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The Complexity of Thyroid Transcription Factor 1 with Both Pro- and Anti-oncogenic Activities*

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After the original identification of thyroid transcription factor 1 (TTF-1 or NKX2-1) biochemical activity as a transcriptional regulator of thyroglobulin in 1989, the bulk of the ensuing research has concentrated on elucidating the roles of NKX2-1 in the development of lung and thyroid tissues. Motivated by its specific expression pattern, pathologists adopted the NKX2-1 immunoreactivity to distinguish pulmonary from nonpulmonary nonthyroid adenocarcinomas. Interestingly, the concept of NKX2-1 as an active participant in lung tumorigenesis did not take hold until 2007. This minireview contrasts the recent advancements of NKX2-1-related observations primarily in the realm of pulmonary malignancies.

In lung cancer, NK2 homeobox 1 (*NKX2-1*) was proposed to be an appealing candidate lineage-survival oncogene in 2006 (1). This was a logical conjecture based on the frequent NKX2-1 overexpression in lung adenocarcinomas (ADs)[2] (2, 3) and the requirement of NKX2-1 for branching morphogenesis of normal lung development as well as the differentiation of lung epithelial cells (4–6). In 2007, four studies uncovered that *NKX2-1* is recurrently amplified in human lung cancer (7–10), implying that NKX2-1 is likely functionally relevant to the pulmonary tumorigenic process, beyond just a marker of lung ADs. Indeed, a race was on to tease out the oncogenic mechanism of NKX2-1. In parallel, a much unexpected finding related to *Nkx2-1* was reported by Winslow *et al.* (11): *Nkx2-1* prevents primary tumors from metastasizing. This anti-metastatic activity of *Nkx2-1* conceptually contradicts the functional ramification of the *NKX2-1* gene amplification seen in human lung cancer. Nevertheless, the latest data from a number of investigators provide a deeper glimpse of the mechanistic intricacy to the anti-oncogenic function of NKX2-1. Evidently, *NKX2-1* joins an expanding list of cancer genes with both pro- and anti-oncogenic activities. These genes include *MYC* (12), *AKT1* (13), *MDM2* (14), *WT1* (15), *REST* (16), and others. In this minireview, I succinctly retrace and contrast the studies from the original discovery of *NKX2-1* gene amplification to the multifunctionalities of NKX2-1 in cancers.

Genetic Alterations of *NKX2-1* in Lung Cancer

NKX2-1 is located at the 14q13.3 region in the human genome. Earlier studies using lower resolution genomic tools had identified 14q13 amplification (17). In 2006, this region was again reported to undergo focal and wide DNA copy number increases in a panel of human lung adenocarcinoma cell lines using array-based comparative genomic hybridization (aCGH) (18). The minimal amplified area across all of the cancer cell lines with the 14q13.3 amplicon contains nine known genes: *PSMA6*, *NFKBIA*, *INSM2*, *BRMS1*, *MBIP*, *NKX2-1*, *NKX2-8*, *PAX9*, and *SLC25A21*. However, the target gene of amplification in this amplicon was not pursued by functional analyses at that time. Within 2 years of this 2006 study, four independent reports documented the gene amplification of *NKX2-1* from different angles. Prompted by the fact that NKX2-1 is a reliable marker of the terminal respiratory unit type of lung ADs (19), Tanaka *et al.* (9) investigated the potential involvement of NKX2-1 in the pathogenesis of lung ADs. After obtaining the evidence that RNAi-induced knockdown of *NKX2-1* in NKX2-1$^+$ lung adenocarcinoma cell lines retarded cell growth, they searched for genetic alterations of *NKX2-1* in AD specimens. No somatic point mutation was uncovered; nevertheless, 2.3% of lung ADs contained an *NKX2-1* gene copy number increase in their analysis. Curiously, they also detected a higher frequency of increased *NKX2-1* gene copies at metastatic sites compared with primary sites. However, the 14q13.3 minimal amplified area, *i.e.* the core amplicon, was not studied, and thus, it was not known how many other genes were coamplified with *NKX2-1* (9).

In the next two studies, Kendall *et al.* (7) and Weir *et al.* (10) employed different types of aCGH-based tools to profile the genomic DNA copy number landscapes of human lung cancer. Although both studies identified the 14q13.3 amplicon as the most recurrent focal amplicon not containing an apparent lung oncogene, the sizes and boundaries of the core amplicon varied from 413 kb (covering three genes: *NKX2-1*, *NKX2-8*, and *PAX9*) (7) to 480 kb (covering two genes: *MBIP* and *NKX2-1*) (10). It is not clear why the core amplicon boundaries varied. Presumably, it is a reflection of the intrinsic sample differences. Alternatively, it may be related to the actual methodology in scoring/determining the amplified region. Kendall *et al.* used quantitative PCR to refine the amplicon boundaries, whereas Weir *et al.* used a statistical method (genomic identification of significant targets in cancer, or GISTIC (20)) to score amplicons. Nevertheless, *NKX2-1* was found to be amplified in both studies. More importantly, the *NKX2-1* amplicon occurs only in lung cancer, in line with the lung cell lineage link of *NKX2-1*. Defining the core amplicