of p300 specifically impairs AE/AE9a-induced transcriptional activation and leukemogenesis, but does not affect the development of MLL–AF9-induced leukemia. Acetyl-AEK43 is present in blast cells isolated from *t*(8;21) leukemia patients and these leukemia cells, but not normal human $CD34^+$ cells, are sensitive to growth inhibition by p300 inhibitors. AE and p300 co-localize at the regulatory regions of many AE upregulated genes, which includes the regulators of self-renewal (e.g., *Id1*, *p21*, and *Egr1*). AE and p300 can cooperate in the transcriptional regulation of these target genes. Several TFIID subunits that specifically bind to a K43 acetylated AML1 peptide but not to the identical non-acetylated peptide. Furthermore, these results establish a novel link between post-translational modification of a non-histone protein (by a "histone modifying enzyme") and transcriptional regulation, which has crucial implications for the study of cancer and the regulation of gene expression. As ETO is thought to be a key component of co-repressor complexes, its interactions with the co-activator p300 in cells is surprising and raises a question about whether "bipotential" complexes, like bivalent histone marks, may allow cells to turn on or off a given set of genes in response to certain internal or external signals. Thus, acetylation of an oncogenic fusion transcription factor itself can promote gene activation independent of any effect on histone acetylation, and can be essential for leukemia stem cell to self-renewal and malignant transformation (4, 61).

Tip60

Tip60 is Required for an Oncogene-Induced DNA-Damage Response

Tip60 regulates several transcription factors, which can promote or suppress carcinogenesis (e.g., p53 and Myc). *Tip60* regulates DNA-damage response (DDR) signaling induced by oncogenes, which can prevent cancer progression. Loss of one allele of *Tip60* inhibited Myc-induced DDR but had no effect on DDR in normal B cells. In the *Tip60* heterozygous knockout mice, *Tip60* inhibits Myc-induced lymphomagenesis at the pre-tumoral stage. The

mono-allelic loss of *Tip60* occurs often in lymphomas with concomitant reduction in mRNA levels; this often coexists with p53 mutations and is related to the disease grade. Thus, *Tip60* functions as a tumor suppressor in a haplo-insufficient manner, and *Tip60* is essential for controlling Myc-induced DDR in cancer cells (62).

Tip60 is Involved in c-Myb-Driven Leukemogenesis

TIP60 interacts with c-Myb, which requires the transactivation domain of c-Myb and the HAT domain of *TIP60*. Coexpression of *TIP60* impairs c-Myb-induced transcription activation. *TIP60* can bind to the promoters of c-Myb target genes, which is dependent on c-Myb. Furthermore, c-Myb interacts with HDAC1 and HDAC2, which are associated with *TIP60* and cause transcriptional repression. *TIP60* negatively regulates the transcription activity of c-Myb through interacting with HDACs in human hematopoietic cells. Consistently, knockdown of *Tip60* increased the expression level of c-Myc. It has been found that the expression level of *Tip60* is much (~60%) lower in the patients with AML. These findings suggest that *TIP60* regulates the function of c-Myb and that dysregulated *TIP60* could be involved in c-Myb-driven leukemogenesis (63).

MOZ/MORF

The MOZ-TIF2 Fusion Protein is Associated with AML

MOZ–TIF2 is associated with AML chromosomal abnormalities at inv(8)(p11q13). MOZ–TIF2 contains the CBP interaction domain (CID) of TIF2 and the HAT domains of MOZ and TIF2 (64–67). In a murine bone marrow transplant assay, MOZ–TIF2 causes AML that could be serially transplanted (67). It has been found that the interaction between MOZ–TIF2 and CBP through the CID domain and the C2HC nucleosome recognition motif in MOZ are essential for transformation (68). MOZ–TIF2 dominantly inhibits the transcription activity of CBP-dependent activation (e.g., p53 and nuclear receptors), which requires the CID domain. The nuclear localization of MOZ–TIF2 is abnormal, which is dependent on the MOZ portion of this fusion protein. CBP expression is decreased in the cells expressing MOZ–TIF2, which results in the depletion of CBP from PML bodies. The critical characteristics of MOZ–TIF2 are to disrupt the normal activity of CBP/CBP-dependent activators in acute myeloid leukemia (69). MOZ–TIF2 binds to the promoter of RARβ2, leading to the dissociation of CBP/p300, the abnormal histone acetylation and the downregulation of RARβ2. MOZ–TIF2 was recruited to AML1 target promoters and upregulated transcription mediated by AML1. Both MOZ and MOZ–TIF2 were found to co-localize with AML1 (70). MOZ–TIF2 impaired retinoic acid-mediated transcription activation of C/EBPβ/CD11b, and inhibited nuclear receptor-induced gene activation through aberrant recruitment of CBP, which required both the MOZ and TIF2 domains of this fusion protein (71).

In a transgenic zebrafish model, spi1 promoter-driven MOZ–TIF2 expression induced the development of AML in 2 of 180 embryos expressing MYST3/NCOA2, in which kidney invasion by myeloid blast cells was observed (72). MOZ–TIF2 interacted with PU.1 to stimulate the expression of M-CSFR, and PU.1 is required for the establishment and maintenance of LSCs by MOZ–TIF2. Loss of CSF1R impairs MOZ–TIF2-induced leuekmogenesis in mouse models and CSF1R inhibitors delay the development of MOZ–TIF2-induced AML (73). MOZ–TIF2 can cooperate with FLT3–ITD to transform hematopoietic cells. STAT5 signaling is required for MOZ–TIF2-induced leukemogenesis. Deletion of STAT5 led to the differentiation of MOZ–TIF2-transduced fetal liver cells, and these cells lost their replating ability. The recipient mice transplanted with $Stat5^{-/-}$ MOZ–TIF2 leukemia cells have longer latency and incomplete penetrance. STAT5 is essential for the self-renewal of leukemia stem cells in MOZ–TIF2 driven leukemia (74). Overexpression of HOXA9, HOXA10, and MEIS1 was observed in AML patients with MOZ fusions. MOZ–TIF2 forms a stable complex with bromodomain-PHD finger protein 1 (BRPF1), and MOZ–TIF2/BRPF1 associate with Hox genes in the MOZ–TIF2 driven leukemia cells. BRPF1 depletion disrupts the localization of MOZ on the Hox genes, which led to the inhibition of MOZ–TIF2-induced transformation. Moreover, mutant MOZ–TIF2 lacking HAT activity could not deregulate HOX genes or initiate AML. Thus, MOZ–TIF2/BRPF1 induces leukemogenesis through the upregulation of HOX genes, which is regulated by MOZ-dependent histone acetylation (75).

MOZ–CBP and MOZ–p300 are Generated by Chromosomal Translocations in AML

The recurrent translocation *t*(8;16)(p11;p13) leads to the MOZ–CBP fusion gene, which contains the MOZ finger motifs and HAT domain and a mostly intact CBP (76). This *t*(8;16)(p11;p13) translocation occurs in the M5 subtype AML, which is characterized by erythrophagocytosis and a poor prognosis. The CBP–MOZ mRNA is not in-frame, which suggested that MOZ–CBP is the critical fusion for the initiation of leukemia (77, 78). MOZ, CBP, and MOZ–CBP are all able to acetylate the transcription factor AML1. The level of MOZ–AML1 complex upregulates when M1 myeloid cells differentiate into macrophages/monocytes. This finding suggests that the MOZ–AML1 complex could have important functions in the differentiation of myeloid cells. MOZ–CBP inhibits the differentiation of M1 myeloid cells, and could induce the development of leukemia through impairing AML1-induced transcription activation (79). MOZ–CBP cooperates with steroid receptor co-activator-1 to activate transcription, and the CBP portion of MOZ–CBP is required for the transcription activity of this fusion protein. It has been found that the interaction between MOZ–CBP and NF-κB could also have critical functions in leukemogenesis (80). MOZ–CBP inhibits p53-mediated transcription, and the impairment of MOZ/p53-induced transcription contributes to the development of leukemia (81).

The translocation *t*(8;22)(p11;q13) in acute myeloid leukemia generates the fusion gene MOZ–p300, and the MOZ zinc finger/HAT domain are fused to a mostly intact p300. Thus, MOZ–p300 has two HAT domains from MOZ and p300 portions, and may play an important role in the development of leukemia through deregulation of histone acetylation (82). It has been found that MOZ is fused with an unknown partner at 2p23 in one patient with tMDS. Therefore, MOZ might lead to the abnormal histone acetylation and promote the pathogenesis of myeloid malignancies (83).

GCN5/PCAF

The *t*(1;19) translocation was found in pediatric pre-B cell ALL, which leads to the fusion of E2A and PBX1 and the generation of a *E2A–PBX1* fusion protein. *E2A–PBX1* is able to induce the transformation of hematopoietic cells. It has been found that SPT3–TAFII31–GCN5L acetylase (STAGA) and its HAT subunit *GCN5* directly binds to the E2A portion of *E2A–PBX1*. *GCN5* can acetylate and stabilize the *E2A–PBX1* fusion protein (23). AML1/MDS1/EVI1 (AME), a transcription repressor generated by translocation *t*(3;21) in human leukemia, binds to P/CAF and *GCN5* through two binding sites, with one of the binding sites being in the Runt domain. *GCN5* and P/CAF are able to acetylate AME, and either P/CAF or *GCN5* can cooperate with AME to impair the repression of *AML1*-dependent transcriptional activation (84, 85).

The Important Role of p53 Acetylation in Hematological Malignancies

The transcription factor p53 was the first non-histone substrate discovered to be acetylated by HATs (5). Levels of p53 acetylation are associated with the activation and stabilization of p53 (86–93); acetylation of p53 also stimulates its sequence-specific DNA-binding (94–97). The seven different lysine (K164, K305, K370, K372, K373, K381, and K382) in the C-terminus of p53 are acetylated by CBP and PCAF (98). Acetylation of p53 is crucial for the recruitment of CBP/p300 to the promoters of its target genes. CBP and p300 are able to promote p53-mediated transcription activation (99). TIP60/hMOF acetylates p53 at position 120 (100, 101), which can be induced by DNA damage or oncogenic stress-mediated p19ARF activation (102). Mutation of p53 lysine 120 to arginine inhibited p53-induced transcription activation. p53 can also be acetylated at one lysine outside its C-terminus, which is critical for the activation of the proapoptotic genes PUMA and BAX. In the p53 acetylation-deficient knockin mouse, the expression of p53 target genes is decreased after DNA damage (103). The deletion of the lysine residue at position 164 and other acetylation sites in p53 blocked p53-mediated transactivation of p21 and inhibition of cell growth (104). These findings suggest that p53 acetylation may play a critical role in the pathogenesis of hematological malignancies.

Third Generation HDAC Inhibitors for the Therapy of Hematological Malignancies

The FDA approved drug, Beleodaq, is used for the therapy of patients with peripheral T cell lymphomas (PTCL), an aggressive disease, which accounts for ~15% of all non-Hodgkin lymphomas. Beleodaq inhibits HDAC and it is the third drug to receive FDA approval for PTCL. In the trial that led to the FDA approval, Beleodaq's overall response rates were comparable to those of Folotyn and Istodax; 10.8% of patients experienced a complete response and 15% had a partial response. The response rate was even higher in patients with angioimmunoblastic T cell lymphoma, which suggests that targeting acetylation is a promising therapeutic strategy for the therapy of hematological malignancies.

The Potential Therapeutic Effects of Histone Acetyltransferase inhibitor in Hematopoiesis

The HAT Inhibitor, Garcinol, Induces the Expansion of Hematopoietic Stem/Progenitor Cells

The HAT inhibitor, Garcinol, is derived from plants. It has been identified as a stimulator of human HSPCs expansion in the screening of natural products. During a 7-day culture of $CD34^+CD38^-$ HSCs or $CD34^+$ HSPCs, Garcinol was able to induce the expansion of HSPCs, and this ability is associated with its inhibitory effect on HATs. The derivatives of Garcinol, which can expand HSPCs, are also able to inhibit HAT activity and histone acetylation. Altogether, the Garcinol effects suggest that targeting HATs could be a promising strategy for expanding HSPCs (105).

Inhibition of p300 Impairs Antitumor Immunity

$Foxp3^+$ Treg cells can not only regulate immune homeostasis/autoimmunity but also limit the immune response of hosts to tumors. Thus, targeting $Foxp3^+$ Treg cells could be a promising strategy to improve antitumor immunity. Conditional deletion or pharmacological inhibition of p300 was able to increase T cell receptor-induced apoptosis in $Foxp3^+$ Treg cells and abrogate the suppressive functions of Treg cells. Inhibition of p300 can also impair the induction of peripheral Treg cells and tumor growth in the immunocompetent mouse model. Collectively, p300 is critical for the homeostasis and function of $Foxp3^+$ Treg cells, and targeting p300 could be a new approach for cancer immunotherapy (54).

The p300 Inhibitor, C646, Acts selectively on *t*(8;21) leukemia Cells

The HAT p300 can enhance the self-renewal ability of leukemia stem cells through acetylating AE and activating the target genes of AE, which indicated that p300 could be a promising drug target for *t*(8;21) leukemia. C646, a selective and competitive p300 inhibitor, can inhibit the proliferation and colony formation of *t*(8;21) leukemia cells, and induce apoptosis and G1 phase cell cycle arrest in *t*(8;21) leukemia cells and primary cells isolated from patients with *t*(8;21) leukemia. C646 does not significantly affect the normal HSPCs mobilized by GCSF. In particular, $AML1^-ETO^+$ AML cells are more sensitive to C646 compared with $AML1^-ETO^-$ AML cells. The growth inhibition of $AML1^-ETO^+$ leukemia cells induced by C646 are associated with the decreased acetylation of histone H3 and downregulation of Bcl2/C-kit, suggesting that C646 could be a promising drug candidate for the treatment of $AML1^-ETO^+$ leukemia (4, 106).

The HAT inhibitor, Epigallocatechin-3-Gallate, inhibits B cell Transformation

Epigallocatechin-3-gallate (EGCG) has been found as a HAT inhibitor in natural compound screening. EGCG can block p300-mediated acetylation of p65, impairing its translocation to the nucleus, and it can upregulate the amount of IκBα in the cytoplasm, thus inhibiting NF-κB activity in several ways, and decreasing the expression of NF-κB target genes. EGCG impairs B cell transformation by EBV, perhaps via suppression of NF-κB acetylation (107), and it can inhibit the binding of p300 to the IL-6

promoter and block cytokine gene expression. Thus, EGCG could be a potential therapy for B cell malignancies.

Leukemia and Lymphoma Cell Lines are sensitive to p300 Inhibitor L002

p300 plays an important role in signal transduction pathways that promote the proliferation and survival of malignant cells. Therefore, p300 represents a promising drug target for hematological malignancies, and libraries of compounds have been screened for p300 inhibitors. One candidate, L002, inhibits p300 *in vitro*, with an IC50 of ~2 μM. L002 can block histone acetylation and p53 acetylation, and can inhibit the activation of STAT3. Biochemical testing of a series of related compounds revealed functional groups that may impact the inhibitory potency of L002 against p300. Interestingly, these analogs show inhibitory activities against CBP, PCAF, and GCN5, but against several other acetyltransferases (KAT5, KAT6B, and KAT7), HDACs and HMTases. Among the NCI-60 panel of cancer cell lines, leukemia, and lymphoma cell lines were extremely sensitive to L002. Thus, this new acetyltransferase inhibitor, L002, is a potential anticancer agent (108).

Anacardic Acid Derivatives Inhibit PCAF and induce Apoptosis in Chronic Myeloid Leukemia Cells

The different acetylation of proteins correlates with the development of BCR–ABL-positive leukemia. A derivative of anacardic acid – small molecule MG153, which is developed to have stronger HAT inhibitory ability, is a potent inhibitor of PCAF. The inhibition of PCAF decreases proliferation and induces apoptosis, which correlates with loss of the mitochondrial membrane potential and DNA fragmentation. Importantly, cells expressing BCR–ABL are more sensitive to PCAF inhibition compared to parental cells without BCR–ABL. Moreover, inhibition of PCAF in BCR–ABL-expressing cells breaks their resistance to DNA damage-induced cell death. Targeting the PCAF alone or in combination with DNA-damaging drugs shows cytotoxic effects and should be considered as a prospective therapeutic strategy in chronic myeloid leukemia (CML) cells. Moreover, anacardic acid derivative MG153 is a valuable agent and further studies validating its therapeutic relevance should be performed (109).

GCN5 Inhibitors have Anti-Leukemia Effects

The a-methylene-g-butyrolactone 3 (MB-3) is a cell-permeable inhibitor against GCN5, and is able to decrease the levels of histone H3 acetylation and non-histone substrate (a-tubulin) acetylation. GCN5 acetylates E2A–PBX1, and MB-3 reduces the levels of E2A–PBX1 acetylation and E2A–PBX1 protein in a dose-dependent manner. RCH–ACV cells are derived from the bone marrow cells of a patient with pre-B cell acute lymphoid leukemia, and has *t*(1;19) translocation, which generates the *E2A–PBX*1 fusion gene. E2A–PBX1 acetylation was inhibited by MB-3, and the level of E2A–PBX1 and GCN5 protein was decreased when RCH–ACV cells were treated with MB-3. The E2A–PBX1 half-life was shorter in the cells treated with MB-3, indicating GCN5-dependent acetylation can affect the stability of E2A–PBX1 protein. A reduction in Wnt16, an E2A–PBX1 target gene were also observed in RCH–ACV cells cultured with MB-3, indicating the importance for the pathogenesis of *t*(1;19)-positive pre-B cell leukemia. Additionally, the expression of E2A–PBX1, E2A, and Wnt16 were significantly decreased in the primary *t*(1;19) pre-B ALL cells treated with MB-3. MB-3 does not affect the expression of Pol II or Tubulin, which suggests that MB-3 can destabilize certain proteins probably through the inhibition of GCN5-dependent histone or non-histone substrate acetylation. These findings indicate that GCN5 inhibitors have potential value as therapeutic agents for ALL (23). Some recently identified GCN5 inhibitors, such as (thiazol-2-yl)hydrizones (110, 111), might also be able to target hematological malignancies.

Conclusions

Lysine acetylation acetylation occurs not only at the histone tails but also in the non-histone proteins. LATs are catalytic subunits of multiprotein complexes, whose biochemical and molecular characterization have yielded much important information about the function and regulation of acetyltransferase activity. Importantly, HATs have the catalytic/non-catalytic and histone/non-histone effects on the hematopoietic cells, which confer HAT the ability to control a variety of cellular events in normal and malignant hematopoiesis. Genetic approaches are very useful to study how protein acetylation controls a variety of cellular events in normal and malignant hematopoiesis. The study on the gene knockout mouse models shows that p300 and CBP play distinct roles in hematopoiesis; GCN5 but not PCAF is essential in early embryonic development. Thus, such an *in vivo* approach will generate the new findings on the role and mechanism of LATs. One major finding in the mechanism study on the function of protein acetylation is that bromodomain can bind to the acetylated lysine. Since a lot of proteins have bromodomain, it would be interesting to understand whether all bromodomains can recognize the acetylated lysine and how bromodomains specifically recognize the acetylated lysine. MOZ, MORF, p300, and CBP are involved in the leukemia-associated chromosomal translocation, which can generate leukemogenic fusion genes. Thus, LATs have critical functions in the pathogenesis of hematological malignancies. The direct involvement of LATs in hematological malignancies indicates that compounds with the ability to regulate the activity of LATs are the potential drugs for the treatment of hematological malignancies. Thus, the studies on the function and mechanism of histone/non-histone proteins acetylation will not only shed new light on how lysine acetylation controls a variety of cellular events in normal and malignant hematopoiesis but also provide critical insights into the development of new therapeutic strategies for the therapy of hematological malignancies.

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A subset of nuclear receptors are uniquely expressed in uveal melanoma cells

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Uveal melanoma (UM) is recognized as the most common intraocular malignancy and the second most common form of melanoma. Nearly 50% of UM patients develop untreatable and fatal metastases. The 48-member nuclear receptor (NR) superfamily represents a therapeutically targetable group of transcription factors known for their regulation of key cancer pathways in numerous tumor types. Here, we profiled the expression of the 48 human NRs by qRT-PCR across a melanoma cell line panel including 5 UM lines, 9 cutaneous melanoma (CM) lines, and normal primary melanocytes. NR expression patterns identified a few key features. First, in agreement with our past studies identifying RXRg as a CM-specific marker, we found that UM cells also exhibit high levels of RXRg expression, making it a universal biomarker for melanoma tumors. Second, we found that LXRb is highly expressed in both UM and CM lines, suggesting that it may be a therapeutic target in a UM metastatic setting as it has been in CM models. Third, we found that RARg, PPARd, EAR2, RXRa, and TRa expressions could subdivide UM from CM. Previous studies of UM cancers identified key mutations in three genes: GNAQ, GNA11, and BRAF. We found unique NR expression profiles associated with each of these UM mutations. We then performed NR-to-NR and NR-to-genome expression correlation analyses to find potential NR-driven transcriptional programs activated in UM and CM. Specifically, RXRg controlled gene networks were identified that may drive melanoma-specific signaling and metabolism. ERRa was identified as a UM-defining NR and genes correlated with its expression confirm the role of ERRa in metabolic control. Given the plethora of available NR agonists, antagonists, and selective receptor modulators, pharmacologic manipulation of these NRs and their transcriptional outputs may lead to a more comprehensive understanding of key UM pathways and how we can leverage them for better therapeutic alternatives.

Keywords: nuclear receptor expression, uveal melanoma, cutaneous melanoma, NCI-60, profiling

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Introduction

Uveal melanoma (UM) is the most common form of intraocular cancer in adults and comprises about 5% of all malignant melanoma diagnoses (1). UM tumors differ from cutaneous melanomas (CM) in that they arise from melanocytes of the choroid, ciliary body, and iris, defined as the uvea. Although there are effective therapeutic approaches for treating primary uveal tumors, more than 50% of patients exhibit hematogenous spread and metastatic disease, most often to the liver

(~80–90% of cases) (2). Once UM disease has disseminated, therapeutic options are severely limited and average survival rates range from 2 to 8 months (3).

One of the major factors hampering development of therapeutic options for metastatic UM is the lack of discernible driver mutations. Unlike CMs, which frequently harbor $BRAF^{V600E}$ or NRAS mutations, only ~5% of UMs (specifically, only UMs originating from the iris) exhibit $BRAF^{V600E}$ mutations, and NRAS mutations are typically not observed in UM tumors (4). Recent mutational profiling studies of UM have identified mutually exclusive, activating mutations in two G protein coupled receptor alpha subunits, GNAQ and GNA11, in more than 80% of profiled UM tumors (5). These mutations appear to be relatively UM specific and are only found in about 5% of cases in other tumor types (6).

The nearly ubiquitous presence of the GNAQ and GNA11 mutations in UM suggests that they would make an effective therapeutic target, but functional studies of these mutations have noted them to be relatively weak oncoproteins that require other genetic alterations (including p53 and p16/CDK4/RB1 pathway inactivation) to transform immortalized melanocytes (7). Some success has been seen with targeting of downstream targets of GNAQ/GNA11 (specifically combined PKC and MEK inhibition), suggesting that indirect targeting of these mutations may be more effective (8). Recent advances have been made in understanding the underlying mechanism of the GPCR alpha subunit's oncogenic activity, specifically the identification of the transcriptional coactivator yes-associated protein 1 (YAP 1) as a pro-proliferative oncogene and potential therapeutic target (9).

The nuclear receptor (NR) superfamily of transcription factors includes 48 members, most of which activate complex transcriptional programs via ligand binding (10). NRs regulate numerous physiological programs including developmental, homeostatic, proliferative, reproductive, and metabolic pathways (11). In a cancer context, NRs have been validated as pro-proliferative and oncogenic drivers in many tumor types including breast, ovarian, prostate, endometrial, and hematological malignancies (12, 13). In these diseases, NRs have proven to be effective therapeutic targets with numerous drugs targeting many NRs including estrogen receptor (ER) in breast, ovarian, and endometrial cancers, androgen receptor (AR) in prostate tumors, and glucocorticoid receptor (GR) in some hematological malignancies (14). In previously published work studying lung cancer, NR expression analysis has been successfully used to develop a prognostic signature for both survival and progression free survival and to identify potential therapeutic drug targets in pre-clinical models (15).

Nuclear receptors have also been noted as having tumor suppressive functions including VDR's protective function in colon cancer (16); PPARg's activation in reducing tumorigenicity in many cancer tissue types (17); TR4 and RARb as tumor suppressors in prostate (18, 19), and NUR77 and NOR1 as tumor suppressors in AML (20). Given the extensive roles that NRs play in the maintenance of normal development and physiology as well as the emerging understanding of NRs in oncogenic pathways, we set out to investigate how NR expression and activity may be leveraged to discover novel diagnostic, prognostic, and therapeutic alternatives in UM.

The expression and activity of NRs in UMs have been, to date, completely unstudied. To address this issue, we have used high-throughput qRT-PCR to profile the expression of the 48 members of the NR superfamily in various UM cell lines derived from both primary and metastatic lesions and in a normal melanocyte cell line. Based on these results, we report UM-specific NR expression patterns as well as pharmacologically targetable NR-regulated gene networks that could be driving proliferative or oncogenic signaling in UM.

Materials and Methods

Cell Lines and RNA Extractions

All UM cell lines were a kind gift from Dr. Jerry Niederkorn at UT Southwestern and were grown as originally described (21). CM cell lines were received from the NCI, NIH. Cell line identity was confirmed by fingerprinting and compared to standards when available. Primary adult human melanocytes were purchased from Cascade Biologics and were grown per the company's instructions. Cell pellets were processed for RNA extraction using the RNeasy kit, according to the manufacturer's protocol. Extracted RNA was quantified, aliquoted, and stored at −80°C and used to make the corresponding cDNA with Invitrogen's (Carlsbad, CA, USA) First Strand kit.

qRT-PCR and Data Analysis

Analysis of NR expression (mRNA) was performed in triplicate using the TaqMan-based efficiency-corrected cycle threshold method with 12.5 ng cDNA per reaction for 50 cycles in an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described (22). NR mRNAs with cycle times >35 were determined to be below detection. Primer concentrations were 75 nM for 18S rRNA and 300 nM for NR primers; probes were added at 250 nM. The sequences of the validated primer/probe sets for the 48 human NRs are available at www.nursa.org under the rapid release tab. Universal cDNA standards generated from human adult RNA (BD Clontech, Palo Alto, CA, USA) were used for analysis of all receptors except CAR, FXRb, PXR, SHP, DAX-1, ERb, LRH-1, PNR, SF-1, and TLX, which were too limited in expression to use the universal RNA set. For these receptors, commercially available tissue-specific total RNA standards derived from cell lines or adult organ donors were used from liver, ovary, eye, adrenal, and brain, as appropriate. qRT-PCR data were analyzed using ABI instrument software SDS2.1. Baseline values of amplification plots were set automatically, and threshold values were kept constant to obtain normalized cycle times and linear regression data. Because PCR efficiencies for each receptor primer set vary, individual receptor PCR efficiencies were determined to permit receptor-to-receptor comparisons. PCR efficiencies were calculated from the slope of the resulting standard curves as reported previously (11). Normalized mRNA levels are expressed as arbitrary units and were obtained by dividing the averaged, efficiency-corrected values for NR mRNA expression by that for 18S RNA expression for each sample.

Microarray Data

All microarray data were obtained from published datasets available at the GEO Database (http://www.ncbi.nlm.nih.gov/gds/) (23–25). The data were generated using two different platforms, Affymetrix Human Genome U133 Plus 2.0 Array (63 UM tumors and nine NCI-60 melanoma cell lines) and Illumina HumanHT-12 V4.0 expression beadchip (three UM cell lines). Data were compiled and a consensus list of 17,700 unique genes was further analyzed as described in the manuscript text.

Statistical Methodologies

Dendrograms were generated using R Statistical Software. Analysis parameters included distance calculations using a Manhattan methodology and Ward's method for cluster aggregation. Correlation coefficients for comparisons between data were calculated using Pearson Correlation. *p*-Values for comparisons between groups of measurements were performed using Student's *t*-tests. Bonferroni Corrections were used to account for multiple-hypothesis testing as appropriate.

Gene Ontology Analysis

Correlation coefficients (Pearson) were calculated for each gene and the NR in question (either ERRa or RXRg). Lists were then culled to retain the most significantly positively correlated genes (cutoff of $r > 0.6$ was used). Culled lists were input into gene ontology (GO) analysis tool GOrilla and GO analyses were performed as described (26).

Results

NR Expression in Melanomas

To investigate the expression levels of the human NR superfamily ($n = 48$) in UM, we performed high-throughput qRT-PCR expression analysis across a cell line panel consisting of five UM cell lines, nine CM cell lines from the NCI-60 (27), and one primary melanocyte control (**Figure 1**). A heat map was generated to display the results and it was seen that several receptors including SF-1, SHP, TLX, PR, and HNF4a are either expressed at very low levels or completely unexpressed across the panel, suggesting that they do not play a large role in either CM or UM. Other receptors, such as COUPT-FII, LXRb, and RXRg, were found to be strongly expressed across all the samples analyzed. GR, NOR1, NURR1, PPARa, TR2, and TR4 were also expressed in all samples, but at more moderate levels.

Data quality was assessed by comparison to 63 previously published microarray profiles generated from patient UM samples (Figure S1 in Supplementary Material) (24). In this comparison, it was seen that NRs unexpressed in the qRT-PCR dataset reported here were also unexpressed in the patient dataset. Likewise, NRs found highly expressed in the qRT-PCR dataset were generally well expressed in the UM patient samples. Examples of these NRs are shown on Figure S1 in Supplementary Material. Overall, Spearman rank correlation of the expression levels of the 48 NRs between the two datasets was found to be 0.619 ($p < 0.0001$), suggesting that the data presented here for UM lines are representative of findings in clinical UM samples.

Hierarchical Clustering of Cell Lines by NR Expression

To visualize relationships within the dataset, unsupervised hierarchical clustering analyses were performed on the qRT-PCR dataset for both the 13 cell lines and the 48 NRs (**Figure 1**). For these analyses, distance matrices were calculated using the Manhattan distance methodology and clusters were aggregated based on Ward's method. First, we observed that the expression of the 48 NRs can properly segregate the UM lines from the CM lines. The known mutational spectrum of CM and UM would suggest that these two types of melanoma differ from each other, and the unique NR expression signatures seen here, support this idea. As would be expected, NR expression does subdivide the normal melanocyte from the tumor cell lines, but interestingly places the melanocyte in closer proximity to the uveal cluster.

Analysis of the clusters generated by aggregation of the NR probes found several levels of distinctions. Primary subdivisions include a large group of receptors that are either lowly expressed or fairly uniformly expressed, and a second group of receptors that are differentially expressed between CM and UM samples. Interestingly, two NRs (LXRb and RXRg) were particularly distinct from the other NRs in the dataset due to their exceptionally high expression. Previously published work from our group noted that RXRg expression is very high in CM cell lines while essentially unexpressed in every other tissue type in the NCI-60 cancer cell line panel (27). High levels of RXRg expression are similarly seen in UM. When the UM samples were clustered together with the previously published NCI-60 NR expression data, it was found that RXRg expression defined a "melanoma cluster," which contained both UM and CM samples (Figure S2 in Supplementary Material).

Differential Expression of NRs in UM Compared to CM

We next examined NRs differentially expressed between UM and CM (**Figure 2**). First, three NRs (RARg, PPARd, and EAR2) were found to have significantly lower expression levels in CM than in UM cell lines. Comparison to the melanocyte control suggested that the UM samples had retained "normal" expression of these receptors while expression had been lost in the CM samples. Conversely, it was found that RXRa expression was lower in UM than in CM or in the melanocyte control, suggesting UM had specifically lost RXRa expression. Analysis of TRa levels found that UM lines retained expression of this NR similar to that of the melanocyte, but that expression of TRa was significantly higher in CM. By contrast, REVerb an expression appears to be lost specifically in UM. As was previously mentioned, LXRb is highly expressed across all melanoma samples, but is expressed significantly higher in UM samples. This finding is particularly notable since LXRb agonists have been shown to reduce proliferative and metastatic potential in CM pre-clinical mouse models (28).

Clusters of Co-Expressed NRs

To better understand the relationships between the NRs themselves within the melanoma panel, we calculated correlation coefficients (performed as before) for all possible pairwise combinations of the 48 NRs. The results of this unsupervised clustering

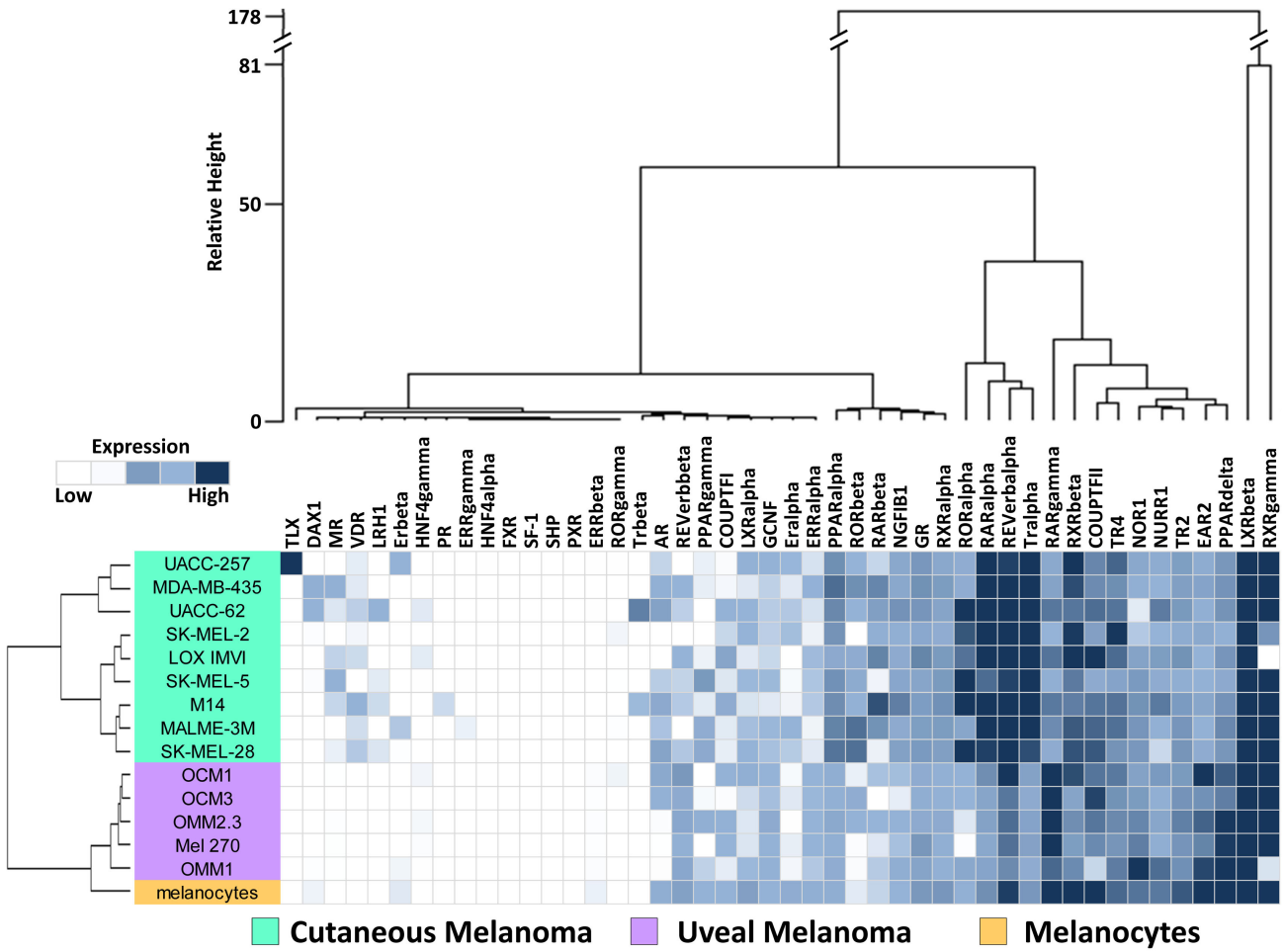

FIGURE 1 | Nuclear receptor expression in melanomas. Clustered heat map representation of mRNA expression levels of the 48 human NRs in a panel of human uveal melanoma ($n = 5$) and cutaneous melanoma ($n = 9$) cell lines as measured by qRT-PCR. Additionally, a melanocyte was included as a normal comparator. Unsupervised hierarchical clustering (Distance calculations: Manhattan, Aggregation method: Ward's) was performed on the NRs (columns) and cell line samples (rows, colored according to key). qRT-PCR data were quantified using standard curves and normalized to 18S as described in Section "Materials and Methods." Data are color coded such that relatively highly expressed NRs are dark blue while lowly or unexpressed receptors (cycle time >35) are white.

analysis are shown as a heat map in **Figure 3**. Several clusters of strongly positive correlations could be seen, including a cluster containing receptors identified as differentially regulated between CM and UM. The CM-specific cluster included EAR2, REV-ERb, RARg, NOR1, and GCNF while the UM-specific cluster contained LXRb, ERRb, TR2, and ERRa. These pockets of strong correlation suggest transcriptional and/or functional interconnections within these receptor subgroups and within specific melanoma subtypes.

Differential Expression of NRs Across UM

Finally, we compared NR profiles across the different UM cell lines (**Figure 4**). Because UM metastatic disease is essentially fatal, we were particularly interested in identifying metastasis-specific NR expression patterns. Initially, we chose to compare NR expression in the cell line pair MEL270 (primary) and OMM2.3 (metastasis), both of which were derived from the same patient. Several genes were found differentially expressed between the pair with the most significant, liganded NRs highlighted in **Figure 4A**. ERa and GR expressions were found to be lost in the metastasis-derived OMM2.3 versus the primary MEL270. Conversely, LXRb and PPARg expressions were either significantly lower (LXRb) or completely undetected (PPARg) in the primary while robust expression was seen in the metastatic line, suggesting that these genes were up-regulated during the metastatic process and may be essential for retention of proliferative capacity or for survival at distant anatomical sites.

Within the UM panel, three cell lines were derived from primary tumors (OCM3, MEL270, and OCM1) and two cell lines were derived from metastatic lesions (OMM1 and OMM2.3). Comparison between these two groups found that NGFIB was up-regulated in the metastatic lines versus the primary tumor lines and the melanocyte control line (**Figure 4B**). There were several other NRs that were up-regulated in the metastatic group, but the small sample size and heterogeneity among primary tumor lines precluded statistical significance.

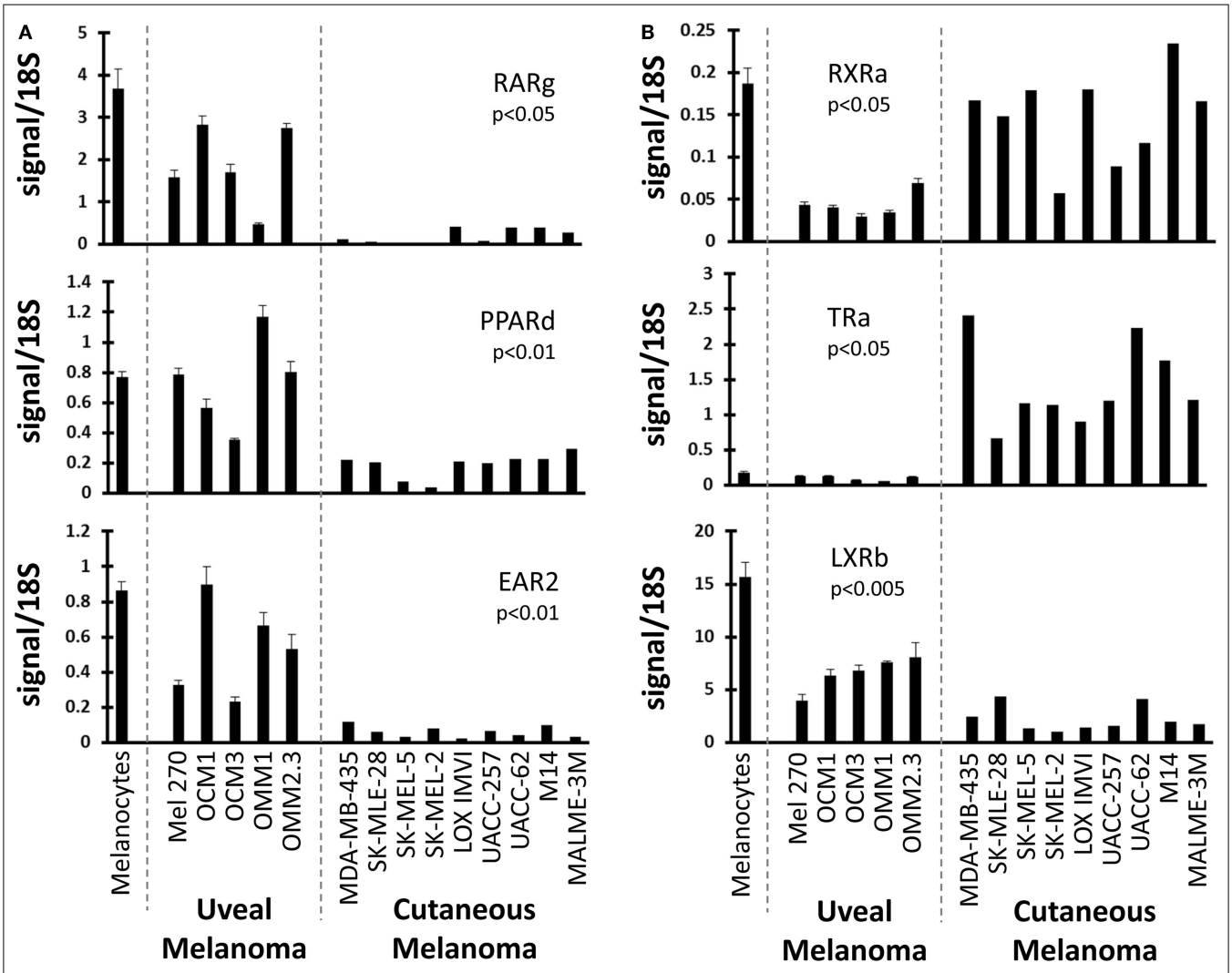

FIGURE 2 | Uveal versus cutaneous melanoma NR expression patterns. Patterns of mRNA expression across the cell line panel for select receptors. *p*-Values are shown for the comparison of the expression level of the indicated nuclear receptor between uveal and cutaneous melanoma cell lines. Calculations were performed using a Student's *t*-test and were Bonferroni-corrected for to account for multiple-hypothesis testing. **(A)** Three receptors (RARg, PPARd, and EAR2) are lost in cutaneous melanoma but retained in the uveal melanoma samples (versus melanocyte expression). **(B)** (Top) RXRa is lost in uveal melanoma samples. (Middle) TRa is overexpressed by cutaneous melanomas. (Bottom) LXRb expression is higher in uveal melanoma samples.

Examples of all three of the key UM mutations are represented in the cell line panel (**Table 1**). Because GNAQ, GNA11, and BRAF mutations are mutually exclusive in clinical samples (5), we identified potentially mutation-specific NR expression patterns (**Figure 4C**). First, we found that the GNA11 mutant cell line OMM1 showed significant overexpression of NOR1 versus the rest of the panel. Comparisons between the BRAF mutant UM lines OCM1 and OCM3 and the rest of the panel found that the BRAF mutants retained expression levels of AR comparable to the melanocyte while the other cell lines had lost AR expression. Finally, it was found that both OMM2.3 and MEL270 (GNAQ mutants) had almost completely lost RORa expression while the other cell lines maintained RORa expression at levels comparable to the primary melanocyte line.

NR-Driven Gene Networks in UM

Finally, because NR activity has been largely unstudied in UM, we examined publically available microarray profiles generated from both UM and CM cell lines (23, 25) to identify gene networks that might be NR-regulated in the melanoma context. For these analyses, we selected two liganded NRs (ERRa and RXRg) that are expressed across the melanoma panel and that correlated strongly with our qRT-PCR data for these same cell lines ($r = 0.82$ for ERRa and 0.7 for RXRg). Lists of microarray probes positively correlated with either ERRa or RXRg expression patterns were generated, culled to the top 400 genes correlating with each receptor (Table S1 in Supplementary Material), and subjected to gene ontology (GO) analysis using publically available GO analysis tool Gorilla (26). Top GO terms associated with ERRa and RXRg are shown

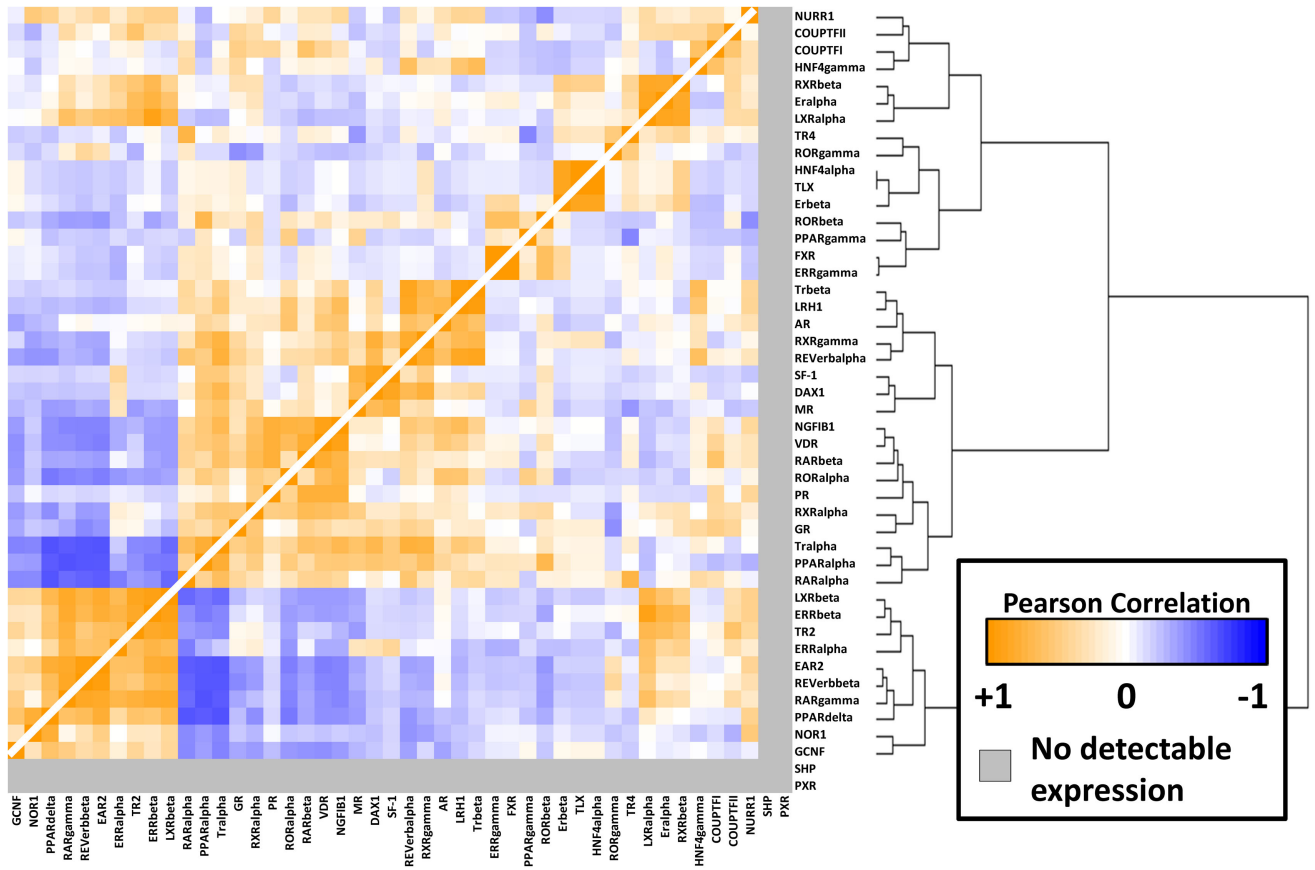

FIGURE 3 | NR-to-NR pairwise correlation comparisons. Pairwise Pearson correlation coefficient (PCC) analysis of the nuclear receptors. Hierarchical clustering analysis was performed as in **Figure 1**. Positive correlations are depicted in orange with the strongest intensities corresponding to higher correlations (0.95 was the highest pairwise correlation). Similarly, negative correlations are depicted in blue tending toward white as they become less intense. The range of positive correlations (0–0.95) was greater than the range of negative correlations (0 to −0.64).

in **Tables 2** and **3**, respectively. For RXRg (the main differentiator between melanomas and other cancers), top GO terms included numerous lipid and basal metabolism associated functions as expected (10), suggesting RXRg may be regulating these functions within a melanoma context. Furthermore, four GO terms associated with RXRg pertained to peroxisome activity [RXRs and PPARs have a well-established relationship (10)], suggesting RXRg could acquire activities within a melanoma setting. One of the genes defining the UM cluster was ERRa, a known regulator of metabolic pathways (29). Predictably, 11 of the top 12 GO terms involved metabolic regulation, indicating that ERRa may contribute to regulation of metabolism in UM, a role that may be pharmacologically targetable via inverse agonists of ERRa.

Discussion

In this study, we measured expression levels of the 48 human NRs by qRT-PCR across a panel of UM cell lines and a normal melanocyte control to expand on our original work across the NCI-60 panel. We demonstrated that UM (like CM) is distinguished from other cancer cell lines by high expression of RXRg, an NR that we previously reported separates melanomas from other cancers. Furthermore, UM and CM can be differentiated based solely on their NR expression profiles with several NRs differentially expressed between the two (including RARg, PPARd, EAR2, RXRa, and TRa). Our results confirm the distinction of UM and CM as separate diseases in line with their differing mutational profiles. We also examined whether there are NRs preferentially expressed in the different mutational subtypes of UM ($GNA11^{Q209L}$, $BRAF^{V600E}$, and $GNAQ^{Q209P}$) and identified receptors (NOR1, AR, and RORa, respectively) exhibiting mutation-specific expression patterns.

Because of the particular importance of metastases within UM, we compared primary-derived and metastasis-derived UM cell lines to identify NRs that might be playing specific roles within the metastatic context. We discovered that NGFIB was up-regulated in UM metastatic cell lines versus primary-derived UM cell lines. Although this was the only NR that met our statistical threshold, it is notable that two other members of the NR4 family (NOR1 and NURR1) also showed generally higher expression in the metastasis-derived UM cell lines. The role of NR4 family members in cancer is controversial as it has been noted to be pro-proliferative in some contexts and tumor suppressive in others (20). One other notable NR that trended toward higher expression

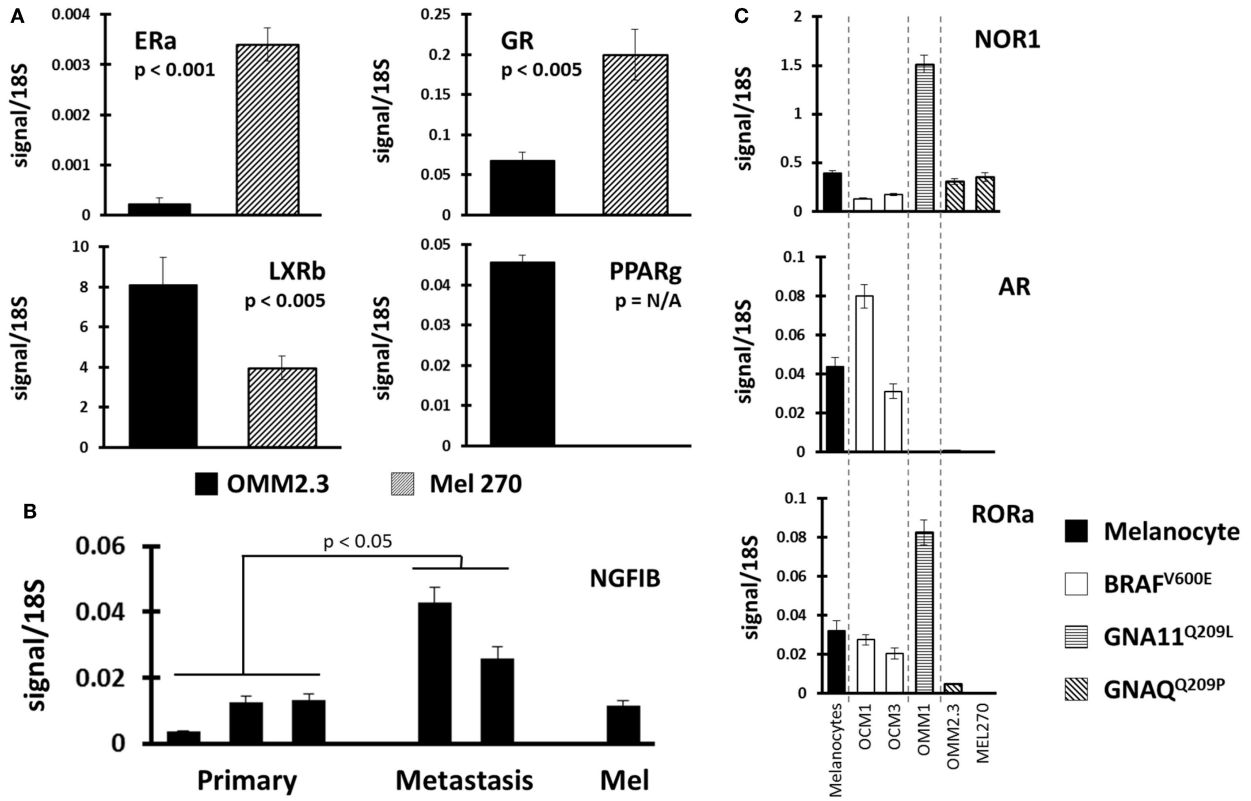

FIGURE 4 | Identification of NR expression patterns within uveal melanoma subtypes. **(A)** Comparisons were made between replicate data generated from UM cell line pair OMM2.3 (metastasis-derived) and MEL270 (primary-derived) with *p*-values calculated through a Bonferroni-corrected Student's *t*-test. ERa and GR expressions were lost in the metastatic line (top) while LXRb and PPARg expressions were higher in the metastasis (bottom). **(B)** NGFIB expression was found higher in the metastasis-derived UM cell lines versus the primary-derived UM lines (*p*-value from corrected *t*-test). **(C)** Receptor expression was compared between the different mutational states of UM. (Top) NOR1 was found overexpressed in the GNA11 mutant, (middle) AR expression was retained in the BRAF mutants, and (bottom) RORa expression was lost in the GNAQ mutant cell lines.

TABLE 1 | Mutation status of cell lines studied.

Cell line	Gq mutant	G11 mutant	BRAF mutant
Melanocytes	WT	WT	WT
OCM1	WT	WT	V600E
OCM3	WT	WT	V600E
OMM1	WT	Q209L	WT
OMM2.3*	Q209P	WT	WT
Mel270*	Q209P	WT	WT
LOXIMVI	WT	WT	V600E
M14	WT	WT	V600E
MALME-3M	WT	WT	V600E
MDA-MB-435	WT	WT	V600E
SK-MEL-2	WT	WT	V600E
SK-MEL-28	WT	WT	V600E
SK-MEL-5	WT	WT	V600E
ACC-257	WT	WT	V600E
UACC-62	WT	WT	V600E

**Indicates cell lines from same patient.*
The cell lines used in this study are listed, along with their mutation status for $GNAQ^{Q209P}$ (Gq), $GNA11^{Q209L}$ (G11), and $BRAF^{V600E}$ (BRAF). WT, wild type.

in the metastasis-derived uveal lines was PPARg, which has been noted in many cancer models to be anti-proliferative upon ligand activation (17).

Finally, we examined NR-to-NR correlation patterns as well as NR-to-genome correlation patterns to identify receptors that might be interacting with each other and to identify networks of genes that certain, key NRs might be controlling within the UM context. ERRa was one of the NRs that differentiated the UM cluster from the CM cluster, and is particularly important given its role as a therapeutic target in other cancers (particularly breast cancer) and the growing availability of ERRa-targeted therapeutics (30) including inverse agonists, which lower the receptor's constitutive activity.

Analogous studies conducted previously by our group and others have successfully utilized NR expression profiles to subdivide between different cancer types. It has been demonstrated that expression profiles of the 48 NRs alone can properly distinguish between cancers of vastly different origins (within the NCI-60 panel) (27), between small-cell and non-small-cell lung cancers (15), and between different types of thyroid cancers (31). Here, we further add the differentiation by NRs between UM and CMs, suggesting that the use of NR expression patterns may be broadly applicable as a tool to categorize different cancers and histological groupings. Given their key roles in regulation of global cellular signaling processes and in cellular development pathways, it is not surprising that NRs play such a central role

TABLE 2 | ERR alpha associated gene ontology (GO) terms.

GO term	Description	*p*-Value	FDR *q*-value
GO:0044238	Primary metabolic process	2.35E-05	3.07E-01
GO:0071704	Organic substance metabolic process	5.93E-05	3.88E-01
GO:0044260	Cellular macromolecule metabolic process	8.73E-05	3.81E-01
GO:0044237	Cellular metabolic process	1.03E-04	3.37E-01
GO:0043170	Macromolecule metabolic process	1.93E-04	5.05E-01
GO:0090304	Nucleic acid metabolic process	2.28E-04	4.98E-01
GO:0006139	Nucleobase-containing compound metabolic process	2.59E-04	4.84E-01
GO:0016070	RNA metabolic process	4.45E-04	7.28E-01
GO:0046483	Heterocycle metabolic process	4.85E-04	7.05E-01
GO:0006725	Cellular aromatic compound metabolic process	5.08E-04	6.65E-01
GO:0006366	Transcription from RNA polymerase II promoter	6.71E-04	7.98E-01
GO:0009058	Biosynthetic process	9.59E-04	1.00E + 00

Gene ontology descriptions of genes positively correlated to ERR alpha expression levels in UM are shown, along with the corresponding p-value and false discovery rate q-values.

TABLE 3 | RXR gamma associated gene ontology (GO) terms.

GO term	Description	*p*-Value	FDR *q*-value
GO:0044255	Cellular lipid metabolic process	4.39E-06	5.75E-02
GO:0006629	Lipid metabolic process	1.36E-05	8.89E-02
GO:0008610	Lipid biosynthetic process	3.67E-05	1.60E-01
GO:0006631	Fatty acid metabolic process	3.87E-05	1.27E-01
GO:0032787	Monocarboxylic acid metabolic process	1.20E-04	3.15E-01
GO:0071616	Acyl-coA biosynthetic process	1.26E-04	2.75E-01
GO:0035384	Thioester biosynthetic process	1.26E-04	2.36E-01
GO:0019752	Carboxylic acid metabolic process	2.26E-04	3.70E-01
GO:0006082	Organic acid metabolic process	2.81E-04	4.09E-01
GO:0032868	Response to insulin	3.98E-04	5.21E-01
GO:0006625	Protein targeting to peroxisome	5.38E-04	6.40E-01
GO:0072663	Establishment of protein localization to peroxisome	5.38E-04	5.87E-01
GO:0072662	Protein localization to peroxisome	5.38E-04	5.42E-01
GO:0043436	Oxoacid metabolic process	5.56E-04	5.20E-01
GO:0006790	Sulfur compound metabolic process	6.29E-04	5.48E-01
GO:0043574	Peroxisomal transport	6.57E-04	5.38E-01
GO:0006633	Fatty acid biosynthetic process	8.21E-04	6.32E-01

Gene ontology descriptions of genes positively correlated to RXR gamma expression levels in UM are shown, along with the corresponding p-value and false discovery rate q-values.

to the identities of these cell types, even within a dedifferentiated cancer state.

It is of significant interest that several NRs are differentially expressed between UM and CM tumors. Particularly, the findings RARg, PPARd, EAR2, TRa, and LXRb that are retained in UM at levels comparable to the melanocyte while being reduced or lost in CM suggests that there may be opportunities for NR-directed therapeutic interventions in UM that are not available in CM due to CM-specific loss of these receptors. It is also worth noting that the UM-specific loss of RXRa expression. Because RXRa is a heterodimeric binding partner for many NRs, lower RXRa expression might suggest indirect downregulation of NR signaling in UM tumors.

It has recently been reported that metastatic CM can be inhibited by administration of LXRb agonists in pre-clinical models of CM (28). Here, we report that LXRb receptor levels are even higher in UM samples than the levels observed in CM samples. Given the known differences between UM and CM tumors, it will be important for future studies to examine whether or not LXRb-directed therapies or other NR ligand strategies will be effective in controlling UM metastatic disease *in vivo*.

Melanoma is an aggressive, highly metastatic disease that is notoriously difficult to treat using standard cytotoxic agents (32). Mechanistic studies have found that CM cells achieve their hallmark chemoresistance through genome-scale reprogramming of proliferation and survival pathways during disease progression. Given these findings, it is not surprising that many modern therapeutic strategies involve induction of wholesale changes in the transcriptome of CM cells through epigenetic modulation to overcome these anti-apoptotic and pro-proliferative pathways (33, 34). Although much work has been done in CM, far fewer studies have investigated ocular-derived UMs where metastatic disease is equally as fatal (3).

Mutational profiling of UM has identified mutually exclusive, UM specific, activating mutations in two paralogs (GNAQ and GNA11) in more than 80% of UM cases. Although these mutations would seem obvious targets for therapeutic intervention, GNAQ/11 mutations have not been amenable to therapeutic development in UM and recent work has instead focused on inhibiting downstream events and gene networks driven by these mutations (9, 35). As an example, combination therapy with inhibitors of GNAQ/11 downstream target protein kinase C (PKC) and MEK has been shown to inhibit the *in vitro* growth of GNAQ/11 UM mutant cell lines (8). Another recently identified downstream target of GNAQ/11 mutants is YAP1 and a YAP1 inhibitor, verteporfin, has also been shown to be effective inhibiting UM growth in xenograft models (35). However, as was pointed out in a recent preview opinion from Field and Barbour (36), it is important to note that these inhibitors alone will likely be insufficient for treating UM metastases as GNAQ/11 mutations are only weakly oncogenic being unable to transform immortalized melanocytes without additional, cooperating mutations (7). Recent clinical trial results using the MEK inhibitor selumetinib in metastatic UM patients underscore their opinion as there was no overall survival benefit (37).

As transcription factors, activated NRs are extremely effective in eliciting widespread physiologic changes in cells through alteration of the transcriptional output and architecture of the genome (38). One of the most striking examples of ligand-mediated NR activity comes from studies of estradiol's effects on the transcriptome of an ER-positive breast cancer cell line. They report nearly 23,000 transcripts (equivalent to more than 25% of total cellular transcriptomic output) that are altered during ER activation (39). Other ligand/receptor combinations known to elicit broad-scale expression changes include mifepristone/progesterone receptor in endometrial tissue (40) and T0901317/liver X receptors in the human monocytic cell line THP-1 (41). Defining which NRs

might be playing a role in transcriptional reprogramming during UM onset and progression, as we have begun to do here, should catalyze a better understanding and targeting of this disease. Overall, it will be interesting to see how NR expression patterns correlate with clinical disease progression in the future release of the UM TCGA dataset, to then design NR-driven therapeutic strategies.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2015.00093

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Global characteristics of CSIG-associated gene expression changes in human HEK293 cells and the implications for CSIG regulating cell proliferation and senescence

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Cellular senescence-inhibited gene (CSIG), also named as ribosomal_L1 domain-containing 1 (RSL1D1), is implicated in various processes including cell cycle regulation, cellular senescence, apoptosis, and tumor metastasis. However, little is known about the regulatory mechanism underlying its functions. To screen important targets and signaling pathways modulated by CSIG, we compared the gene expression profiles in CSIG-silencing and control HEK293 cells using Affymetrix microarray Human Genome U133 Plus 2.0 GeneChips. A total of 590 genes displayed statistically significant expression changes, with 279 genes up-regulated and 311 down-regulated, respectively. These genes are involved in a broad array of biological processes, mainly in transcriptional regulation, cell cycle, signal transduction, oxidation reduction, development, and cell adhesion. The differential expression of genes such as ZNF616, KPNA5, and MAP3K3 was further validated by real-time PCR and western blot analysis. Furthermore, we investigated the correlated expression patterns of Cdc14B, ESCO1, KPNA5, MAP3K3, and CSIG during cell cycle and senescence progression, which imply the important pathways CSIG regulating cell cycle and senescence. The mechanism study showed that CSIG modulated the mRNA half-life of Cdc14B, CASP7, and CREBL2. This study shows that expression profiling can be used to identify genes that are transcriptionally or post-transcriptionally modified following CSIG knockdown and to reveal the molecular mechanism of cell proliferation and senescence regulated by CSIG.

Keywords: CSIG/RSL1D1, senescence, cell cycle, gene expression, microarray

Introduction

Cellular senescence, a natural barrier to cancer progression, is causally implicated in generating age-related phenotype (1–5), but the fundamental mechanisms that drive senescence remain largely unknown. Using a suppressive subtractive hybridization, we have identified and cloned a

cellular senescence-inhibited gene (CSIG) (GenBank accession No. AY154473, http://www.ncbi.nlm.nih.gov) (6). CSIG is a Ribosomal_L1 Domain-Containing Protein and therefore was also named as RSL1D1 in the Human Genome Organization (HUGO) Nomenclature Committee Database. CSIG is abundantly expressed in early-passage fibroblasts, but its expression declines during cellular senescence. CSIG modulated cell cycle progression, in turn promoting cell proliferation (7). Moreover, overexpression of CSIG significantly delayed the progression of replicative senescence, while knockdown of CSIG expression accelerated replicative senescence (7). Our findings indicate that CSIG acts as a novel regulatory component of replicative senescence. Consistently, Meng et al. and Zhu et al. reported that CSIG/RSL1D1 could regulate the activity of nucleostemin which delays the aging progression in mouse fibroblasts (8, 9). In addition, Li et al. found that CSIG is required for p33ING1 to induce apoptosis under UV irradiation (10). Moreover, emerging evidences have indicated that CSIG might implicate in various biological processes such as breast cancer metastasis (11), tumor cell survival (12), inflammation (13), and bone formation (14).

According to informatics analysis (available at http://www.expasy.org), CSIG is evolutionarily conserved and human CSIG protein contains part of Ribosomal L1p/L10e consensus sequence (residues 30–260) in the N-terminus and a long Lys_rich domain (residues 280–485) in the C-terminus, suggesting that it may participate in ribosome biosynthesis or act as a transcriptional co-factor. Our previous studies have identified CSIG as a nucleolus protein accumulated in ribosome (7). Although the non-ribosomal functions of CSIG to regulate proliferation, apoptosis, and senescence progression have been established (7, 10); however, the molecular basis underlying is poorly understood.

In this investigation, we have compared differential gene expression patterns in HEK293 cells between CSIG knockdown and control samples using a fold change (FC) $\geq$1.5 as a cutoff to define CSIG-related gene expression and profile changes. We have demonstrated that gene expression changes associated with CSIG knockdown in 293 cells impact transcription regulation, cell cycle, development, and certain critical signal transduction pathways. Furthermore, we have identified candidate genes for further in depth analysis. Changes in expression patterns provide further evidence and a molecular basis for CSIG to regulate cell proliferation and senescence.

Materials and Methods

Cell Culture

Human embryonic kidney cell (HEK293) was cultured in DMEM medium, containing 10% fetal bovine serum. Human diploid fibroblasts 2BS cell line from human female embryo lung was established at the National Institute of Biological Products (Beijing, China) and has been fully characterized (6, 7). The expected replicative life span of 2BS cells is about 70 population doublings (PDs). 2BS cells were considered to be young (early-passaged) at PD 30 or below and fully senescent at PD 55 or above. Senescent cells are characterized by an irreversible growth arrest and accumulated p16INK4a. 2BS cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO_2.

CSIG Knockdown in 293 Cells/Plasmids and Transfection

The pcDNA3.1-CSIG and control vectors were purified with QIAGEN Plasmid Maxi Kits. Cells were transfected with plasmids coated by Lipofectamine 2000 (Invitrogen) following the manufacturer's indications. To transiently silence CSIG, siRNA targeting CSIG (siCSIG) and control siRNA were synthesized (Genema), respectively. siRNAs were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. Cells were collected 48 h after transfection for further analysis. The siRNA sequences were as follows:

- CSIG siRNA: 5′-AGAAGGAACAGACGCCAGA-3′
- Control siRNA: 5′-TTCTCCGAACGTGTCACGT-3′

Western Blotting

Cells were washed with PBS, collected, and lysed on ice for 30 min with RIPA (Applygen Technologies Inc., Beijing, China) containing a protease inhibitor mixture (Fermentas). Cell lysates were then centrifuged for 10 min at 15,000 × *g* at 4°C. The supernatant was collected, and the protein concentration was determined using the BCA Protein Assay Reagent (Pierce). Total protein (20 ~ 40 μg) was subjected to 10 ~ 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to nitrocellulose membranes (Millipore). After blocking in 5% non-fat dry milk in TBST (10 mm Tris-Cl, pH 7.5, 150 mm NaCl, 0.05% Tween 20), the membranes were incubated with primary antibodies overnight at 4°C. The membranes were then washed three times with TBST and then incubated with HRP-conjugated secondary antibodies (Zhongshan Biotechnologies Inc., China) for 1 h at room temperature. Proteins were visualized using chemiluminescent substrate (Millipore) according to the manufacturer's instructions. Blots were probed with the following antibodies: anti-CSIG [used as previously described (7)], anti-p16 (sc-759, Santa Cruz), anti-ESCO1 (ab128312, Abcam), anti-Cdc14B (sc-374572, Santa Cruz), anti-KPNA5 (ab81450, Abcam), anti-MAP3K3 (ab40750, Abcam), anti-Cdc2 (E53, Epitomics), and anti-PCNA (BS1289, Bioworld).

RNA Extraction

Total RNA was isolated from HEK293 cells and 2BS cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The quality of the RNA samples was examined by quantifying the A260:A280 ratio (the minimal acceptable ratio is 1.7) and the 28S/18S by visualizing rRNA bands in agarose gel (the minimal acceptable ratio is 1.5).

Affymetrix cDNA Microarray

The microarray screen was performed in triplicate using Affymetrix microarray Human Genome U133 Plus 2.0 chips containing 38,500 genes. Briefly, 15–20 g of biotin-labeled cRNA was fragmented by incubating in a buffer containing 200 mmol/l Tris acetate (pH8.1), 500 mmol/l KOAc, and 150 mmol/l MgOAc at 95°C for 35 min. The fragmented cDNA was hybridized with a

TABLE 1 | DNA sequences of the primers used for quantitative real-time PCR.

Identity/gene	Nucleotide sequences
ZNF367	Forward: 5′-AACCGCCACTGTCCGAAGCA-3′
	Reverse: 5′-CCTTTCAAAGTGGGGGTGCGCT-3′
ZNF616	Forward: 5′-TGGAAATGCCTGGAGCCTGTGC-3′
	Reverse: 5′-GGCCCGATGAAAGGCTTTGCCA-3′
KPNA5	Forward: 5′-GCAGACGTGTGTTGGGCCCTTT-3′
	Reverse: 5′-TCCATTGGTGCTTCCTGCTGCT-3′
CASP7	Forward: 5′-AAATGCCGCCTGCTTCGCCT-3′
	Reverse: 5′-TGGAGCAGAGGGCTTGCACA-3′
PPM1A	Forward: 5′-CGGCTGTGATCGGTTTGCCA-3′
	Reverse: 5′-GCCAGAGAGCCATTCACACGCT-3′
SETD7	Forward: 5′-TGAACGGTCCAGCCCAGGAA-3′
	Reverse: 5′-ACTGCTCTCAGGGTGCGGAT-3′
CREBL2	Forward: 5′-CGTGGTCGGAAGCCAGCCAAAA-3′
	Reverse: 5′-TTCGGGCTCGGCATTCTCTTGC-3′
NOLC1	Forward: 5′-AGCCCAAGGCGACTGCCAAA-3′
	Reverse: 5′-GCTGCCCCGCTTCTTCTTGGTT-3′
TRAK1	Forward: 5′-ACGGCAGCGACATAGGCAAC-3′
	Reverse: 5′-AGCAGAAATGCCCCGCTCCT-3′
CCDC115	Forward: 5′-CTGGAGGGGAAACGAACGGTGT-3′
	Reverse: 5′-ATGCGGTTCTGGAGGCTGGCTA-3′
C11orf24	Forward: 5′-TCAGCACAGCCCTCGCACAA-3′
	Reverse: 5′-ACCTTGTGCTTGGGGACGCA-3′
MTA2	Forward: 5′-AAGGAACGGCTACGACCTGGCT-3′
	Reverse: 5′-AACAGGAAGCACAGAGGCGGCA-3′
PCK1	Forward: 5′-AAGGTTGAGTGCGTCGGGGA-3′
	Reverse: 5′-TTCCCAGTAAACGCCCCCGT-3′
ESCO1	Forward: 5′-ACGAAACGAAACCTGTGCCTGT-3′
	Reverse: 5′-AGGCACTGATGGCTGTGGACT-3′
SEH1L	Forward: 5′-GCTCTCGTGCTCATTCCCCCAT-3′
	Reverse: 5′-GGCAGTGTCAGCATCGCAAGAGT-3′
RAB31	Forward: 5′-GGGGACACTGGGGTTGGGAAAT-3′
	Reverse: 5′-AGGTCGCACTTGTTTCCAGCG-3′
STAT1	Forward: 5′-TGGAGTGGAAGCGGAGACAGCA-3′
	Reverse: 5′-TCACCACAACGGGCAGAGAGGT-3′
TMEM109	Forward: 5′-ACACTGGATGCCTGGATTGGGC-3′
	Reverse: 5′-AAGCCGAGGAGCAGAGACAGCA-3′
KIAA1549	Forward: 5′-AGCGTGCCCTCCGTGTTCAT-3′
	Reverse: 5′-TGCCTCTGCTTGGCGGGATT-3′
UBE2I	Forward: 5′-TCCGTGGGAAGGAGGCTTGT-3′
	Reverse: 5′-TGGCTTGTGCTCGGACCCTT-3′
MAP3K3	Forward: 5′-ACGAATGTCCCGTGCCCAGA-3′
	Reverse: 5′-TTCCATAGCCCTCGCCGCTGAT-3′
YWHAH	Forward: 5′-CGCTATGAAGGCGGTGACAGAG-3′
	Reverse: 5′-AGGGTGAGGTTGTCTCGCAGCA-3′
ITGB8	Forward: 5′-GCCTCGTTCCTCTGGGCAGC-3′
	Reverse: 5′-TTCTGGACCCAGCGCAAGGC-3′

pre-equilibrated Affymetrix chip at 45°C for 14–16 h. The hybridizations were washed in a fluidic station with non-stringent buffer (6× SSPE, 0.01% Tween 20, and 0.005% antifoam) for 10 cycles and stringent buffer (100 mmol/l 2N-morpholino-ethanesulfonic acid, 0.1M NaCl, and 0.01% Tween 20) for 4 cycles and stained with strepto-avidin phycoerythrin. This was followed by incubation with biotinylated mouse antiavidin antibody and restained with strepto-avidin phycoerythrin. The chips were scanned in an Agilent ChipScanner (Affymetrix Inc., Santa Clara, CA, USA) to detect hybridization signals.

Baseline analyses were done with AGCC to identify statistically significant gene expression alterations between samples derived from HEK293 cells transfected with siCSIG and siNC, respectively. Because samples were analyzed in triplicates, these results were additionally screened for consistent P by the Student's *t*-tests ($P < 0.05$) to eliminate random sampling errors.

Quantitative Real-Time PCR

Real-time PCR analysis was performed in triplicate using the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7300 sequence detector (Applied Biosystems). Each PCR was assembled using 96-well MicroAmp Optical plates (Applied Biosystems) with a total volume of 15 μl containing 1.5 μl cDNA templates, 1 μM of each primer, and 7.5 μl of 2× SYBR Green Master Mix and brought to final volume with RNase-free water. Thermal reaction cycles of 50°C for 2 min, 95°C for 10 min, and 40 repetitions of 95°C for 15 s and 60°C for 1 min were used. The data were analyzed using the ΔΔCT method, normalizing the C_t values of the indicated gene to the C_t values of GAPDH relative to a control sample. The GAPDH gene served as an endogenous control for normalization. Gene-specific primers were designed using Primer 5. The primer sequences used in this study are shown in **Table 1**.

Flow Cytometry

When cells reached 70–80% confluence, they were washed with PBS, detached with 0.25% trypsin, and fixed with 75% ethanol overnight. After treatment with 1 mg/ml RNase A (Sigma) at 37°C for 30 min, cells were resuspended in 0.5 ml of PBS and stained with propidium iodide in the dark for 30 min. Fluorescence was measured with a FACScan flow cytometry system (BD Biosciences).

mRNA Stability Assay

Experiments were carried out during logarithmic phase of cultured 293 cells. We treated 293 cell lines from time 0 with actinomycin D

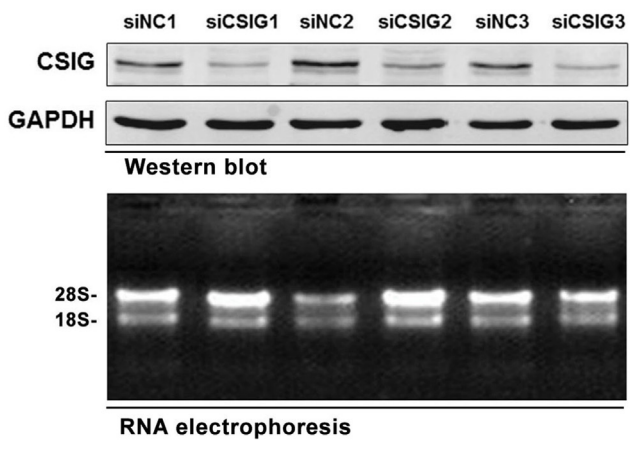

FIGURE 1 | The RNA samples prepared for Affymetrix microarray experiment. Upper panel, western blot analysis of CSIG expression in siCSIG and siNC transiently transfected HEK293 cells. Total protein was extracted, and immunoblotting was performed using specific antibodies against CSIG as indicated. GAPDH served as a loading control. Bottom panel, the intactness of the RNA samples was tested using RNA electrophoresis. The three parallel experiments, indicated as 1, 2, and 3, respectively, were performed with the same siRNA.

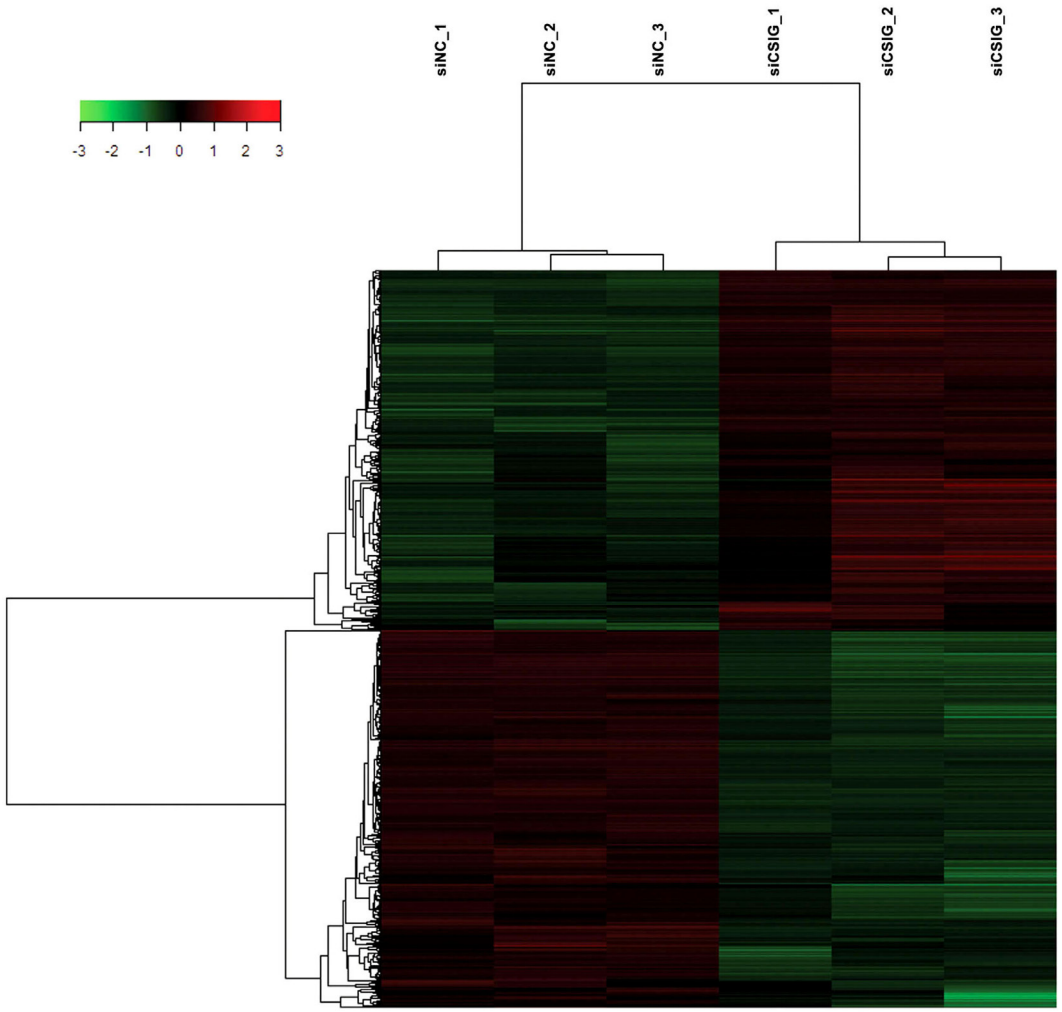

FIGURE 2 | Hierarchical clustering heat map of the 841 genes with significant differentially expressed changes following CSIG knockdown with *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥1.5, and FDR <5%. Each column represents a sample; each row refers to a gene. Gene expression changes with respect to median changes are denoted by: red, up-regulated (ratio ≥1.5); green, down-regulated (ratio <1/1.5); and black, unchanged.

TABLE 2 | The fold-change distribution of gene expression changes following CSIG knockdown.

	FC ≥ 1.5	1.5 ≤ FC < 2	2 ≤ FC < 3	3 ≤ FC
Total genes	841	721	116	4
Up-regulated	411	355	55	2
Down-regulated	430	366	62	2
Percentage of FC genes in total genes	100	85.7	13.8	0.5

The FC distribution of gene expression changes following CSIG knockdown. Shown is the number of genes in each FC size range for genes with FCs ≥1.5 and adjusted P-values <0.05; FC = fold change (detailed gene list, Table S1 in Supplementary Material).

(10 μg/ml) for the indicated times. Then washed cultures in PBS, extracted RNA with RNA Extraction Kit, and analyzed RNA by quantitative RT-PCR (qRT-PCR).

Data Analysis

Microarray scan data were analyzed using the significance analysis of microarrays (SAM) R-package. To compare the results of different hybridization experiments, the signal intensity of each gene on different arrays was normalized versus the total intensity of all genes in the array. Corresponding normalized signals on different arrays were then compared to identify differential regulation in the gene expression between samples. Relative gene expression changes ≥1.5-fold were considered meaningful to represent up-regulation or down-regulation. Gene ontology (GO) analysis was performed to identify significantly enriched biological processes and molecular functions.

Statistical Analysis

All values are expressed as means ± SD in the figures. Statistical significance was assessed using Student's *t*-test, and *P*-values of <0.05 were considered significant.

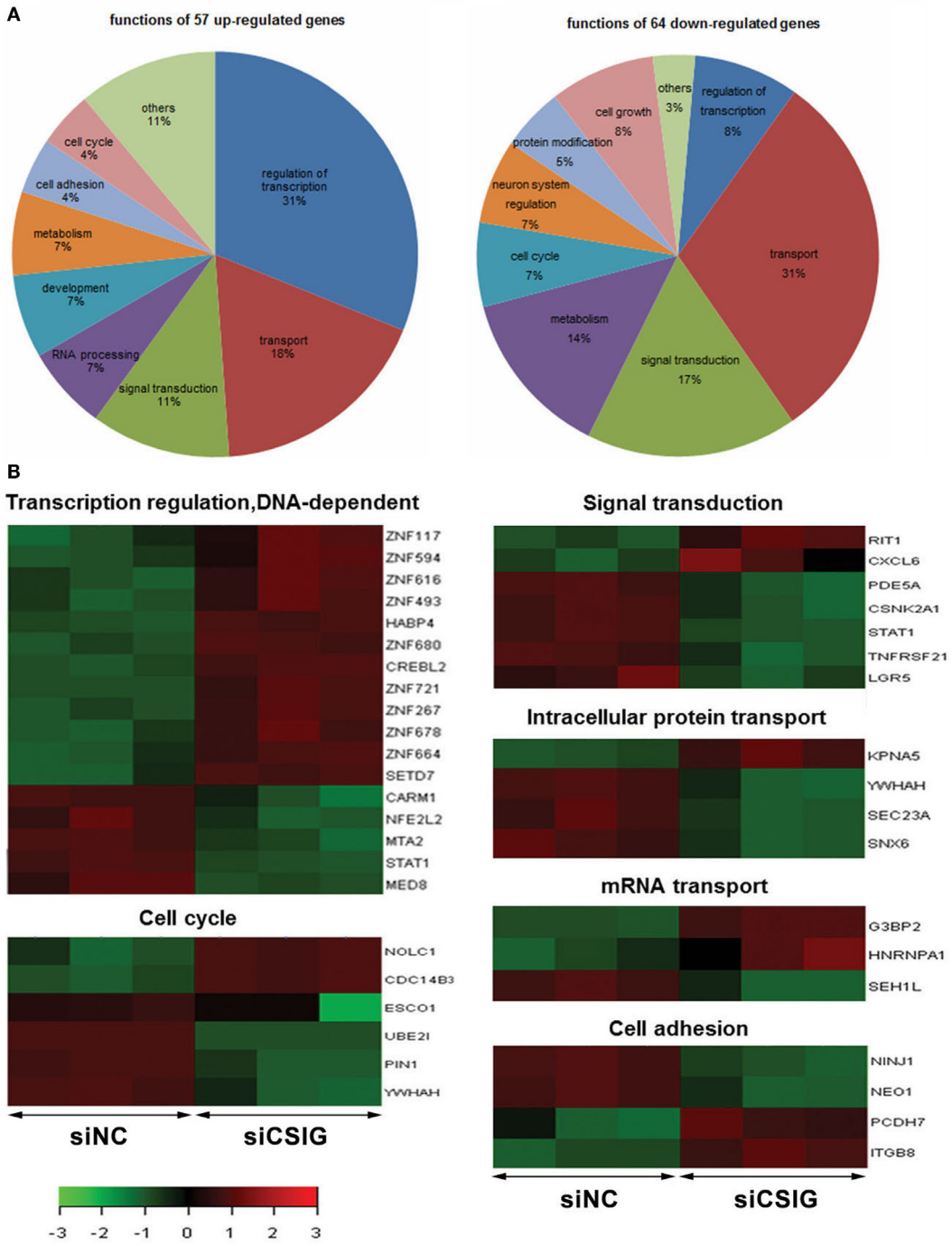

FIGURE 3 | **Gene ontologies and functional analysis of the differentially expressed genes with *t*-test adjusted *P*-values <0.05, a fold-change cut off ≥2. (A)** Gene ontologies with significantly over-represented differentially expressed genes following CSIG knockdown and the Ratio of genes implicated in various processes. **(B)** The differentially expressed genes implied in various functions by red–green hot spots. The color bar shows the fold change and corresponding color depth.

Results

Affymetrix cDNA Microarray Analysis of Gene Expression Profiles Following CSIG Knockdown

To preparing RNA samples for microarray analysis, the small interfering RNA (siRNA) specifically targeting CSIG (siCSIG) and the control siRNA (siNC) targeting none of the human genes were transiently transfected into HEK293 cells, respectively, and cells were collected for analysis after 48 h. First, a small amount of samples were subject to western blot analysis

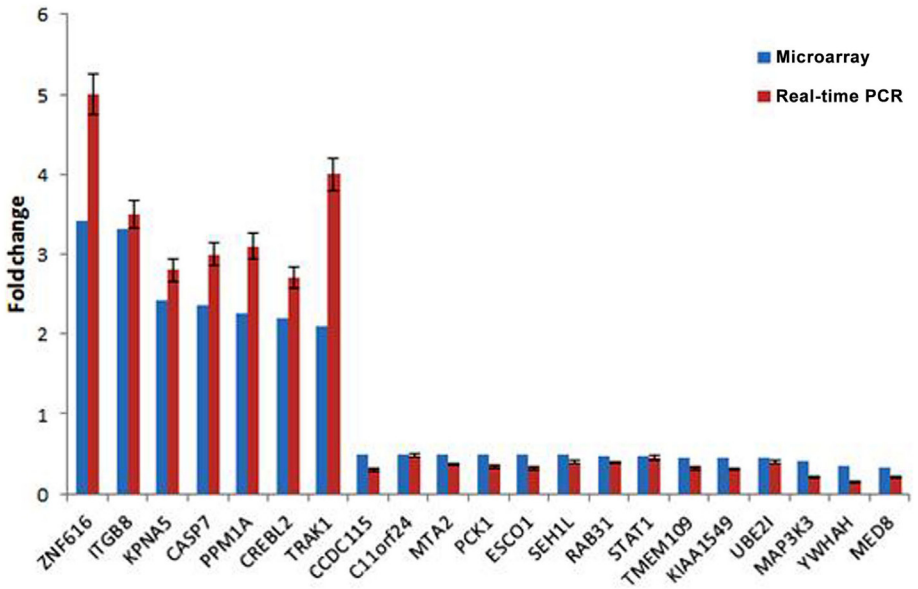

FIGURE 4 | Agreement between microarray and real-time quantitative RT-PCR data. The blue block represent microarray data, the red block represent real-time PCR results. The results are mean ± SEM and the *P*-values are all <0.05.

of the gene knockdown efficiency. Comparing with control cells, CSIG siRNA transfection induced a more than 60% of decrease of CSIG level (**Figure 1**, upper panel). And then total RNAs from CSIG siRNA and control siRNA transfected cells were extracted from the remaining cultures in the same dish, respectively. Subsequently, two groups of samples in triplicate were subject to RNA integrity and purification examination. According to the RNA agarose gel analysis, the ratio of 28S:18S is 2:1 on the whole (**Figure 1**, bottom panel), which indicated the intactness of the RNA samples. And the ratio of A260:A280 is basically in the range of 1.7 ~ 2.0, which proved the purification of sample. Totally, the detection results indicated that the quality of RNA samples we prepared ultimately meets the requirements of Affymetrix cDNA microarray experiment.

To screen important targets and signaling pathways modulated by CSIG, in this study, we analyzed the differential expression of genes in HEK293 cells from three CSIG knockdown and the corresponding control samples using six Affymetrix GeneChip Human Genome U133 plus 2.0 microarrays. The Affymetrix cDNA microarray analysis of the gene expression was performed as described in Section "Materials and Methods." The global genome gene expression profiles are analyzed and listed as mean values from triplicate GeneChips in supporting Table S1 in Supplementary Material. Differential expression analysis between CSIG knockdown and control cells was carried out with the SAM R-package software. We identified a total of 841 probe sets associated with 590 genes of known function – representing 4.7% of the 12487 well characterized human genes measurable on the microarray – that were expressed differentially between these two groups. The following criteria were used for gene selection: adjusted $P < 0.05$ and FC $\geq$1.5. Of these 590 genes, 311 (53%) were down-regulated and 279 (47%) were up-regulated (**Figure 2**). The majority of the selected genes showed moderate (yet significant) alterations in expression of between 1.5- and 2.0-fold (**Table 2**; for all genes, see Table S1 in Supplementary Material). Using adjusted $P < 0.05$ and FC $\geq$2 as a cutoff, there were totally 121 genes showing differential expression following CSIG knockdown, with 57 genes up-regulated (more than 2-folds increase) and 64 genes down-regulated (<0.5-folds decrease), respectively (**Table 2**).

According to GO analysis and pathway analysis, the differentially expressed genes are implicated in a variety of process. By analysis using adjusted $P < 0.05$ and FC $\geq$2 as a cutoff, there are 31% of up-regulated genes implicated in regulation of transcription, and 31% of down-regulated genes involved in transport, respectively (**Figure 3A**). Notably, there are nine ZNF genes among them showing increased levels all together (**Figure 3B**). There are seven genes involved in signal transduction process with RIT1 and CXCL6 up-regulated while LGR5, PDE5A, CSNK2A1, STAT1, and TNFRSF21 down-regulated, six genes involved in cell cycle progress with NOLC1 and Cdc14B3 up-regulated while ESCO1, UBE2I, PIN1, and YWHAH down-regulated (**Figure 3B**). There are four genes participate in intracellular protein transport with KPNA5 up-regulated while YWHAH, SEC23A, and SNX6 down-regulated, and another four genes implicated in cell adhesion process with PCDH7 and ITGB8 up-regulated while NINJ1and NEO1 down-regulated (**Figure 3B**). There are three genes including G3BP2, HNRNPA1, and SEH1L involved in mRNA transport (**Figure 3B**).

Validation of the Differential Expression of Selected Genes

To validate the result of Affymetrix microarray screen, we performed real-time qRT-PCR analysis in triplicate for 21

TABLE 3 | Verification of microarray data by quantitative real-time PCR.

Symbol	Description	Fold (genechip)	Fold (qPCR)
ZNF616	Zinc finger protein 616	3.41	5.0
ITGB8	Integrin, beta 8	3.317	3.5
KPNA5	Karyopherin alpha 5 (importin alpha 6)	2.4291	2.8
CASP7	Caspase 7, apoptosis-related cysteine peptidase	2.3629	3.0
PPM1A	Protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	2.2666	3.1
CREBL2	cAMP responsive element binding protein-like 2	2.1951	2.7
TRAK1	Trafficking protein, kinesin binding 1	2.0994	4.0
CCDC115	Coiled-coil domain-containing 115	0.4988	0.35
C11orf24	Chromosome 11 open reading frame 24	0.4952	0.48
MTA2	Metastasis associated 1 family, member 2	0.4949	0.4
PCK1	Phosphoenolpyruvate carboxykinase 1 (soluble)	0.49	0.36
ESCO1	Establishment of cohesion 1 homolog 1 (*S. cerevisiae*)	0.49	0.35
SEH1L	SEH1-like (*S. cerevisiae*)	0.4894	0.4
RAB31	RAB31, member RAS oncogene family	0.4715	0.4
STAT1	Signal transducer and activator of transcription 1, 91 kDa	0.4707	0.45
TMEM109	Transmembrane protein 109	0.456	0.35
KIAA1549	KIAA1549	0.45	0.35
UBE2I	Ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	0.4518	0.43
MAP3K3	Mitogen-activated protein kinase kinase kinase 3	0.4086	0.2
YWHAH	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, eta polypeptide	0.3572	0.17
MED8	Mediator complex subunit 8	0.3653	0.25

selected genes using cDNA from CSIG knockdown and control 293 cell samples. Specific primers were designed and synthesized as described in Section "Materials and Methods." The examined genes includes CCDC115, PPM1A, PCK1, KIAA1549, SETD7, ESCO1, CASP7, TMEM109, CREBL2, SEH1L, C11orf24, TRAK1, ZNF367, RAB31, STAT1, KPNA5, MAP3K3, NOLC1, HNRNPA1, YWHAH, ZNF616, MTA2, and UBE2I. CSIG siRNA and control siRNA were transiently transfected into HEK293 cells and cells were collected after 48 h for RNA extraction and reverse transcription. Consistent with the Affymetrix microarray data, real-time PCR result confirmed that the expressions of ZNF616, ITGB8, KPNA5, CASP7, PPM1A, CREBL2, and TRAK1 are increased (**Figure 4**), while expressions of CCDC115, C11orf24, MTA2, PCK1, ESCO1, SEH1L, RAB31, STAT1, TMEM109, KIAA1549, UBE2I, MAP3K3, YWHAH, and MED8 are decreased following CSIG knockdown, respectively (**Figure 4**). The CSIG-related changes in gene expression measured by qRT-PCR were in agreement with microarray data (Table 3). The microarrays thus provided a reliable comparison of gene expression in 293 cells following CSIG knockdown.

To further confirm the differential expression of above genes, we carried out real-time PCR analysis in CSIG-overexpressed HEK293 cells. On the contrary, following CSIG overexpression, the expressions of ZNF616, ITGB8, KPNA5, CASP7, PPM1A, CREBL2, and TRAK1 are decreased, while expressions of CCDC115, C11orf24, MTA2, PCK1,ESCO1, SEH1L, RAB31, STAT1, TMEM109, KIAA1549, UBE2I, MAP3K3, YWHAH, and MED8 are induced (**Figure 5A**). As predicted, by comparison with the expression patterns following CSIG-silencing, CSIG overexpression induced an inverse expression alterations, which validated the microarray data on the contrary. Western blot analysis of Cdc14B, ESCO1, KPNA5, and MAP3K3 was further carried out in CSIG-silencing and CSIG-overexpressed HEK293 cells. Following CSIG knockdown, Cdc14B, ESCO1, and MAP3K3 expressions are inhibited, while KPNA5 is increased (**Figure 5B**). Inversely, when CSIG overexpressed, Cdc14B, ESCO1, and MAP3K3 were

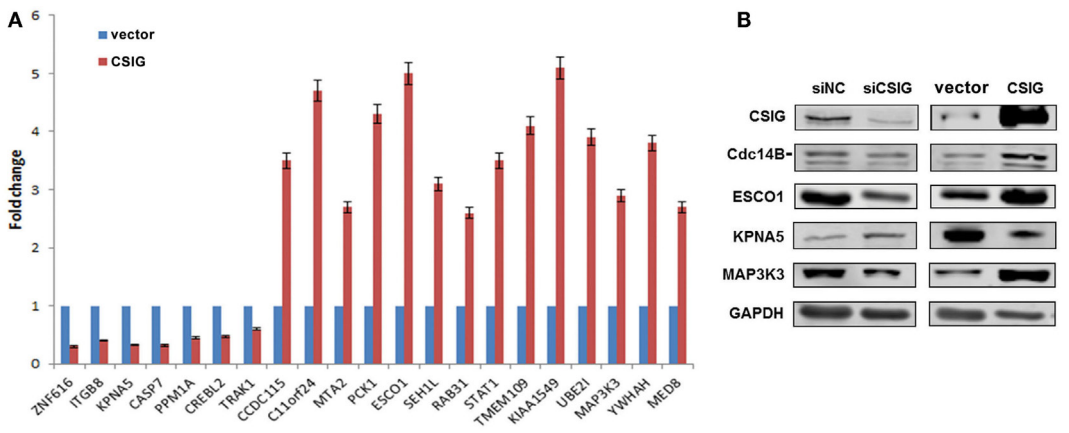

FIGURE 5 | The expressions of differentially expressed genes in CSIG overexpressed or silenced HEK293 cells. **(A)** Real-time PCR analysis of gene expressions in CSIG-transfected and control HEK293 cells. The results are mean ± SEM and the *P*-values are all <0.05. **(B)** Western blot analysis of Cdc14B, ESCO1, KPNA5, and MAP3K3 expressions in HEK293 cells following CSIG knockdown or overexpression.

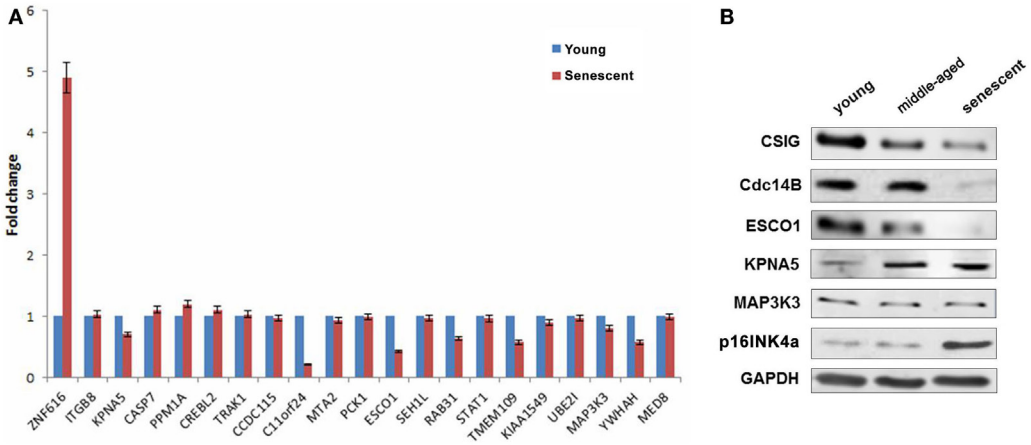

FIGURE 6 | Explore the correlation of differentially expressed genes with CSIG in senescence. **(A)** Real-time PCR analysis of gene expressions in early-passaged (young) and senescent 2BS cells. The results are mean ± SEM and the *P*-values are all <0.05. **(B)** Western blot analysis of Cdc14B, ESCO1, KPNA5, and MAP3K3 expressions in early-passaged (young), middle-aged, and senescent 2BS cells. p16INK4a served as a senescence marker. And GAPDH served as negative control.

induced, and KPNA5 is decreased correspondingly (**Figure 5B**). The observations are consistent with the real-time PCR results, indicated a positive expression correlation of Cdc14B, ESCO1, MAP3K3 with CSIG.

Explore the Correlation of Differentially Expressed Genes with CSIG in Senescence

As described previously, CSIG is abundant in early-passage (young) cells but declined during senescence. To explore the correlation of differentially expressed genes with CSIG in senescence, the expressions of the selected 21 genes were detected by real-time PCR during senescence using the diploid fibroblast 2BS senescence model cells. As shown, comparing with the expression in young cells, ZNF616 was significantly induced in senescent cells while KPNA5, PPM1A, and CREBL2 were slightly increased (**Figure 6A**). In contrary, expressions of C11orf 24, ESCO1, TMEM109, and YWHAH were abundant in young cells, but decreased in senescent cells (**Figure 6A**). Furthermore, we analyzed the expressions of Cdc14B, ESCO1, KPNA5, and MAP3K3 in young (~18 PDs), middle-aged (~40 PDs), and senescent (~57 PDs) 2BS cells and found the decreased expressions of Cdc14B, ESCO1, and increased expressions of KPNA5 with the increasing PDs of 2BS cells, while no obvious changes for MAP3K3 (**Figure 6B**). The results suggested a CSIG-modulated expression pattern of these genes during senescence.

Explore the Correlation of Differentially Expressed Genes with CSIG in Cell Cycle

As GO and pathway analysis showed that several differentially expressed genes were implicated in cell cycle progression. It will be of great interest to study whether CSIG regulate senescence through regulating cell cycle-associated proteins. To explore the correlation of differentially expressed genes with CSIG in cell cycle, we examined the expression patterns of them during cell cycle phases. Young 2BS cells were synchronized by serum starvation for 56 h and then regained to culture with DMEM containing 10% FBS. Cells were collected at different time points for flow cytometry analysis and western blot analysis. According to cell cycle distribution shown in **Figure 7A**, following serum starvation treatment, more than 90% cells were synchronized at G_1 phase (**Figure 7A**). When recovered by addition of 10% FBS, cells reentered into normal cell cycle process gradually. The percent of S phase cells reached to the highest 40.58% at 18 h following recovery, while the percent of cells in G_2/M phase is nearly 0 (**Figure 7A**). At 24 h point, the percent of G_2/M phase cells was increased to 47.36% with very low percent of S and G1 phase cells, accordingly (**Figure 7A**).

As known, cyclin D1 is accumulated in G_1 phase, cyclin B1, and Cdc2 in transition of S phase to G_2/M phase and G_2/M phase, and PCNA in early G_1 phase and S phase (**Figure 7B**). Thus, we simultaneously detected the expressions of cell cycle proteins as the positive control for specific cell cycle phases. Western blot analysis showed that CSIG expression is fluctuated during cell cycle and accumulates in S phase (~18 h) and G_2/M phase (~24 h) of cell cycle, and Cdc14B is abundant in G_1 phase, S phase (~18 h) and G_2/M phase (~24 h), hnRNP A1 is induced in G_2/M phase (~24 h), and KPNA5 in S phase (~18 h), respectively (**Figure 7B**). Meanwhile, we observed no expression alteration of ESCO1 and MAP3K3 during cell cycle (**Figure 7B**).

CSIG Modulates CREBL2, Caspase7, and Cdc14B mRNA Turnover

As a RNA-binding protein previously reported by our and other labs we supposed that CSIG might mainly play a role on post-transcriptional level (7, 15), affecting mRNA degradation or translation. To detect whether the CSIG-associated mRNA level alterations are resulted from the mRNA degradation, we performed the pulse-chase experiment to examine the half-life of several selected mRNA. As shown in **Figure 8**, following CSIG knockdown,

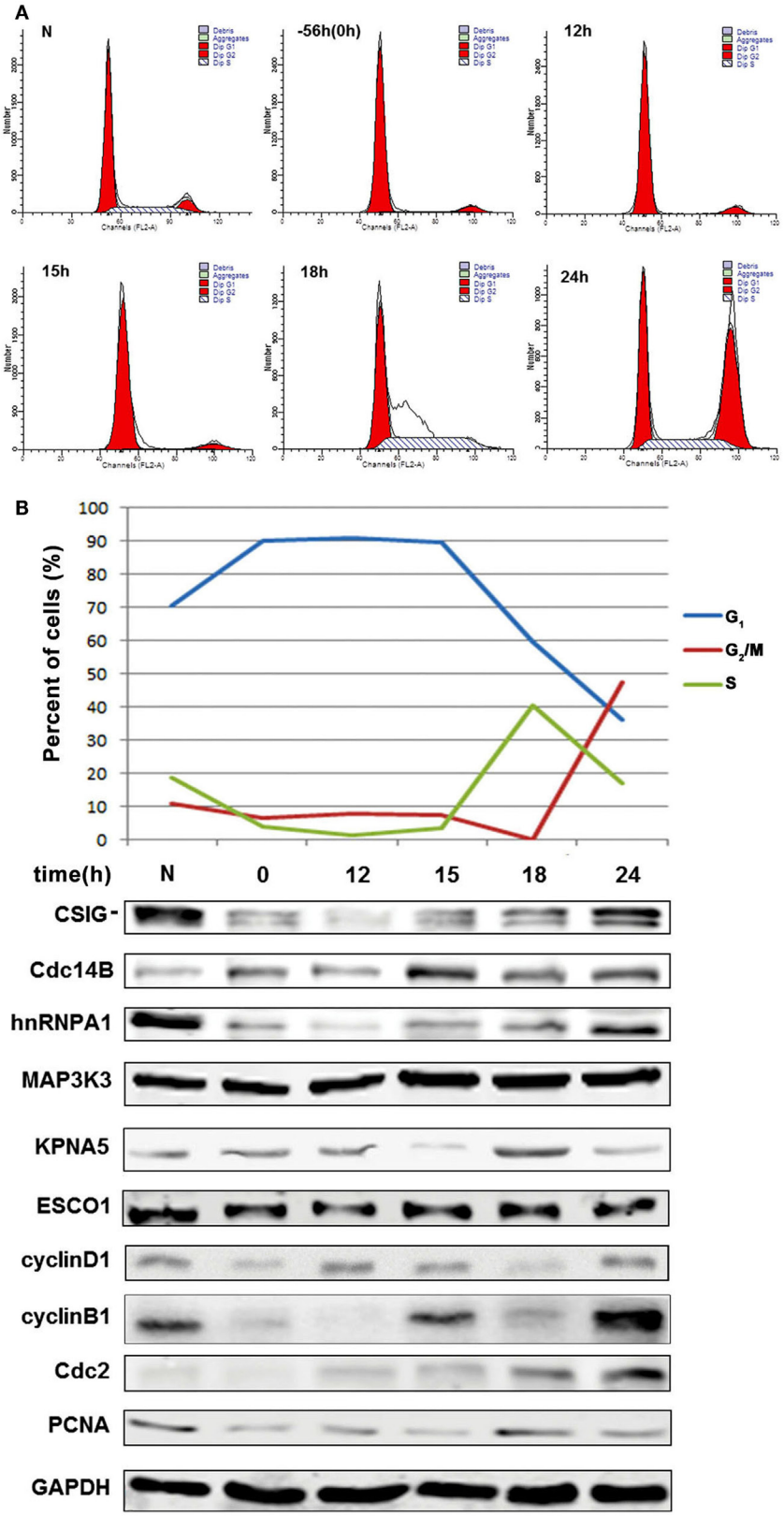

FIGURE 7 | Explore the correlation of differentially expressed genes with CSIG in cell cycle.

(*Continued*)

FIGURE 7 | Continued
(A) Flow cytometry analysis of cell cycle phases following cell synchronization. Early-passaged 2BS cells (18 PDs) were serum-starved for 56 h and then cultured in normal medium with 10% FBS. Cells were collected at different time points for cell cycle analysis by flow cytometry. **(B)** Upper panel, Sketch map of cell cycle phases (G_1, G_2/M, and S phase) according to values of cell cycle distribution at different time points. Bottom panel, western blot analysis of the expressions of CSIG, Cdc14B, hnRNPA1, MAP3K3, KPNA5, and ESCO1 during cell cycle. N represents normal 2BS cells. 0 represents a time point when cells starved for 56 h. cyclinD1, cyclinB1, Cdc2, and PCNA served as positive control (cell cycle phase marker proteins). And GAPDH served as negative control.

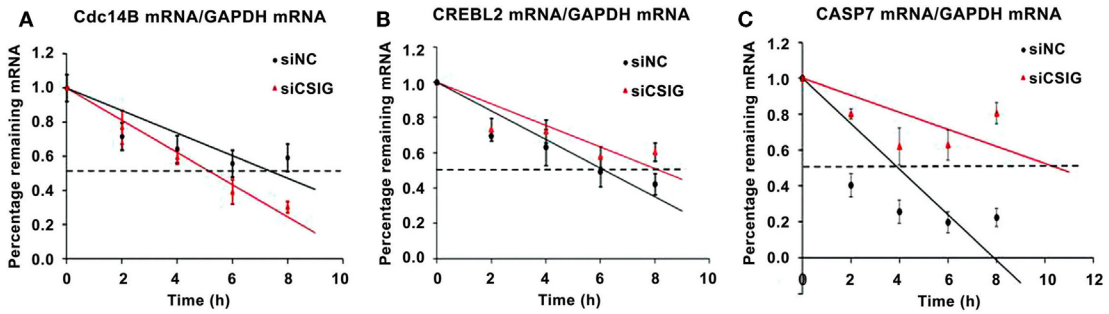

FIGURE 8 | CSIG regulates the stability of Cdc14B, CREBL2, and Caspase7 mRNA in HEK293 cells. RNA was isolated at the indicated times after actinomycin D application to HEK293 cell lines, and the stability of Cdc14B, CREBL2, and Caspase7(CASP7) mRNA was normalized to the values for GAPDH mRNA. **(A)** Cdc14B mRNA half-life is shortened following CSIG knockdown. **(B)** CREBL2 mRNA half-life is prolonged following CSIG knockdown. **(C)** CASP7 mRNA half-life is prolonged following CSIG knockdown.

the half-life of Cdc14B mRNA is shortened by ~2 h (**Figure 8A**), while the half-life of CREBL2 and Caspase7 mRNA is prolonged by 2 and 6.5 h, respectively (**Figure 8B,C**), indicating CSIG implication in mRNA turnover.

Discussion

The present study stemmed from our previous findings. Using a suppressive subtractive hybridization system, we previously identified a CSIG (also known as RSL1D1), which is involved in important processes including senescence, cell cycle regulation, stress response, and tumor metastasis. Here, we set out to explore the regulatory mechanism underlying. To screen important targets and signaling pathways modulated by CSIG, we investigated the differential gene expression profiles following CSIG knockdown in HEK293 cells using the Affymetrix GeneChip microarray. Of the 12,487 genes represented on the microarray, 279 genes, including ZNF616, ITGB8, CASP7, and PPM1A, were up-regulated and 311 genes, including STAT1, UBE2I, MAP3K3, MED8, PCK1, and ESCO1, were down-regulated in CSIG knockdown 293 cells. The differentially expressed genes are involved in a broad array of biological processes, mainly in cell cycle, signal transduction, transcriptional regulation, development, and cell adhesion (**Figure 3**), which offers the possibility of CSIG participation in these processes. Notably, according to GO and pathway analysis, 17 of the changed genes including 9 ZNF genes were implicated in DNA-dependent transcription regulation. Among them, ZNF616 showed the most significant increase following CSIG knockdown. Consistent to the senescence-inhibited expression of CSIG, we observed the senescence-induced expression (low in young cells, while increased in senescent cells) of ZNF616, which suggested the potential CSIG–ZNF616 pathway during senescence progression.

The differential expression of genes such as ZNF616, ESCO1, KPNA5, and MAP3K3 was further validated by real-time PCR and western blot analysis. We further demonstrate the correlated expression patterns of ESCO1, KPNA5, and MAP3K3 with CSIG during cell cycle and senescence progression. According to real-time PCR analysis, ZNF616, c11orf24, ESCO1, and YWHAH exhibit differential expression during senescence. Furthermore, western blot analysis showed a cell age-dependent expression of Cdc14B, ESCO1, and KPNA5 (**Figure 6**). Cell division cycle 14B (Cdc14B), a bidirectional phosphatase, is involved in cell cycle (yeast), DNA damage response, DNA repair, and aging process (16–18). Establishment of cohesion 1 homolog 1 (ESCO1), belonging to a conserved family of acetyltransferases (19, 20), is mainly involved in sister chromatid cohesion (21) and DNA damage repair (22). KPNA5 belongs to the importin α protein family and is thought to be involved in nuclear localization signals (NLS)-dependent protein import into the nucleus (23). The expression and function correlations with CSIG suggested that these proteins might act as potential downstream effectors or mediators of CSIG to regulating cell proliferation and senescence. Further studies on link between CSIG-regulated genes and senescence will be a significant work. Recently, we observed one of CSIG-regulated genes, Cdc14B, really modulates senescence progression (unpublished). It is likely that CSIG is also involved in the regulation of *in vivo* aging. In this regard, studies to develop a knock-out system and to further validate the cellular targets of CSIG during tissue aging are underway in our laboratory. The results of these studies will hopefully provide a more complete understanding of the role of CSIG and its mechanisms of action.

Our previous results showed that CSIG predominantly localized in the nucleolus, the major site for synthesizing and assembling ribosomal subunits. As a nucleolus protein, CSIG is expressed extensively and abundantly in cells. And it has been proved by our practical work that the effect of CSIG knockdown is better than

CSIG overexpression. Therefore, we adopted the gene knockdown strategy. According to our previous study (7) and report (15), CSIG may be an mRNA-binding protein, which means that CSIG might regulate gene expression mainly on post-transcriptional level (mRNA turnover and translation). Therefore, we examined the mRNA half-life change following CSIG knockdown. Consequently, our observations proved the presumption, and the mechanism exploring is under way in our lab.

Together, the analysis of differentially expressed genes following CSIG knockdown provides important clues for the regulatory mechanisms of CSIG in proliferation and senescence. This differential expression profile in response to CSIG knockdown should prove useful for identification of target genes and for elucidating the molecular mechanism responsible for the regulation of cell proliferation and senescence by CSIG.

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Supplementary Material

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REVIEW
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Transcriptional control of mitosis: deregulation and cancer

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Research over the past few decades has well established the molecular functioning of mitosis. Deregulation of these functions has also been attributed to the generation of aneuploidy in different tumor types. Numerous studies have given insight into the regulation of mitosis by cell cycle specific proteins. Optimum abundance of these proteins is pivotal to timely execution of mitosis. Aberrant expressions of these mitotic proteins have been reported in different cancer types. Several post-transcriptional mechanisms and their interplay have subsequently been identified that control the level of mitotic proteins. However, to date, infrequent incidences of cancer-associated mutations have been reported for the genes expressing these proteins. Therefore, altered expression of these mitotic regulators in tumor samples can largely be attributed to transcriptional deregulation. This review discusses the biology of transcriptional control for mitosis and evaluates its role in the generation of aneuploidy and tumorigenesis.

Keywords: mitosis, aneuploidy, cancer, transcription, mutation

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Introduction

The propagation of eukaryotic life is orchestrated by the generation of descendent cells through the biological process of cell division. While mitosis controls the propagation of somatic cells, generation of germ cells is controlled by meiosis. The fidelity of mitosis determines the equal division of duplicated chromosomes to the two daughter cells. The first phase of mitosis, that is, nuclear division or karyokinesis is divided into four sub-phases. Prophase marks the initiation of mitosis bringing about chromosome condensation, separation of duplicated centrosomes, and recruitment of some mitotic checkpoint proteins to the kinetochores. Following this, disassembly of the nuclear envelope (NE) marks the entry into metaphase (prometaphase). Subsequently, the release of chromosomes into cytoplasm activates the spindle assembly checkpoint (SAC) at each unattached kinetochore. After microtubule capturing of each chromatid pair at their kinetochores and alignment at the midzone, silencing of the SAC occurs and cell overcomes the "wait anaphase" signal. During anaphase, sister chromatids are completely separated to the two opposite poles of the cell and the invagination of plasma membrane around the spindle midzone becomes visible. Telophase ends with chromosome decondensation and reassembly of the NE around polar chromosomes. Cytokinesis or cytoplasmic division giving rise to two daughter cells follows soon after. Intriguingly, each of these events is sequentially organized in a manner that minimizes any segregational errors. Therefore, defects in the operation of any mitotic event may lead to the generation of chromosomal instability (CIN), a hallmark of cancer (1–5). Having said this, precision and efficiency of the mitotic cell division depends on proper regulation of the expression and function of mitotic proteins. Indeed, most of these proteins show mitotic phase specific activity. This activity is chiefly regulated by post-translational modifications, namely, phosphorylation and ubiquitination, and some other mechanisms (6). Notably, transcription also plays a key role in the maintenance of cell cycle specific

protein levels (7). However, little has been summarized about the transcriptional control of the mitotic phenomenon. In this review, we will discuss the role of transcription in mitotic regulation and provide evidence for transcriptional anomalies underlying abnormal mitotic events that lead to CIN and tumorigenesis.

Mitosis and Aneuploidy

Errors in chromosome partitioning often give rise to aneuploidy. There are several roads that lead to aneuploidy through mitotic errors (4, 8, 9). The first and foremost reason of mitotic aneuploidy is a faulty SAC. The SAC monitors bipolar segregation of duplicated chromosomes during metaphase to anaphase transition (10). Prior to anaphase, sister chromatids remain held together by the cohesin complex (11). At the onset of anaphase, securin gets ubiquitinated by the E3 ubiquitin ligase, anaphase promoting complex/cyclosome (APC/C). This degradation of securin, in turn, makes separase (a protease) free and active. The latter then cleaves cohesin and the chromosomes begin to separate (10, 12). In the presence of any unattached kinetochore or lack of amphitelic attachment SAC is activated. A number of proteins are involved in the tasks executed by the SAC (10). At the molecular level, the mitotic checkpoint complex (MCC) comprised of the Mad and Bub families of proteins, sequesters APC/C adapter protein Cdc20 (13), and APC/C remains inactive until the defects get corrected. After the completion of proper bipolar attachment Cdc20 is ubiquitinated by mitotic ubiquitin carrier protein UbcH10 and gets free from inhibitory MCC (14). Concordant with that, the SAC antagonist protein p31comet binds to the MCC component Mad2 and modulates extraction of Mad2 from MCC. This, in turn, causes disassembly of MCC and blocks further sequestration of Cdc20 (15, 16). Free Cdc20 activates APC/C, which then degrades anaphase inhibitors and cells progress through mitosis. The stepwise functioning of these events depends on the balanced level of the SAC proteins. While mutations in the SAC genes are infrequent in human cancers, their altered expressions are often reported in various cancers and have been associated with defective SAC-mediated aneuploidy (4). Hence, the balanced levels of different SAC proteins are important determinants of SAC behavior. The cell cycle specific transcriptional regulation of SAC proteins might be an elemental reason in maintaining this balance, deregulation of which might be involved in altered levels of the SAC molecules.

In search of other CIN inducing mitotic phenomena, genetic screens have identified cohesion defects as contributors to the onset of aneuploidy (3, 4, 8). Glitches in the machinery monitoring sister chromatid cohesion might promote aneuploidy. Consistent with this, a recent study identified mutations in *STAG2* (which encodes a protein subunit of the cohesion complex) in a number of aneuploid primary tumors and cancer cell lines (17). Also, overexpression of securin and separase, two key regulators of cohesion, is reported to promote aneuploidy and tumorigenesis (18, 19). Chromosome missegregation may also occur in case of merotelic attachment where a single kinetochore attaches to microtubules emanating from both poles of the spindle (20, 21). Several molecular components, for example, Aurora kinase B, kinesin-13 proteins, MCAK, INCENP, Survivin, and Shugoshin are associated in this phenomenon and their overexpression are reported in cancers of various origins (21). A final source of aneuploidy is the prevalence of aberrant centromeres and multipolar mitosis (2–4). Centrosomes provide mitotic spindle poles and concurrently, presence of more than two centrosomes might produce multipolar spindles. Additionally, aberrant chromosome numbers and multipolarity are associated with CIN in various cancers (22). A number of cellular proteins, including Aurora kinase A, Plk1, Chk1, Chk2, Cyclin B1, and Cdk1, regulate centrosome duplication and the abnormal upstream regulation of these proteins is found in various cancers (2).

Molecular Control of Mitosis: Regulation of Mitotic Proteins

Mitosis is a complex event performed by multiple factors with distinct phase specific responsibilities. Regulation at the protein level plays a crucial role in the mitosis specific performances by these factors. These regulations can occur through several routes (**Figure 1**). First, ubiquitination-mediated protein degradation is believed to be pivotal. The mitotic ubiquitin ligase, APC/C promotes ubiquitination of various protein substrates in a spatial manner (23). By ubiquitinating and consequently targeting mitotic inhibitors for proteasomal degradation, this cellular phenomenon controls mitotic progression in a unidirectional manner. Second, phosphorylation controls functional activities of a number of mitotic proteins in a time-dependent manner. Mitotic cyclin dependent kinase Cdk1, in association with Cyclin A or B, phosphorylates more than 70 substrates involved in various steps of mitosis (24). Some other mitotic kinases like Aurora, Polo, and Nek families also participate in phosphorylation-mediated mitotic regulation (24). As a third mechanism, microRNA (miRNA)-mediated regulation of mitotic proteins is also currently emerging (25–30). In this list of regulatory pathways, the control of expression at the transcription level could be considered as momentous.

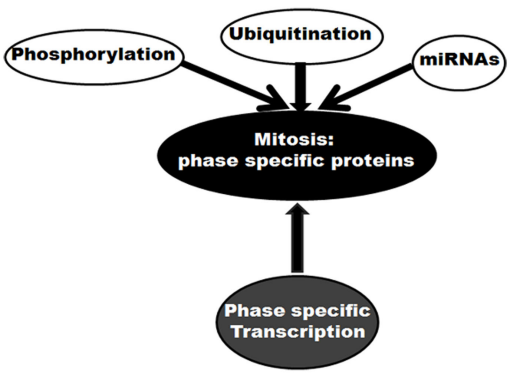

FIGURE 1 | Molecular control of mitosis: regulation of mitotic proteins. Different regulatory mechanisms are shown.

Roads to Chromosomal Instability: Contribution of Mutation Versus Transcription of Mitotic Genes

Most of the tumors are reported to acquire a number of mutations in proto-oncogenes and tumor suppressor genes. Mutation of a gene may alter its product, qualitatively or quantitatively. Extensive search has shown mutations in >1% of candidate genes causally related to oncogenesis, termed as cancer genes (31). Given the fact that mitotic protein levels are pivotal in proper execution of mitosis, the mutational defects can be assumed prime factors in deregulation of mitosis. Simultaneously, a few reports have identified mutations in SAC as well as other mitotic genes in cancers of different origins (32–34). For example, a biallelic germline mutation of the SAC gene *BUB1B* has been diagnosed with mosaic variegated aneuploidy, a rare recessive condition of childhood cancer (35). The genetic alterations, such as gene amplification or depletion, also play a key role in the regulation of many mitotic genes. For example, the genes expressing Aurora-A and Ect2 are amplified in several types of tumors (36–38). Interestingly, despite these reports, mutations directly affecting a mitotic gene are not frequent among cancer types. In an *in silico* approach, we analyzed the mutation status of 526 genes from a list of 572 validated mitotic genes (39) using COSMIC v67 database[1] (Table S1 in Supplementary Material). The percent mutation for each of these genes was obtained from the percentage of unique mutated samples out of total samples studied. The extracted dataset showed <1% of mutations in 84% (441) of the genes. On the other hand, only 5% of the genes showed mutations in >3% of the samples (**Figure 2**). In a separate approach, we tried to find out the expression status of validated mitotic hits (39). Using ONCOMINE 4.4 research edition database[2] cancer versus normal expression patterns were obtained for 557 mitotic genes in 7 head and neck squamous cell carcinoma (HNSCC) datasets (Table S2 in Supplementary Material). Among these, 15% (82) of the genes were found to be overexpressed and 1% was found to be down-regulated in >60% of datasets (**Figure 2**). To find out whether mutations are responsible for the altered expression of these genes we correlated these two analyses (Table S3 in Supplementary Material). The analysis revealed that 73% of the overexpressed mitotic genes have mutations in <1% of the samples. Only 19% of the overexpressed genes were detected with mutations in 1–2% samples and 8% of the genes were detected with mutations in >3% of the samples. Among the down-regulated genes, 87% of the genes showed mutations in <1% of the samples while 13% of the genes showed mutations in 1–2% of the samples (**Figure 2**). This data clearly negate the involvement of mutations in regulating the expression of mitotic genes. The probable reasons behind these findings could be (a) mutation in any one of the mitotic genes including SAC regulators may weaken the checkpoint or other mitotic regulations; (b) mutation leading to complete inactivation of any crucial mitosis regulatory gene would be fatal and be eliminated by death of the affected cell(s). For instance, germline deletion of the SAC gene *MAD2* is associated with the loss of pregnancy (40). Indeed, negligible cancer-associated mutations are reported for Aurora kinase B, Cdk1, Cyclin B, Nek2, and Pin1, proteins involved in initial events of mitosis (Table S1 in Supplementary Material) (6). Also, mutations in mitotic checkpoint genes themselves are not found responsible for abnormal checkpoint in cancer cells (8) and infrequently reported for core SAC proteins like Cdc20, Bub3, and Mad2, and SAC-associated proteins like Borealin, Zwint, Hec1, and Aurora kinase B (6).

[1]cancer.sanger.ac.uk/cancergenome/projects/cosmic/
[2]http://www.oncomine.org/resource/login.html

Transcriptional Control of Mitotic Genes

Mitosis, like any other pathway, is essentially interplay among various protein molecules with tightly regulated phase specific functional activities. A number of mitotic genes show peak level of transcription when the cell passes through the G2 phase (**Figure 3**) (41). Promoters of these genes remain repressed during G0 and G1 phase. The relief from repression starts at the S-phase and peaks after reaching the G2 phase. The transcription factor, NF-Y is crucial in this timely expression (41). A number of mitotic genes contain two or three CCAAT boxes. These sites are recruited by hetero-trimeric NF-Y in association with histone acetyltransferase p300. This dynamic recruitment brings upon transcriptional activation of mitotic genes at the late phase of cell cycle (42). Two consensus sites, namely, cell cycle dependent element (CDE) and cell cycle genes homology region (CHR), have been extensively described in the global regulation of genes having mitosis specific expression (41). Transcriptional repression remains maintained during G0 and G1 phase through the binding of repressor proteins in CDE and/or CHR elements. The release from repression starts at the S-phase. Following this, activation of these promoters mostly occurs through CCAAT boxes after binding of NF-Y in combination with the co-activator, p300. Promoters of mitotic genes, namely, CCNA (Cyclin A), CCNB1 (Cyclin B1), CCNB2 (Cyclin B2), CDC2/CDK1, CDC25C, CKS1, MKLP1, PLK1, and TOME-1, with well documented mitotic phase specific regulation by CDE/CHR elements, are found to be activated through their CCAAT consensus elements (41). On the other hand, p53 has been associated with the repression of several mitotic genes through CDE/CHR elements (41). A number of mitotic genes, like CDC20, CKS1, CCNB1, CCNB2, and CDC2/CDK1, are repressed by p53 (43–46). However, cell cycle specific repression of some other genes without CDE/CHR has also been documented (41). Toward that, besides CDE/CHR site driven effect, a direct p53 binding element has been identified to regulate Cdc20 expression (47).

Beside this, several other transcription factors have also been reported to control the expression of genes in mitosis specific manner. As, for example, Forkbox M1 (FoxM1) has been identified as a master regulator of mitosis. Laoukili et al. have elegantly shown a transcriptional cluster to be regulated by FoxM1 (48). Another study conducted by Wang et al. also ended up with similar observation for FoxM1 as a master regulator of mitotic genes, like CDC25A, AURKA, AURKB, Survivin, CENPA, CENPB, CKS1, SKP2, and PLK1 (49). Subsequently, Fu et al. have shown that mitotic serine/threonine kinase protein, Polo like kinase 1 (PLK1), a target of FoxM1 itself, interacts with and phosphorylates FoxM1. This phosphorylation, indeed, regulates the transcriptional program driven by FoxM1 (50), thereby

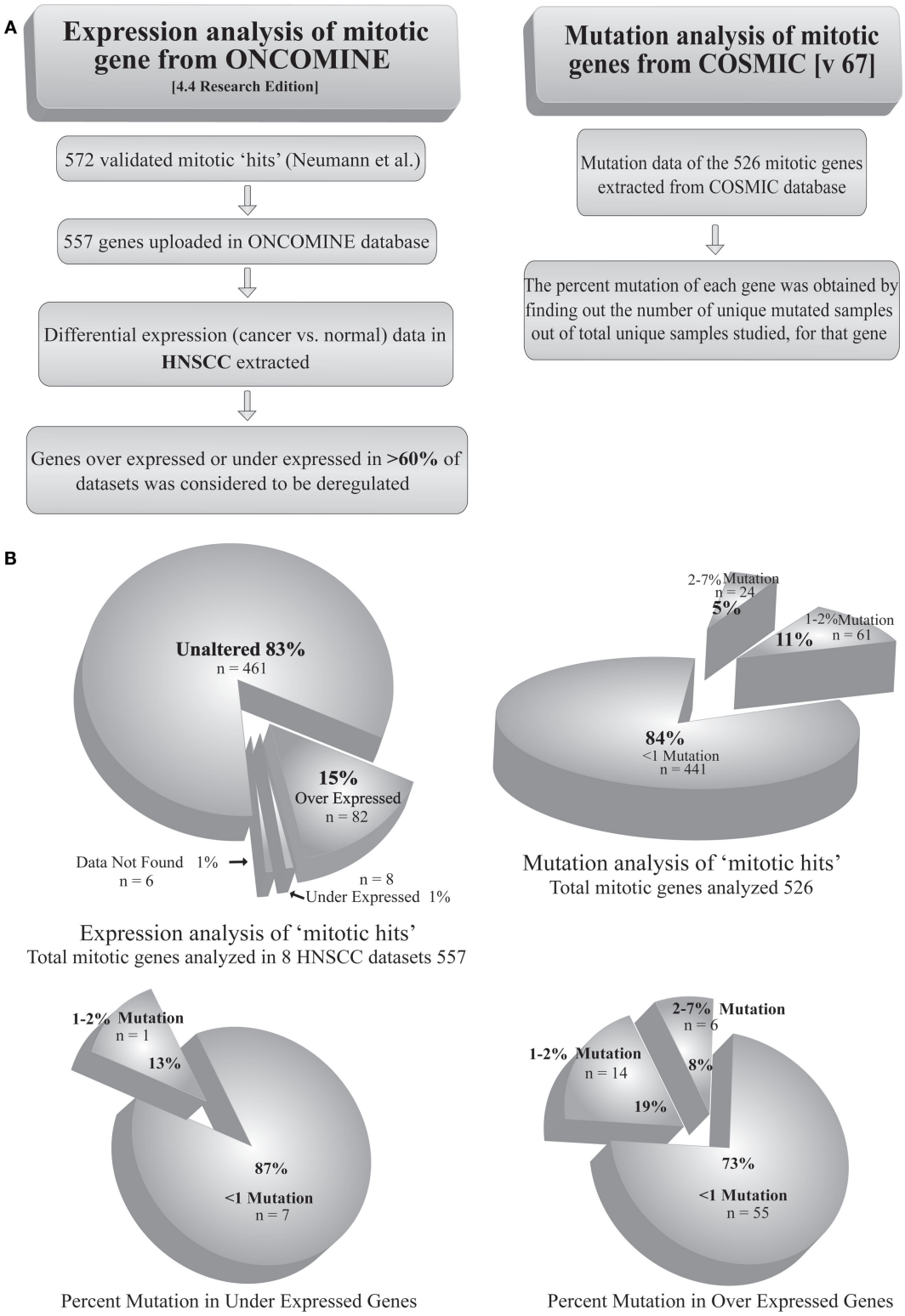

FIGURE 2 | Analysis of mutation and transcriptional alteration in mitotic genes. **(A)** Expression analysis of mitotic genes was done using ONCOMINE (4.4 research edition) database and mutation analysis was done using COSMIC (v67) according to the given workflow. **(B)** The two analyses were correlated to obtain the percentage of mutations in overexpressed and downregulated genes.

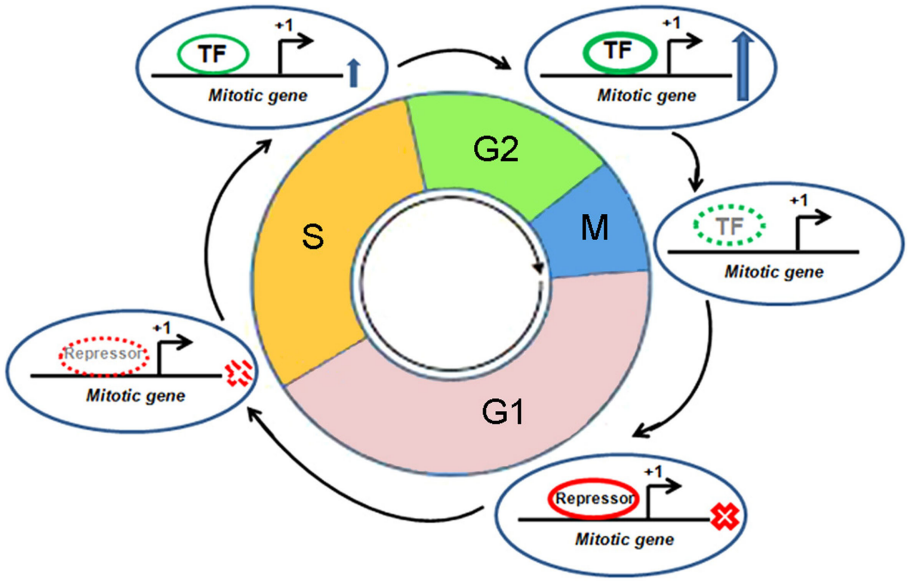

FIGURE 3 | Cell cycle specific transcriptional regulation of a mitotic gene. Different cell cycle phases, mitotic gene, transcription start site, transcription factor (TF), and repressor are shown. "X" mark indicates "transcription off" condition and up arrow indicates "transcription on" condition. Dotted appearance indicates gradual reduction of recruitment of TF or repressor.

indicating a positive feedback loop as a driving force in mitotic transcriptional regulation.

In the last decade, E2F transcription factor family, well-known regulator of S-phase specific trans-regulation, has also been identified in transcriptional control of mitotic genes (51–57). The initial finding of E2F targets from microarray analysis was validated in more than one way and was followed up with identification of target genes involved in chromosome condensation and segregation, SAC functioning, centrosome organization and duplication, and cytokinesis. For instance, core SAC protein Mad2, mitotic ubiquitin carrier protein UbcH10 and PTTG1, a subunit of chromosome cohesion regulator Securin are shown in extensive detail to be G2/M specific E2F targets (58–60). Zhu et al. further showed that recruitment of an activator E2F to the promoter of mitotic cyclin-dependent kinase gene CDC2/CDK1 requires an adjacent CCAAT consensus site pre-occupied by NF-Y. Furthermore, the authors reported that the association of Myb family transcription factor, b-Myb to the promoter of CDC2 and CCNB1 depends on an intact E2F binding site, suggesting a co-operative nature of trans-factor binding in determining mitotic gene activation. Interestingly, b-Myb, itself being an early phase E2F target, links the E2F driven early phase (G1/S) and late phase (G2/M) transcription cascade. Cdc2, Cyclin A2, and Cyclin B1, three important regulators of mitotic entry and progression, were found to be under control of b-Myb-E2F mediated transcription (61).

Human MuvB core complex, comprising of Lin9, Lin37, Lin52, Lin54, and RBBP4, was also identified to regulate transcription of the genes required for the progression into mitosis. Knockdown of the members of this complex led to downregulation of mRNA levels of mitotic proteins including Plk1, Aurora kinase A, Bub1, CENP-E, Lap2, Cyclin A2, Cyclin B1, Cep55, Survivin, and Cdc2 (62, 63). Following this, Sadasivam et al. (64) explains the association and interplay among these master regulators of transcription during the course of cell cycle. They showed that DREAM complex (comprising of *D*P1, *R*b-related protein p130, *E*2F4, *a*nd *M*uvB core complex) functions as a global repressor of mitotic genes during quiescence or G0 phase. Following entry of a cell in G1 phase after quiescence, this DREAM complex dissociates from MuvB core complex. The MuvB core complex then associates with b-Myb and gets recruited to the promoters of late phase mitotic genes (64). Subsequently, MuvB and b-Myb together facilitate the binding of FoxM1 to these promoters during G2 phase to promote the transcription of mitotic proteins like, Cyclin B1, Plk1, Cdc6, Aurora kinase A, and RacGAP1. The cell cycle regulated expression of three other mitotic genes, namely ECT2, MgcRacGAP, and MKLP1, also showed CHR dependent repression throughout G1 phase (65). These genes code for three important regulators of Rho GTPases, critical for mitotic progression, and cytokinesis. The cut homeobox 1 (Cux1) transcription factor coordinately induces the expression of these three genes from S-phase. Moreover, E2F1 was shown to be required in this Cux1 dependent trans-activation process (65).

Besides these well-known consensus elements and master regulators of transcription, some gene specific regulations are also documented in influencing the expression of several mitotic genes. For instance, the transcription of Cdc20 is reported to be regulated by E2F through a new element called Cell cycle *Si*te *R*egulating p55Cdc/*F*izzy transcription (SIRF) (66). Surprisingly, a few mitotic proteins are also identified with transcription regulatory activities. A report showed that WD40 domain containing mitotic checkpoint proteins could act as co-repressors during interphase. The WD40 domain containing SAC proteins, Cdc20 and Bub3, form a complex with histone deacetylases (HDAC1 and HDAC2) during the course of repression (67). On the other

hand, we showed that Cdc20, in combination with APC/C and CBP/p300, transcriptionally activates the expression of UbcH10 (68). Furthermore, recruitment of this Cdc20 trans-complex showed dependence on E2F consensus element on the *UBCH10* promoter (60). The mitotic kinase Plk1 was reported to regulate mitotic gene transcription program by phosphorylating FoxM1 (50). Together, these findings clearly indicate a co-ordination of several master regulators of transcription among themselves and with some gene specific co-activators in controlling cell cycle specific expression of mitotic players. This, in turn, points out the importance of transcription in maintenance of mitotic progression.

Transcriptional Alterations of Mitotic Genes and Association with Cancer

On a cellular level, cancer cells are associated with the loss-of-function mutations of tumor suppressors and the gain-of-function mutations of proto-oncogenes. As many of the mitotic genes are transcriptionally regulated by tumor suppressor or proto-oncoprotein trans-factors, the above-mentioned mutational incidences frequently deregulate the transcriptional outcome of the mitotic genes in tumor cells (**Figure 4**). This, in turn, results in the abnormal execution of mitosis and defects in the chromosomal segregation leading to aneuploidy. Concordant with that, abnormal expressions of many mitotic genes are often associated with the occurrence of oncogenesis.

At the initial stages of mitosis (centrosome maturation, chromosome condensation, NE breakdown, and spindle formation), a number of proteins participate in an orchestrated fashion. Among them, expression of Cyclin B, a Cdk1 activator involved in G2/M progression, has been found to be regulated by the tumor suppressor p53 (69). The direct interaction of p53 to the promoter response element downregulates Cyclin B expression upon DNA damage-mediated checkpoint arrest (69). With alteration of p53 pathway, overexpression of Cyclin B has been shown to contribute to the alteration of SAC and occurrence of CIN in cancer samples (70–72). The Ser–Thr kinase, Plk1 (involved in mitotic initiation in more ways than one) showed elevated mRNA levels in a variety of tumors (73). This protein is transcriptionally coordinated during cell cycle, its level being low during interphase and maximum in mitosis (74). The cell cycle-dependent repression of Plk1 is mediated by Rb pathway. During DNA damage-mediated checkpoint activation, tumor suppressors like p53 and BRCA1 are found to influence levels of Plk1 (74, 75). Correlated with the loss of functional tumor suppressors, transcriptional deregulation of Plk1 is reported in various cancers and associated with CIN and oncogenic transformation (76). Furthermore, tumor suppressors like BRCA1 and Rb are reported to regulate the levels of another mitotic kinase Nek2. In co-ordination with the loss-of-function of tumor suppressors, the overexpression of this protein is associated with CIN and cancer (77–80). Cell cycle specific expression of mitotic Aurora kinases (Aurora kinase A and Aurora kinase B) are CDE-CHR element regulated (81, 82). Oncoproteins like EWS-Fli1 and Myc upregulate expression of aurora family proteins through binding on promoter response elements. On the other hand, tumor suppressors like p53, Brd4 also influence expression of Aurora kinases (83–86). In fact, the altered expression of Aurora kinases are potential markers of cancer progression (87). Regulated expression of kinetochore protein Hec1 is directly related to phosphorylation-mediated inactivation of Rb during the course of cell cycle. Beside this, the CREB family of oncoprotein transcription factors has been shown to upregulate the levels of kinetochore protein Hec1 (88). Disrupted pRb function is associated with transcriptional upregulation of Hec1, which may cause aneuploidy and tumor formation (89, 90).

The initial events of mitosis are followed by chromosomal alignment at the equatorial plane of the cell during metaphase. The amphitelic metaphase alignment precedes SAC release, chromosomal segregation, and entry into anaphase. Consistent with their mitotic roles, a number of core SAC and SAC-associated proteins (Mad1, Mad2, Bub1, Cdc20, UbcH10 to name a few) accumulate gradually through the G2 phase with peak levels at mitosis (13, 68, 91, 92). Different transcription factors and chromatin modifiers regulate cell cycle specific promoter activities of these genes (68, 92, 93). The well-known tumor suppressor p53 is reported to control the transcription of *CDC20* and *BUB1B* (46, 47, 94). Upon genotoxic stress, Cdc20 expression is indirectly suppressed by p53 through p21-dependent mechanism (46). On the other hand, a direct p53 binding element has been identified on the *CDC20* promoter and shown to bring about repression of transcription through chromatin remodeling (47).

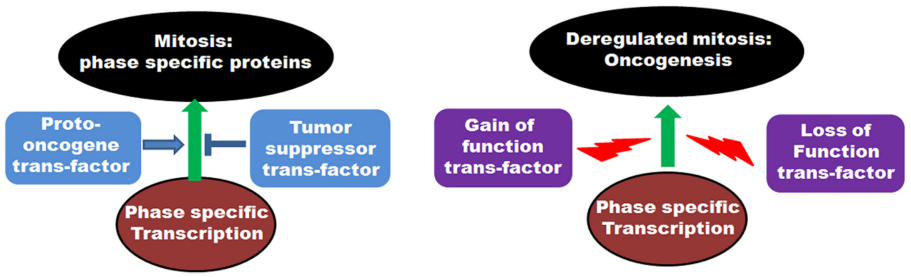

FIGURE 4 | Role of transcription factors in regulation of mitosis: The left panel shows the involvement of various proto-oncogenic trans-factors as well as tumor suppressor transcription factors in regulation of phase specific expression of mitotic proteins. The right panel depicts the deregulation of transcription by gain of function mutations of proto-oncogene trans-factors as well as loss of function mutations of tumor suppressor trans-factors and onset of oncogenesis.

TABLE 1 | Role of proto-oncoprotein and tumor suppressor transcription factors in mitosis and involvement in oncogenesis.

Transcription factor	Mitotic target	Reference
PROTO-ONCOPROTEINS		
c-Myc	Aurora kinase A and Aurora kinase B	(83, 99)
c-Myc	Mad2 and BubR1	(100)
c-Myc	Cyclin B1	(101)
Epstein–Barr virus nuclear antigen 2	Mad2, Plk1	(102)
FoxM1	Cyclin B1, CENP-F, Plk1, Nek2, Aurora kinase B, Cyclin	(48, 103–105)
Mutant p53	Cyclin A, Cyclin B1, Cyclin B2, Cdk1	(106)
Mutant p53	Mad1	(107)
EWS-Fli1	Aurora kinase A and Aurora kinase B	(85)
CREB	Hec1	(88)
CREB	Cyclin A	(108)
TUMOR SUPPRESSORS		
BRCA1	Mad2	(96)
BRCA1	BubR1, Hec1, Stk6, Nek2, Securin, Prc1, Plk, Knls2, Cdc2, and Cdc20	(78)
Rb	Mad2	(58, 109)
Rb	Hec1	(90)
Rb	UbcH10	(60)
p53	Cdc20	(46, 47)
p53	Mad1	(95)
p53	Aurora kinase A, Plk2, Lats2	(110)
p53	Cyclin A1	(111)
p53	Cyclin B	(45)
p53	Emi1	(112)
pVHL	Mad2	(113)

Similarly, direct recruitment of p53 on the promoter consensus element brings about chromatin remodeling and the repression of Mad1 expression (95). Expression of Mad2 is regulated by E2F in a cell cycle-dependent manner. Rb inactivation leads to aberrant Mad2 expression by deregulating E2F activity and contributes to mitotic defects and aneuploidy (58). The tumor suppressor BRCA1 was also reported to regulate Mad2 expression (96). Cancer-associated defects in these tumor suppressors contribute to the abnormal expression of these proteins and a flawed SAC. Indeed, transcriptional abnormalities including differential promoter methylation of these SAC proteins are potential markers of cancers of various origins (97, 98). Their deregulated expressions are associated with CIN phenotype and incidence of cancer (6).

The final stages of mitosis involve cytokinesis and mitotic exit. Along with mitotic kinases like Aurora, Polo, and related families, some other molecular components also regulate this stage of the cell cycle. Protein regulator of cytokinesis 1 (PRC1) and the guanine nucleotide exchange factor, Ect2, the two major molecules of cytokinesis have been related with cancer-associated altered expressions and CIN (6). In conclusion, we have summarized a number of reports from the ever-growing lists of proto-onco gene as well as tumor suppressor trans-factors in regulation of mitosis and their deregulation in tumor background (**Table 1**).

Conclusion

The role of transcriptional regulatory pathways behind the incidence of tumorigenesis remains an enigma. For a number of key cell cycle regulators, the transcriptional control represents an evolutionarily conserved mechanism to precisely maintain their abundance, working in conjunction with miRNA mediated silencing, translational control, and ubiquitin-mediated degradation (23, 26, 114, 115). Among these cell cycle regulators, a defined set of factors stringently control mitotic entry, progression, and exit. The interplay among these factors is naturally adjusted by their abundance. Abnormality in this abundance is associated with the occurrence of aneuploidy, a hallmark of cancer (2, 8, 116). In this review, we have discussed the maintenance of protein levels of the mitotic players whose transcription is regulated in a cell cycle specific manner. We further discussed the deregulation of their transcriptional control, working in concert with cancer onset. In this regard, mutations in various tumor suppressors and proto-oncogenes acting as co-factors of transcription are found to disharmonize the relative protein levels, rather than mutations in the mitotic genes themselves. Besides this, a few mitotic genes are reported to participate in transcriptional control. Furthermore, the list of transcripts whose transcription is affected by certain cell cycle or developmental transitions is being expanded owing to new genome-wide approaches. Answer to many open questions regarding the interplay between transcriptional regulation and mitotic progression will make an important contribution to the understanding of cell cycle control. This, in turn, will help to dissect the involvement of cell cycle progression in the onset of tumorigenesis.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2015.00060

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The tumor cytosol miRNAs, fluid miRNAs, and exosome miRNAs in lung cancer

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The focus of this review is to provide an update on the progress of microRNAs (miRNAs) as potential biomarkers for lung cancer. miRNAs are single-stranded, small non-coding RNAs that regulate gene expression and show tissue-specific signatures. Accumulating evidence indicates that miRNA expression patterns represent the *in vivo* status in physiology and disease. Moreover, miRNAs are stable in serum and other clinically convenient and available tissue sources, so they are being developed as biomarkers for cancer and other diseases. Cancer is currently the primary driver of the field, but miRNA biomarkers are being developed for many other diseases such as cardiovascular and central nervous system diseases. Here, we examine the framework and scope of the miRNA landscape as it specifically relates to the translation of miRNA expression patterns/signatures into biomarkers for developing diagnostics for lung cancer. We focus on examining tumor cytosol miRNAs, fluid miRNAs, and exosome miRNAs in lung cancer, the connections among these miRNAs, and the potential of miRNA biomarkers for the development of diagnostics. In lung cancer, miRNAs have been studied in both cell populations and in the circulation. However, a major challenge is to develop biomarkers to monitor cancer development and to identify circulating miRNAs that are linked to cancer stage. Importantly, the fact that miRNAs can be successfully harvested from biological fluids allows for the development of biofluid biopsies, in which miRNAs as circulating biomarkers can be captured and analyzed *ex vivo*. Our hope is that these minimally invasive entities provide a window to the *in vivo* milieu of the patients without the need for costly, complex invasive procedures, rapidly moving miRNAs from research to the clinic.

Keywords: microRNA, biomarker, lung cancer

INTRODUCTION

Lung cancer is the most common type of cancer worldwide. It is estimated that there are 430,090 men and women living in the United States with a history of lung cancer, and that an additional 224,210 cases will be diagnosed in 2014 (1). Lung cancer is also the leading cause of cancer-related death (2). Because of the lack of validated population-based screening procedures, most patients with lung cancer are diagnosed at an advanced stage. Consequently, the overall 5-year survival rate is only 15% (3). Therefore, there is an urgent need to identify reliable biomarkers of lung cancer, which can then be used for improving accuracy of diagnosis, predicting prognosis, and monitoring disease progression and response to therapy.

MicroRNAs (miRNAs) are small, non-coding RNAs, 19–24 nucleotides in length. They negatively regulate the expression of multiple genes either by inducing translational silencing or by causing the degradation of messenger RNAs (mRNAs) of the targeted gene, both via incomplete base-pairing to a complementary sequence in the 3′-untranslated region (UTR) (4). Since the discovery of the first miRNA, lin-4, in *Caenorhabditis elegans* (5), more than 1,800 human precursor miRNAs have been characterized (6). The accumulating data indicate that miRNAs play important roles in tumorigenesis, metastasis, and drug responsiveness in lung cancer, and can be potential biomarkers for lung cancer (7, 8). Current research has found that the miRNAs can not only be detected in tumor tissues but also in body fluids and even in some extracellular organelles, such as exosomes, all of which have the potential to serve as biomarkers for lung cancer. In this article, we summarize the progress on miRNAs originating from three different sources (tumor tissues, body fluids, and exosomes) as biomarkers for lung cancer.

THE TUMOR CYTOSOL miRNAs, FLUID miRNAs, AND EXOSOME miRNAs IN LUNG CANCER

THE TUMOR CYTOSOL miRNAs IN LUNG CANCER

Many factors, including variations of chromatin, epigenetic factors, hypoxia, and changes in hormone levels, can affect the expression profiles of tumor cytosolic miRNAs. Differences between miRNAs in tumor tissues and normal tissues have been studied extensively and profoundly, and data collected from these studies indicate that miRNAs are involved in several critical processes of lung cancer including the initiation, metastasis, and drug response.

In 2004, Takamizawa et al. (9) identified the first miRNA family, let-7, which was associated with the tumorigenesis of lung cancer.

In their study, they found that introduction of let-7a and let-7f isoforms into A549 cells, a lung adenocarcinoma cell line with low baseline levels of let-7 expression, significantly inhibited the growth of A549 cells. This was further validated clinically, where significantly shorter patient survival after diagnosis was associated with reduced *let-7* expression. Subsequently, many targets of let-7 have been identified, including the RAS family (10), HMGA2 (10–12), c-Myc (13, 14), CDC25A, CDK6, and Cyclin D2 (15), which elucidated the mechanisms by which let-7 exerts its function in tumorigenesis. Since then, many miRNAs were identified as oncogenes or tumor suppressor genes, such as miR-17–92 (16, 17), miR-218 (18), miR-21 (19), and miR-34 family (miR-34a and miR-34b/c) (20–24).

MicroRNAs not only play pivotal role in tumorigenesis of lung cancer but also are involved in tumor metastasis. Several miRNAs including miR-17–92 (25–28), miR-200 family of miRNAs (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) (29), miR-125a-3p/5p (30), miR-21 (31), and miR-106b-25 cluster (miR-106b and miR-93) (32) are reported to be related to the metastasis of lung cancer.

MicroRNAs are also involved in the drug responsiveness of lung cancer cells. It was reported that overexpression of miR-181b could sensitize A549/Cisplatin (CDDP) cells to CDDP-induced apoptosis by decreasing the levels of the anti-apoptotic protein BCL2 (33). Additionally, miR-181a and miR-630 were reported to be modulators of CDDP response in non-small-cell lung cancer (NSCLC) A549 cells (34). In contrast, down-regulation of miR-17-5p expression was associated with paclitaxel resistance by up-regulation of the autophagic protein Beclin 1 (BECN1) expression in NSCLC (35). Similarly, let-7a, miR-126, and miR-145 could sensitize the responsiveness of the large-cell cancer cell line H460 and A549 cells to Gefitinib (36).

BODY FLUID miRNAs IN LUNG CANCER

In addition to tumor tissues, miRNAs are also found in body fluids such as blood, serum, plasma, urine, and cerebrospinal fluid (CSF), as well as in sputum, saliva, and bronchoalveolar lavage (BAL) (37, 38). Several studies indicate that body fluid miRNAs are stable even under extreme conditions, such as repeated freeze-thaw cycles and extreme pHs (e.g., pH = 1 or pH = 13). This feature makes body fluid miRNAs suitable biomarkers for clinical detection (39).

Chen et al. showed that there is a distinct difference between the profile of miRNAs found in sera of healthy individuals and NSCLC patients. Compared to healthy sera, the expressions of 28 different miRNAs were down-regulated and 63 different miRNAs were up-regulated in lung cancer patients. The expression levels of miRNA-25 and miRNA-223, which exhibited the most robust difference in the profile, were further studied in the sera from 152 lung cancer patients and 75 healthy subjects, showing that both of the miRNAs were indeed highly expressed in cancer patient sera. These results indicated that miRNA-25 and miRNA-223 could be used as potential diagnosis biomarkers for NSCLC (40).

Several other miRNAs, including miR-141, miR-155, miR-1254, and miR-574-5p, were identified as potential early diagnostic biomarkers (41, 42). A recent meta-analysis indicated that the early diagnostic value of circulating miR-21 is much better than the plasma miR-21 (43). Roth et al. found that circulating levels of miR-361-3p and miR-625* could be used as blood-based markers for differentiating malignant lung tumors from benign lung tumors (44).

Body fluid miRNAs, especially the circulating miRNAs, can also be promising biomarkers for metastasis and survival time indication. Roth et al. found that high expression levels of miRNA-10b were highly associated with positive lymph node metastasis in lung cancer (41). Hu et al. investigated the circulating miRNA in 303 lung cancer patients and found that the concentrations of 11 miRNAs were elevated more than fivefolds in patients with shorter survival times compared to those whom survived significantly longer. In addition, miR-486, miR-30d, miR-1, and miR-499 were identified as disease fingerprints to predict overall survival in those patients (45). Boeri et al. showed that plasma levels of miR-155, miR-197, and miR-182 could serve as non-invasive biomarkers for early detection and diagnosis of lung cancer. These miRNAs were shown to be significantly elevated in the plasma of the lung cancer patients compared to the cancer free control subjects by greater than 10-folds, and could help discriminate the two groups (46).

EXOSOMAL miRNAs AND LUNG CANCER

Exosomal miRNAs, strictly speaking, are also body fluid miRNAs. However, in contrast to the miRNAs circulating freely in the body fluid, exosomal miRNAs are encapsulated in the cell organelles called the exosomes, which are small (30–90 nm) extracellular vesicles derived from the multivesicular body (MVB) sorting pathway (47). Numerous studies indicate that the expression of miRNAs in exosomes is different in the normal condition and in pathological conditions such as tumor.

Riccardo and colleagues screened 742 miRNAs in circulating exosomes and selected 4 miRNAs (miR-378a, miR-379, miR-139-5p, and miR-200b-5p) as screening markers for segregating lung adenocarcinoma and carcinomas patients from healthy former smokers. They also identified six miRNAs (miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p) for segregating lung adenocarcinoma patients and lung granuloma patients (48).

Guilherme et al. compare 12 specific miRNAs (miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, and miR-214) in peripheral circulation exosome-derived miRNAs and tumor-derived miRNAs in lung cancer patients and healthy people. The results showed that there was no significant difference between peripheral circulation miRNA-derived exosomes and miRNA-derived tumors, and thus the exosome-derived miRNAs can be used as biomarkers for lung cancer (49).

Clearly, a number of specific miRNAs or their families show clinical associations in lung cancer and potential values in cancer stages in clinic (**Table 1**).

THE ISOLATION AND DETECTION OF TUMOR CYTOSOL miRNAs, FLUID miRNAs, AND EXOSOME miRNAs

Tumor cytosol miRNAs can be isolated from fresh tumor tissues or stored formalin-fixed paraffin embedded (FFPE) tissues with Trizol or the conventional phenol/chloroform extraction.

Compared to miRNAs isolated from tumor tissues, the isolation of miRNAs from body fluids requires careful handling in order to

Table 1 | Selected microRNA signatures in lung cancer and their potential value in clinic.

miRNA	Location	Signature	Potential value in clinic	Reference
let-7	Tumor tissues	↓	Diagnosis marker	(7)
miR-17-92		↑	Diagnosis marker	(14, 15)
miR-218		↓	Diagnosis marker	(16)
miR-21		↑	Diagnosis marker and metastasis marker	(17, 29)
miR-34a miR-34b/c		↓	Diagnosis marker	(18–22)
miR-200 family		↑	Metastasis marker	(27)
miR-125a-3p/5p		↓	Pathological stage indicator and metastasis marker	(28)
miR-106b-25 cluster		↑	Diagnosis marker	(30)
miR-181b		↑	Predictor for drug resistance to Cisplatin	(31, 32)
miR-181a				
miR-630				
miR-17-5p		↑	Predictor for drug resistance to paclitaxel	(33)
miR-145		↑	Predictor for drug resistance to Gefitinib	(34)
miR-25	Body fluids	↑	Early diagnostic marker	(38)
miR-223		↑	Early diagnostic marker	(38)
miR-141		↑	Early diagnostic marker	(39)
miR-155		↑	Early diagnostic marker	(40)
miR-1254		↑	Early diagnostic marker	(41)
miR-574-5p		↑		
miR-361-3p		↑	Indicator for malignant lung tumors vs benign lung tumors	(42)
miR-625*		↑		
miRNA-10b		↑	Indicator for positive lymph node metastasis	(43)
miR-486		↑	Predictor for overall survival	(44)
miR-30d				
miR-1				
miR-499				
miR-197		↑	Diagnosis marker for lung cancer patients vs normal people	(41)
miR-182				
miR-378a	Exosomes	↑	Diagnosis marker for lung adenocarcinoma and carcinomas patients vs healthy former smokers	(46)
miR-379				
miR-139-5p				
miR-200b-5p				
miR-151a-5p		↑	Diagnosis marker for the lung adenocarcinoma patients vs lung granuloma patients	(46)
miR-30a-3p				
miR-200b-5p				
miR-629		↑	Early diagnostic marker	(47)
miR-100				
miR-154-3p				

avoid the contaminations of proteins in the body fluids. Chen et al. used phenol/chloroform to remove the serum proteins after using the Trizol for isolating miRNAs from serum (40). Researchers have also added proteinase K to the body fluids during extraction of the miRNAs (50). There are also commercially available kits for isolation the body fluid miRNAs such as the PARIs Kit and PAXGene Blood miRNA Kit (Qiagen). The conventional detection method is also applicable for body fluid miRNAs (51).

For detection of exosomal miRNAs, exosomes have to be harvested. The commonly used methods for isolation of exosomes include ultracentrifugation (52) and polymer-based Exoquick reagent (System Bioscience, Inc.) (53). The exosomes can be identified by Western-blot analysis with the two commonly used exosomal markers, the tetraspanin molecule CD63 and tumor susceptibility gene TSG 101 (54).

The actual detection of tumor cytosol miRNAs, fluid miRNAs, and exosome miRNAs use similar strategies such as Northern blot, RT-PCR, miRNA array, or next generation sequence (NGS) (55).

Although the procedures for isolation of miRNAs from body fluids or exosomes are relatively more complex than isolation of miRNAs from tumor tissues, miRNAs from body fluids or exosomes are considered as better biomarkers for lung cancers because they involve non-invasive procedures compared biopsies for extracting tumor-derived miRNAs. Another advantage of using miRNAs from body fluids or exosomes as biomarkers in lung cancer is that once potential biomarkers are identified, methods can be optimized to detect these specific miRNAs for use in larger studies and every-day practice (**Table 2**).

THE CONNECTIONS AMONG TUMOR CYTOSOL miRNAs, FLUID miRNAs, AND EXOSOME miRNAs

The maturation of miRNAs have been studied extensively and profoundly, and the well-accepted model is that the DNA coding miRNAs are transcribed into the primary miRNAs (pri-miRNAs), and then the pri-miRNAs are processed by Drosha (an RNase type III endonuclease) and specific cofactors to generate the precursor miRNAs (pre-miRNAs). Pre-miRNAs are transported from nucleus into cytoplasm where they undergo processing by Dicer and various cofactors to for mature miRNAs (56). But how the mature miRNAs enter into body fluids and what is the relationship between body fluid miRNAs and exosome miRNAs are still not clear. There are three possible ways for miRNAs to enter body

Table 2 | The isolation and detection of tumor cytosol, body fluid and exosome miRNAs.

Classification	Tumor cytosol miRNAs	Body fluid miRNAs	Exosome miRNAs
Sources	Fresh tumor tissues or FFPE tissues	Whole blood, serum, plasma, urine, CSF, sputum, saliva, or BAL	
Special sample process for miRNA isolation	None	Need to avoid protein contamination	Need to isolate exosomes
Complexity of isolation	+	++	+++
Detection	qRT-PCR, Northern blot, NGS, microarray		
Invasiveness	Yes	None	None
Main function	Diagnosis, prognosis and therapy	Early diagnosis and prognosis	Early diagnosis, prognosis and drug transportation

FFPE, formalin-fixed paraffin embedded; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage; NGS, next generation sequence.

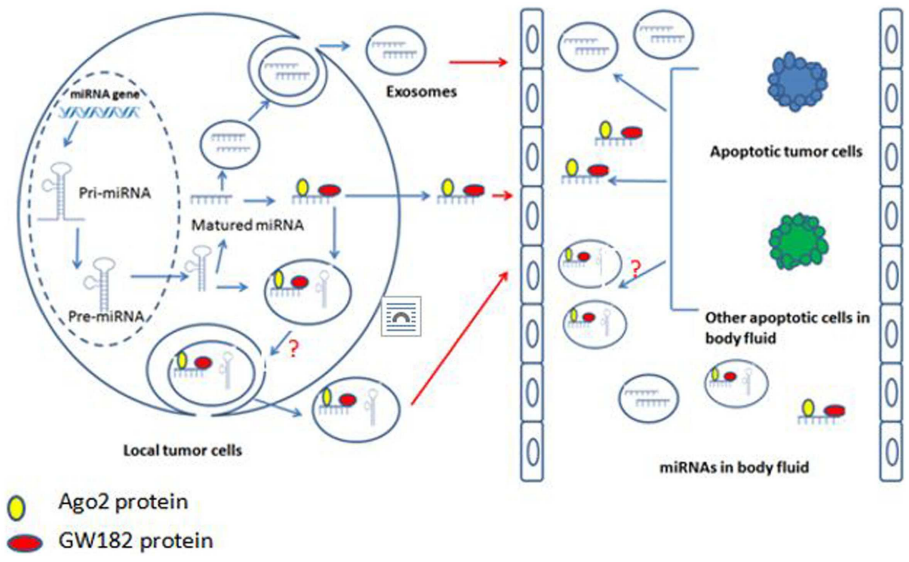

FIGURE 1 | The connection of tumor tissue miRNA, body fluid miRNA, and exosome miRNA.

fluids. First, the tumor cells in the primary location "secret" miRNAs, which combined with Ago2 and GW182 to enter body fluids (57, 58). Second, the miRNAs in the primary tumor cells are packaged into membranous vesicles (MVs) or exosomes and then enter into body fluids (59). However, it is still not clear if the incorporation of miRNAs into exosomes occurs at the pre-miRNA or mature miRNA level. Recently, Villarroya-Beltri et al. reported that exosomes contain mature miRNAs (60). Third, the tumor cells or other cells in body fluids directly release their cytosol miRNAs into body fluids after apoptosis (61) (**Figure 1**).

Currently, there is no consensus regarding the relationships between exosomal miRNAs and the body fluid miRNAs, especially the whole blood miRNAs. Several studies have indicated that the majority of miRNAs found in plasma and serum are present primarily outside the exosomes (62, 63), while other studies found that miRNAs in serum and saliva exist primarily inside the exosomes (54).

CONCLUSION

Over the last decade, great progress has been made in the research of miRNAs and lung cancer. Several miRNAs with differential expression patterns in lung cancer tissues compared to normal tissues have been identified. Furthermore, aberrant expression patterns of miRNAs in lung cancer patients can not only be detected in tumor tissues but also in body fluids and extracellular organelles such as exosomes. All these studies give weight to the conclusion that miRNAs are promising biomarkers for diagnosis and prediction, as well as targets of potential therapeutics for lung cancer. Yet, before they can be effectively integrated into the field of clinical oncology, there are several issues that need to be addressed: (1) Using miRNA array analysis, it is easy to find numerous miRNA candidates whose expressions vary in lung cancer tissue compared to the normal tissue, or whose expressions vary in lung cancer patients body fluids and exosomes compared to the healthy persons. However, there is still no gold standard to evaluate meaningful candidates. It remains a challenge to increase the accuracy of the results from miRNA array analysis and validate meaningful candidates in an efficient manner. (2) Compared to tumor cytosol miRNAs, fluid miRNAs, and exosome miRNAs have attracted more attention as potential diagnostic markers for lung cancer, but there is no standard method for isolating these two kinds of miRNAs, and no reliable endogenous control for evaluation. (3) The quality control of the normal healthy tissue and fluid sample collections is also a key issue in the field, and obtaining a near perfect control at the nano-scale currently remains a major challenge. (4) The up-surging new information in this area brings more and more potential miRNA candidates for lung cancer, but how to interpret and integrate all the information into a network of knowledge for their clinical use as diagnostic and prognostic biomarkers and as potential therapeutic targets represents another attractive area for future investigation. Overall, the study of miRNAs offers a new and exciting angle for us to understand the molecular mechanisms of lung cancer biology. Further studies could provide more accurate biomarkers for both diagnosis and prediction, as well as improved strategies for lung cancer treatment.

AUTHOR CONTRIBUTIONS

All authors participated in conceiving the concept and writing the manuscript.

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Relevance of splicing on tumor-released exosome landscape: implications in cancer therapeutics

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Apart from apoptotic bodies and ectosomes, microvesicles that bud out of the plasma membrane, most types of cells release exosomes when intracellular endosomal microvesicular bodies fuse with their plasma membrane. This last type of vesicles with attributed intercellular communicating capabilities has acquired an enormous attention in the past two decades, especially for their potential as disease biomarkers and/or their use as therapeutic vehicles. The intense study of their molecular composition with special attention to miRNA has led to the development of databases like Exocarta (http://www.exocarta.org/) (1); however, the way exosomes released from tumors (Tex) that may influence cancer patient's health is not yet completely understood.

The observation that exosome production and release is notably increased with tumor progression suggests that they must play an active role in cancer (2, 3). As tumors become more aggressive, heparanase activity is enhanced at least in myeloma, lymphomas, or breast cancer, increasing both the number of exosomes released and the amount of syndecan-1, VEGF, and HGF molecules exposed on their surface (4).

The advantages that Tex present for the delivery of tumorigenic signaling molecules in relation to passive release into the medium are clear, especially for transports across long distances in the body. Firstly, encapsulation of labile molecules such as RNAs and proteins within lipid bilayers offers them protection to degradation; secondly, the surface landscape of the vesicle allows for the possibility of tissue or cell specific targeting and thirdly they are suited for simultaneous multiple message delivery allowing horizontal transfer of complex information from cancer to healthy cells.

In addition to attenuation of antitumor immunity, Tex stimulate angiogenesis, modulate stromal cells activity, and help on the extracellular matrix remodeling contributing to the establishment of a premetastatic niche and generating suitable microenvironments at distant metastatic sites (5). For example, Tex from glioblastoma have the ability to potentiate tumor growth (6) and Tex from melanoma can directly tune a remote lymph node into a microenvironment that facilitates melanoma growth and metastasis even in the local absence of tumor cells (7). These and other similar studies suggest that Tex activities are mediated, at least in part, through the action of particular miRNAs, which presumably down regulate their target transcripts in recipient cells (8, 9). Interestingly enough, Fabbri et al. have recently showed that the Tex miR-21 and 29a molecules can bind to toll-like receptor 7 and 8 (TLR7 and 8) on immune cells and activate them, leading to TLR-mediated NF-kB activation and secretion of prometastatic inflammatory cytokines (10, 11). This offers an alternative mode of action for the miR-mediated Tex paracrine effects in which miR act as aptamer-ligands. So, in addition to Tex internalization Tex protein, lipid, carbohydrate, or nucleic acid surface receptors could interact with target cell receptors to activate intracellular signaling; or, their surface proteins could be cleaved by proteases, and the corresponding soluble fragments act as soluble ligands binding to target cell surface receptors (12). In one case or another, Tex-mediated activities are a threat to patients working toward disease progression. Importantly, anti-cancer agents directed to inhibit DNA replication or microtubule dynamics will result innocuous to Tex. This is, Tex present themselves, together with cancer stem cells, as a therapeutic resistant reservoir for the advancement of the disease, and therefore effective cancer treatments must include targeted inactivation and/or removal of Tex.

On another side, however, exosomes have emerged as potent stimulators of immune responses and from this point of view as agents for cancer therapy. It has been recently showed that induction of HSPs (heat shock proteins)-loaded Tex occurred when hepatocarcinoma cells were treated with chemotherapeutics such as paclitaxel or carboplatin. These Tex released by treated cancer cells conferred superior immunogenicity in inducing HSPs-specific NK cell responses (13). Exosomes can carry a broad variety of immune-stimulatory molecules depending on the cell of origin and *in vitro* culture conditions. In particular, dendritic cell (DC)-derived exosomes (dexosomes) have been shown to carry NK cell activating ligands and can be loaded with antigens to activate invariant NKT cells to induce antigen-specific T and B cell responses. Tumor-antigen-derived dexosomes have been investigated as therapeutic agents against cancer in two phase I clinical trials, with a phase II clinical trial currently ongoing. The results show that although dexosomes were well tolerated, the therapeutic success and immune activation were limited (14, 15). Multiple factors need to be considered in order to improve exosomal immunogenicity for cancer immunotherapy. For example, Tex immunostimulatory effect has been shown

to depend on host's DCs while dexosomes do not (16). Because of their abundant expression of tumor antigens, Tex can be envisaged as an acellular source of antigenic determinants to be exploited in the production of cancer-vaccines and therefore as a therapeutic cancer agent by itself or as a co-adjuvant treatment (17).

Although immune cells, and probably dexosomes, can be found in primary tumor lesions as infiltrating components playing favorable prognostic role (18), in metastatic lesions anti-cancer activities are suppressed due to Tex derived immunosuppressive effects allowing tumor progression. One of the mechanisms responsible for this undesired effect in different types of solid cancers uses endocytic FasL and also TRAIL loaded pro-apoptotic Tex to eliminate overreactive Fas-expressing T cells (19). Thus, Tex in malignant ascites effusions and other cancer patient fluids can eliminate activated T cells through a simple ligand–receptor interaction. In addition, functional CD39 and CD73 Tex are capable of dephosphorylating ATP and AMP to form adenosine to negatively regulate local immune responses (20). Tex can also inhibit DC differentiation, inhibit T-cell proliferation through TGF-β interactions, and promote tumor-immune evasion by interfering with NK cells (19) eventually hijacking the anti-cancer immune response that they might have initiated.

Reached this point, the key question for cancer therapeutics is: will patients benefit by the presence of Tex or effective removal of Tex should be recommended?

The answer does not seem to be straight forward but recent modeling for putative therapeutic implications of exosome exchange between tumor and immune cells may throw some light on it (21). The authors propose the existence of three cancer states: a low cancer load (L) with intermediate immune-level state, and intermediate cancer load (I) with high immune-level state and a high cancer load (H) with low immune-level state. To design and assess possible therapeutic protocols, the authors built a cancer-immunity landscape that includes dynamical states of the cancer-immunity interplay allowing for the visualization of combined protocols as trajectories connecting different states. For example, when the immune recognition is low, the effect of the immune system will not be strong enough to limit cancer progression.

An important observation that should be included in Lu et al. model is that exosome release is controlled by feedback regulatory loops that involve not only cancer cells but also exosome production by normal cells. In this sense, it has been reported that exosome release from normal human mammary epithelial cells and breast cancer cells is not only regulated by the presence of exosomes derived from their own cells but also exosomes from normal mammary epithelial cells inhibit exosome secretion by breast cancer cells in a tissue specific manner, implicating a dynamic equilibrium between exosome release of normal and tumor cells (22).

It is of relevance as well to point out that the different cancer-load-states may refer not only to quantitative traits but also to qualitative cancer-associated features. For example, cancer-specific membrane bound isoforms such as EGFRvIII, generated by defective alternative splicing mechanisms, have been detected on the surface of Tex (6). Such distinctive cancer-associated exons could be used for the design of directional therapeutics, allowing selective inactivation or sequestration of Tex with minimal interference of normal cell exosome activity, reducing possible secondary effects. In addition to antibody-based strategies, synthetic peptides and nucleic acid aptamers have proven not only to bind specifically and tightly to cancer cells but also to home to cancer cells *in vivo* (23–25). Similar approaches could be used to block or sequester Tex through their particular surface landscapes. Targeting through common receptors such as tetraspanins could interfere with normal exosome functions and therefore is not recommended.

The fact that solid tumors are heterogeneous in nature adds yet another degree of complexity to cancer therapeutics. Tumor heterogeneity will be reflected into a multivariety of Tex. Taking into account that the current methods for Tex isolation are based on vesicle size, density, and enrichment of certain common markers (tetraspanins) (26), we can conclude that Tex fractions are far from pure. A possible strategy to study more homogeneous Tex populations would be to establish cell lines from microdissected biopsies so that enough Tex of a particular type can be produced and isolated. This should allow for a better characterization of the Tex complex landscapes, some of them dependent on cancer-associated splicing switches.

Deregulation of splicing is associated to acquisition of cancer advantages, aggressiveness of the tumor, and drug resistance events (27–29). For example, the inclusion of an exon in the pyruvate kinase transcript allows for activation of glycolytic pathway of cancer cells (Warburg's effect), and the KLF6-SV1 variant is associated with poor prognosis and tumor aggressiveness in prostate, lung, and ovarian cancers, associated to EMT (epithelial to mesenchymal transition) (30). In the context of directional targeting of Tex, it would be important to identify specific cancer-associated exons located on the external side of the vesicles. They may or may not be present in the plasmatic membrane of the tumor cell of origin. Toward this end, strategies such as screening of random libraries based on phage display and SELEX (Systematic Evolution of Ligands by EXponential enrichment) technologies allow for the identification of synthetic peptides and aptamers without previous knowledge of the altered splicing events (31, 32). Perhaps, tumor therapies suppressing the splicing machinery itself are advantageous to current systemic cancer treatments.

Tex cancer-associated exon sequences could be used for immunotherapy, to boost DC dexosome release and, at the same time, as targets for Tex inactivation and/or sequestration. If only inactivation/sequestration is applied, the patient would miss the benefit of the immune response against the tumor. On the other side, if the unloading of Tex is not prevented, their cargo will work toward advancement of the disease. This double-approach consisting on blocking Tex function while using their specific cancer-associated exon sequences to induce tumor-immune response could complement current tumor treatments to reduce relapses and to limit metastatic events in immuno-competent patients. In addition, this directional targeting strategy would leave exosome-dependent physiological functions unaffected.

In summary, the study of Tex surface landscape and the detection of

cancer-specific exons on Tex derived from alternative splicing events results essential for the development of Tex targeted therapies. These qualitative switches should be considered in the context of patient immunological and cancer load states for the modeling and the development of improved, personalized cancer therapeutic programs.

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Cancer cell gene expression modulated from plasma membrane integrin αvβ3 by thyroid hormone and nanoparticulate tetrac

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Integrin αvβ3 is generously expressed by cancer cells and rapidly dividing endothelial cells. The principal ligands of the integrin are extracellular matrix proteins, but we have described a cell surface small molecule receptor on αvβ3 that specifically binds thyroid hormone and thyroid hormone analogs. From this receptor, thyroid hormone (L-thyroxine, T_4; 3,5,3′-triiodo-L-thyronine, T_3) and tetraiodothyroacetic acid (tetrac) regulate expression of specific genes by a mechanism that is initiated non-genomically. At the integrin, T_4 and T_3 at physiological concentrations are pro-angiogenic by multiple mechanisms that include gene expression, and T_4 supports tumor cell proliferation. Tetrac blocks the transcriptional activities directed by T_4 and T_3 at αvβ3, but, independently of T_4 and T_3, tetrac modulates transcription of cancer cell genes that are important to cell survival pathways, control of the cell cycle, angiogenesis, apoptosis, cell export of chemotherapeutic agents, and repair of double-strand DNA breaks. We have covalently bound tetrac to a 200 nm biodegradable nanoparticle that prohibits cell entry of tetrac and limits its action to the hormone receptor on the extracellular domain of plasma membrane αvβ3. This reformulation has greater potency than unmodified tetrac at the integrin and affects a broader range of cancer-relevant genes. In addition to these actions on intra-cellular kinase-mediated regulation of gene expression, hormone analogs at αvβ3 have additional effects on intra-cellular protein-trafficking (cytosol compartment to nucleus), nucleoprotein phosphorylation, and generation of nuclear coactivator complexes that are relevant to traditional genomic actions of T_3. Thus, previously unrecognized cell surface-initiated actions of thyroid hormone and tetrac formulations at αvβ3 offer opportunities to regulate angiogenesis and multiple aspects of cancer cell behavior.

Keywords: integrin, thyroid hormone, tetraiodothyroacetic acid, nanoparticle, gene transcription

INTRODUCTION

Integrins are heterodimeric structural proteins of the plasma membrane and are principally involved in cell–cell relationships in tissues and cell–extracellular matrix (ECM) protein interactions (1). The extracellular domain of an integrin binds specific ECM proteins and outside-in transmission of the occurrence of liganding results in the generation of specific signals by the intracellular domain of the integrin. These signals, usually involving various kinases, may result in cellular changes in actin (2, 3) and cell motility (4), modulate endocytosis (5), and affect transcription of specific genes (6–8).

Amply expressed by and activated in cancer cells, integrin αvβ3 interacts with ECM proteins, but has recently been shown to have a panel of specific receptors for non-protein, small molecule ligands (9). Among these are sites for the binding of thyroid hormone (10, 11), dihydrotestosterone (12), and resveratrol (13). The thyroid hormone receptor (TR) on αvβ3 has been well-studied (11, 14). What is now apparent is that this receptor has more complex and coherent effects on cancer-relevant gene expression than had been apparent in analyses of the impact of large molecule (protein) interactions with the integrin. The multiple genes whose expression is modulated from the extracellular domain of αvβ3 by thyroid hormone or its derivative, tetraiodothyroacetic acid (tetrac), relate to angiogenesis, cancer cell proliferation, metastasis, and cancer cell defense pathways (15). The latter include genes relevant to anti-apoptosis, anti-angiogenesis, chemoresistance (MDR1), and repair of double-strand DNA breaks induced by radiation. Within the cell, unmodified tetrac

mimics certain actions of thyroid hormone. At the extracellular domain of αvβ3, in contrast, tetrac blocks binding of L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) – that is, it is a thyroid hormone antagonist. Covalent bonding of tetrac to a nanoparticle prevents cell entry of tetrac and, compared to unmodified tetrac, broadens the spectrum of defensive cancer cell genes whose expression can be desirably regulated from the integrin. This expanded panel includes pro-apoptotic genes and epidermal growth factor receptor (EGFR) gene (see subsequent sections). In addition, the potency of nanoparticulate tetrac as a thyroid hormone antagonist at αvβ3 is greater than that of unmodified tetrac. Thus, without entering the cancer or endothelial cell, thyroid hormone analogs non-genomically initiate important actions on tumor cell and blood vessel cell gene expression. In this review, we survey αvβ3-mediated effects of thyroid hormone and analogs on gene expression in human cancer cells, analyzed by RT-PCR. We also point out that, from its receptor on the integrin, thyroid hormone has adjunctive effects on nuclear receptors for thyroid hormone and for estrogen, regulating the state of phosphorylation or acetylation of such receptors and controlling the formation of complexes within the nucleus of coactivators and receptors.

EARLY EVIDENCE THAT THYROID HORMONE COULD MODULATE GENE EXPRESSION FROM THE CELL EXTERIOR: PROTOONCOGENE EXPRESSION; ANGIOGENESIS

Prior to the discovery of the plasma membrane receptor for thyroid hormone and hormone analogs on integrin αvβ3, agarose-T_4 had been shown to regulate protooncogene expression (16, 17). Agarose-T_4 is a prototypic nanoparticulate formulation of L-thyroxine in which T_4 is covalently bound to a linear polysaccharide polymer; the product is excluded from the cell interior. The thyroid hormone effect on gene expression in these studies was mitogen-activated protein kinase (MAPK)-dependent and was reproduced in cells that lacked the nuclear TR.

Studied in the chick chorioallantoic membrane (CAM) model and also prior to recognition of the hormone receptor on αvβ3, T_4 at physiological free concentrations and T_3 at concentrations that were supraphysiologic were shown to increase vascularity threefold in 72 h (18). The degree of activity was comparable to that of fibroblast growth factor 2 (FGF2; bFGF). Agarose-T_4 also reproduced the pro-angiogenic effect of thyroid hormone. The effects of unmodified thyroid hormone and of agarose-T_4 on angiogenesis were found to be inhibited by tetrac, the hormone analog subsequently shown to block the iodothyronine receptor site on the cell surface. Pharmacologic inhibitors of MAPK (ERK1/2) and of protein kinase C also eliminated thyroid hormone-induced angiogenesis. RT-PCR studies revealed that the hormone-induced transcription of *FGF2* within 6 h, and measurement of FGF2 protein in the medium showed increased release of the angiogenic factor. Thus, the promotion of vascular sprouting (19) and new vessel formation by thyroid hormone was attributable to initiation at the plasma membrane of a non-genomic effect culminating in expression of a specific vascular growth factor gene, manufacture of the gene product and release of the latter protein into the medium.

The cell surface receptor for thyroid hormone and tetrac was shortly thereafter defined on the extracellular domain of integrin αvβ3 and functionally described in the context of angiogenesis (10). Other thyroid hormone agonist analogs, such as GC-1 (20) and diiodothyropropionic acid (DITPA) (21) were also shown to be pro-angiogenic, and tetrac blocked the activity of these analogs at the integrin. However, the anti-angiogenic properties of tetrac expressed at the integrin extend beyond the blockade of binding of T_4 and T_3 to αvβ3. As discussed in the next section, tetrac or its reformulation as a nanoparticulate may affect expression of blood vessel-relevant genes beyond *FGF2* independently of T_4 and T_3. Tetrac and Nanotetrac may also disrupt crosstalk between αvβ3 and adjacent receptors for other vascular growth factors, such as vascular endothelial growth factor (VEGF) and FGF2 (22), and platelet-derived growth factor (PDGF) (Shaker A. Mousa, unpublished observations). However, these effects on crosstalk are unrelated to gene transcription.

TETRAC, NANOTETRAC, AND GENE AND microRNA EXPRESSION THAT IS RELEVANT TO ANGIOGENESIS

As indicated above, unmodified tetrac is taken up by cells and expresses low-grade T_4-like activity and may be converted to triiodothyroacetic acid (triac), which is also thyromimetic (23, 24). To limit the action of tetrac exclusively to integrin αvβ3, we covalently bonded tetrac to a nanoparticle of sufficient size (~200 nm) to preclude cell uptake of the complex (25), thus mimicking agarose-T_4. The polymer we used was biodegradable poly(lactic-co-glycolic acid), in contrast to the physiologically inert agarose. The nanoparticulate formulation involved a stable ether bond of the outer ring hydroxyl group of tetrac to a 6-carbon linker and amide-bonding of the latter to PLGA (25). The amide bond was imbedded in the nanoparticle and thus not readily accessible to circulating or tissue peptidases. The resulting Nanotetrac indeed was restricted to the extracellular space and preserved the previously known actions of tetrac, but it was also found to have desirable additional biologic activities not previously obtained with tetrac.

Microarray studies of two human cancer cell lines showed that tetrac and Nanotetrac downregulated expression of *VEGFA* (26), the gene product of which is a principal inducer of the porous blood vessels associated with cancers (27). These effects are initiated at plasma membrane αvβ3. Tetrac and Nanotetrac also increased transcription of thrombospondin 1 (*THBS1*, *TSP1*). TSP1 protein is an endogenous suppressor of angiogenesis and is invariably suppressed in cancer cells. Nanotetrac also decreased expression of *EGFR*, the gene product of which mediates actions of EGF on angiogenesis. Tetrac lacked this action. Nanotetrac, but not tetrac, downregulates expression of *NFκB* via the integrin and NFκB de-activation is an anti-angiogenic target (28, 29). Finally, thyroid hormone may regulate transcription of the monomeric α*v* gene (30), but it is not known whether this action is initiated at the αvβ3 protein or requires the nuclear TR.

In recent studies of microRNA (miR), we have shown that Nanotetrac increases cellular abundance of miR-15A in breast cancer cells by 10-fold (31) and decreases miR-21 by 50%. miR-21 is pro-angiogenic in certain tumor cells (32) and miR-15A decreases angiogenesis by a VEGF-dependent mechanism (33).

Transcriptional mechanisms involved in the anti-angiogenic activity of Nanotetrac at αvβ3 are summarized in **Table 1**.

Table 1 | Transcriptional mechanisms by which Nanotetrac/tetrac is anti-angiogenic.

Angiogenesis-relevant target	Action	References
bFGF transcription	↓	(18)
VEGFA transcription	↓	(11, 26)
EGFR transcription	↓	(34)
TSP1 (THBS1) transcription	↑	(26, 34)
miR-21 transcription	↓	(31)
miR-15A transcription	↑	(31)
Cellular bFGF abundance	↓	(18)
Cellular Ang-2 abundance	↓	(22)
Cellular MMP-9 abundance	↓	(35)
Pro-angiogenic activity of thyroid hormone	↓	(36)

Measurements of gene transcription were made in breast cancer (34) and medullary thyroid carcinoma cells (26). Protein abundance decreases are presumed to reflect decreased expression of specific genes. The pro-angiogenic activity of thyroid hormone involves non-transcriptional mechanisms as well as actions on specific gene expression shown in this table. Mechanisms that appear to be non-transcriptional are crosstalk between integrin αvβ3 and adjacent vascular growth factor receptors on the cell surface, cell release mechanisms for newly synthesized growth factors and regulation of endothelial cell motility (11, 18, 36).

THYROID HORMONE SUPPORTS CANCER CELL PROLIFERATION AND IS ANTI-APOPTOTIC; TETRAC–NANOTETRAC TRANSCRIPTIONALLY INHIBITS CANCER CELL PROLIFERATION, IS PRO-APOPTOTIC AND DISRUPTS CELL DEFENSE PATHWAY GENE EXPRESSION

A number of laboratories have described the stimulatory effect of thyroid hormone on tumor cells (37–43) and clinical studies have defined thyroid hormone dependence of cancers, in that spontaneous or medically induced hypothyroidism has improved outcomes (44–49). Trophic actions of thyroid hormone on tumor cells were presumed to require a TR isoform and to be genomic in mechanism – that is, to require physical interaction of a TR protein and T_3 – until recognition of the existence in 2005 (10) of the cell surface receptor for thyroid hormone and tetrac on αvβ3, described above (11, 50). Existence of this receptor offered a discrete, non-genomic mechanism for initiation of tumor cell proliferation. TRβ may be involved in certain cancer cell proliferative responses to thyroid hormone (51, 52), but work by Cheng and co-workers indicates that TRβ is a tumor suppressor that, when mutated in the thyroid gland, may be oncogenic (53).

The demonstration that T_4 – including the agarose-T_4 formulation – was a proliferative factor for certain human tumor (breast, thyroid cancer) (42, 54) and animal cells (C6, F98, GL261 glioma cell lines) (43) was accompanied by evidence that unmodified tetrac inhibited the T_4 effect. We had shown that unmodified tetrac blocked non-genomic actions of thyroid hormone on plasma membrane functions (11, 14, 55). The proliferative effect was MAPK-dependent. Interestingly, in human breast cancer (MCF-7) cells, tetrac-inhibitable enhancement of proliferation by thyroid hormone involved Ser-118 phosphorylation of nuclear estrogen receptor-α (ERα); this pathway is identical to that by which estrogen stimulates MCF-7 cell proliferation (54).

In a model of resveratrol-induced apoptosis that involved MAPK phosphorylation of p53 at Ser-15, we showed that T_4 was anti-apoptotic. The hormone prevented the p53 phosphorylation step in several tumor cell lines (42, 56). Tetrac blocked this anti-apoptotic activity of T_4. Additional evidence of the anti-apoptotic activity of T_4 included inhibition of nucleosome liberation by resveratrol, as well as cellular accumulation of the pro-apoptotic $BcLx_s$ protein (56, 57). The hormone did not, however, affect cell accumulation of survival protein $BcLx_l$. The action of thyroid hormone on nucleosome liberation and $BcLx_s$ in tumor cells was prevented by tetrac (58).

Subsequent microarray studies conducted with Nanotetrac in human breast cancer (MDA-MB-231) cells revealed a coherent pro-apoptosis pattern of gene expression. That is, transcription of the X-linked inhibitor of apoptosis (*XIAP*) gene was downregulated and transcription of a set of pro-apoptotic genes – *CASP2*, *CAP8AP2*, *DFFA*, and *BCL2L14* – was stimulated (11, 34).

We would also note that Nanotetrac downregulates expression of 8 of 9 cyclin genes and 1 cyclin-dependent kinase gene (34) and more than 20 oncogenes.

TETRAC–NANOTETRAC ACTIONS ON EXPRESSION OF GENES RELEVANT TO TUMOR INVASIVENESS

Catenins are proteins involved in cell–cell adhesion. β-catenin also has transcriptional functions in the nucleus. Mutation and overexpression of the β-catenin gene occurs in a variety of cancers, including colorectal carcinoma, breast, and ovarian cancer (59, 60). Nanotetrac increases transcription of the *CBY1* gene (34), the gene product of which is an inhibitor of nuclear functions of β-catenins. This is a desirable action of Nanotetrac at αvβ3 in cancer cells. The action would be deleterious in non-cancer cells, but the latter when not undergoing cell division express little αvβ3. Like β-catenins, integrin αvβ3 participates in cellular adhesion complexes.

Nanotetrac also affects α-catenins, downregulating expression of the *CTNNA1* and *CTNNA2* genes. Mutation of *CTNNA2* is associated with tumor invasiveness and thus inhibition of transcription of the gene is desirable, as is downregulation of the non-mutated gene in cancers. The non-mutated gene product of *CTNNA1* can function as a tumor invasion suppressor (61), but mutation is associated with gastrointestinal tract and other cancers (62).

As mentioned above, *MMP-9* expression is induced by thyroid hormone (35). The observations were recently made in myeloma cells and were inhibited by tetrac, thus implicating αvβ3 in this action of T_4. This action of the hormone may contribute to local extension of myeloma in bone and, if documented to be present in solid tumor cells, may presage metastasis. *MMP-2* transcription may also be subject to control by thyroid hormone (63, 64). Several mechanisms may be involved in the hormonal action on *MMP-2*, and it is not yet known whether this effect of the hormone is initiated at integrin αvβ3. The importance of this is that an intact metalloproteinase axis interferes with cell–cell interaction, resulting in tissue destabilization and support of cancer cell invasiveness and metastasis (65).

OVERVIEW OF ANTI-CANCER PROPERTIES OF NANOTETRAC, ACTING AS A SINGLE MODALITY

The anti-cancer actions of Nanotetrac are broadly based in terms of mechanisms, despite initiation at a single target receptor on integrin αvβ3, and in this regard resemble the pluralistic anti-angiogenic actions of the drug. As noted above, the coherence of the effects of the agent on expression of differentially regulated cancer cell genes is remarkable. It is possible that there are effects of Nanotetrac at αvβ3 that may involve integrity of the actin cytoskeleton in cancer cells, and that the drug may influence interactions of the integrin with ECM proteins that may disorient tumor cell movement or interfere with defensive responses (see Conjunctive Radiation and Tetrac/Nanotetrac Treatment of Cancer Cells: Radiosensitization below). However, these possibilities have not yet been examined.

Nanotetrac promotes apoptosis, antagonizes anti-apoptotic (survival) defenses, disrupts control of the cell cycle, and interferes with function of the frequently mutated catenins (11, 26, 34). As noted above in the review of angiogenesis, thyroid hormone and tetrac or its Nanotetrac formulation affect matrix metalloproteinase gene expression. We would also note that thyroid hormone (T_4) has protein-trafficking action on integrin αvβ3, directing internalization of the membrane protein – without the hormone ligand – and nuclear uptake of the αv monomer, but not of β3. In the nuclear compartment, αv is a coactivator (66) involved in transcription of a number of important cancer-relevant genes (see below, Adjunctive Modifications of Nuclear Hormone Receptors that Originate at the Hormone Receptor on αvβ3; Nuclear Uptake of αv Monomer).

Some of these actions of Nanotetrac/tetrac are summarized in **Table 2**.

CHEMOSENSITIZATION BY TETRAC OF CANCER CELLS RESISTANT TO OTHER CANCER CHEMOTHERAPEUTIC AGENTS

P-glycoprotein (P-gp; MDR1; ABCB1) is a plasma membrane efflux pump whose ligands include a number of cancer chemotherapeutic agents (72). The pump is a principal component of cancer cell chemoresistance. Thyroid hormone causes transcription of *MDR1* (73–75) and increases function of the P-gp protein (75). Thus, ambient thyroid hormone may be viewed as a support mechanism for chemoresistance (76). It is not known what the molecular basis is for regulation by iodothyronines of P-gp function or *MDR1* gene expression, i.e., microarray studies have not been conducted to establish whether the induction of *MDR1* gene expression is dependent upon the hormone receptor on integrin αvβ3. However, tetrac increases the intra-cellular retention time of doxorubicin by doxorubicin-resistant breast cancer cells (67), an effect attributed to action of tetrac–Nanotetrac on pump function of P-gp or on gene expression (76).

CONJUNCTIVE RADIATION AND TETRAC/NANOTETRAC TREATMENT OF CANCER CELLS: RADIOSENSITIZATION

Hercbergs and co-workers have defined the potentiation of radiation exposure by tetrac in animal glioma (C6) cells (68) and human glioblastoma (U87MG) cells (69), and Nanotetrac in human prostate cancer (PC3, LNCaP) cells (70). *In vitro* studies revealed that at a 4 Gy x-radiation dose 1 h after exposure to tetrac, there is a 60% reduction in cell survival, compared to control (68). The mechanism of action of tetrac and Nanotetrac is interference with cancer cell repair of double-strand DNA breaks (neutral comet assay/mean tail moment) (69). What components of the DNA break repair process – and, specifically, transcription of what specific genes – are affected by tetrac/Nanotetrac is not yet known.

ADJUNCTIVE MODIFICATIONS OF NUCLEAR HORMONE RECEPTORS THAT ORIGINATE AT THE HORMONE RECEPTOR ON αVβ3; NUCLEAR UPTAKE OF αV MONOMER

The above discussion relates to regulation of transcription of specific cancer cell genes by thyroid hormone analogs that act at the cell surface via integrin αvβ3. Relevant additionally to the end result of modulation of transcription of specific genes from integrin αvβ3 is the adjunctive input from the integrin to the state of

Table 2 | Mechanisms of selected cancer chemotherapeutic actions of Nanotetrac.

Action	Example	References
Chemosensitization	Decreased efflux of doxorubicin, P-gp effect; increased effectiveness of other chemotherapeutic agents	(67)
Radiosensitization	Disordered repair of radiation-induced double-strand DNA breaks; prevention of radiation-induced activation of integrin ávâ3	(68–70)
Disabling of cell survival pathway gene expression	Decreased expression of anti-apoptotic *XIAP*, *MCL1*; enhanced expression of pro-apoptotic *CASP2*, *BCL2L14*, *TP53*, *PIG3*, *BAD*; disruption of catenin pathways via increased expression of *CBY1*, decreased expression of *CTNNA1*, *CTNNA2*; decreased expression of pro-oncogenic *miR-21*, increased expression of pro-apoptotic *miR-21*; decreased expression of matrix metalloproteinase genes, e.g., *MMP-9*; decreased expression of stress-defense genes, e.g., *HIF-1α*, decreased expression of multiple *Ras* oncogenes	(11, 26, 34, 35, 71)
Cell cycle	Downregulation of multiple cyclin, cyclin-dependent protein kinase genes	(11, 34)
Disordering of growth factor pathways	Suppression of *EGFR* gene expression, disabled function of EGFR	(11, 34)
	See **Table 1** for other activities vs. other vascular growth factors, receptors	

nuclear TRs. We have recently reviewed this subject (77). In brief, trafficking of cytoplasmic TRβ1 to the cell nucleus is directed by T_4 at the integrin via MAPK, and the importing by the nucleus of TRα1 is promoted by T_3 via activation of phosphatidylinositol 3-kinase (78). Two discrete binding domains exist at the TR site on αvβ3: the S1 site binds T_3 exclusively and S2 binds both T_4 and T_3. Tetrac–Nanotetrac interferes with hormone binding at both domains. In the case of TRβ1 trafficking, translocation of the receptor into the nucleus occurs as a complex with activated MAPK; specific phosphorylation of the receptor (activation) is a consequence (79, 80). An example of specific gene transcription that occurs as a result of this trafficking/phosphorylation is expression of hypoxia-inducible factor-1α (*HIF1α*) in response to T_3 at the S1 site (78). The complex process of stimulating cancer cell or endothelial cell proliferation occurs via the S2 domain.

Integrin αvβ3 may be internalized by cells as a result of the protein's liganding of T_4 (13). The αv monomer is imported by the nucleus as a result of this process and has been shown to be a coactivator protein that binds to the promoter region of a number of genes, including *ERα*, *HIF-1α*, cyclooxygenase-2 (*COX-2*), and *TRβ1*. ERα protein is important to breast, ovarian, and certain lung cancers. We have implicated nuclear COX-2 protein in the pharmacologic induction of apoptosis (58). HIF-1α protein is a cell survival factor that triggers angiogenesis and cellular conversion to anaerobic metabolism (81). The αv monomer does not import thyroid hormone and the β3 monomer is not taken up by the nucleus. This remarkable process was an unexpected consequence of studies of small molecule actions at the integrin and offers a novel mechanism for regulation of gene expression from the cell surface and integrin.

CONCLUSION

Integrin αvβ3 controls a variety of intra-cellular and transcellular functions. It is a transmembrane structural protein that is differentially expressed/activated in tumor cells and dividing blood vessel cells. The definition of the specific thyroid hormone-tetrac receptor site on αvβ3 (10, 11, 14) enabled recognition of the existence of control from a single locus of expression of differentially regulated, angiogenesis-relevant genes as well as modulation of function of adjacent vascular growth factor receptors. Nanotetrac is a prototypic anti-angiogenic and anti-cancer agent focused on a single, specific small molecule receptor site on the extracellular domain of αvβ3. From this site, Nanotetrac blocks actions of VEGF, FGF2, and PDGF at their plasma membrane receptors, inhibits expression of *VEGFA* and *EGFR*, stimulates transcription of *TSP1*, decreases endothelial cell abundance of Ang-2 without affecting Ang-1, selectively regulates miRNAs that control angiogenesis and decreases endothelial cell motility (**Table 1**).

From the standpoint of anti-cancer activity, Nanotetrac desirably disrupts gene expression critical to cell cycling in αvβ3-bearing tumor cells and dividing endothelial cells and interferes with a substantial group of cell survival pathways so that apoptosis is advanced, and defensive anti-apoptosis pathways are disordered (**Table 2**). Nanotetrac reverses chemoresistance and confers radiosensitivity. This novel and extensive spectrum of actions makes Nanotetrac an attractive anti-angiogenic and anti-cancer agent for further development. The agent has been shown to be an effective anti-proliferative, pro-apoptotic agent in a variety of human cancer cell lines (25, 31, 40–43, 71), to be effective against human cancer xenografts (26, 57, 70, 82–84) and to include important downregulation of tumor-associated angiogenesis (22, 26, 36, 57, 82, 84).

In the absence of an agent such as Nanotetrac with anti-thyroid hormone activity at integrin αvβ3, a reduction in circulating thyroid hormone, notably T_4, that is either spontaneous or medically induced appears to be effective in slowing clinical growth of certain solid tumors. These include breast (45), glioblastoma multiforme (44), head-and-neck cancers (47), and renal cell carcinoma (46). We can postulate that such reductions in systemic levels of T_4 largely affect tumors via the examples of gene expression reviewed above. Several of the current authors have recently confirmed clinically that systematic reduction in circulating T_4 (euthyroid hypothyroxinemia) may arrest growth of certain cancers (85).

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Corrigendum: "Cancer cell gene expression modulated from plasma membrane integrin αvβ3 by thyroid hormone and nanoparticulate tetrac"

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In Table 2, the section designated "Disabling of cell survival pathway gene expression," line 5 incorrectly states ". . .pro-apoptotic miR-21"; the correct statement is ". . .pro-apoptotic miR-15A." The issue is correctly discussed in the body of the published text, p. 2, right column, paragraph 4.

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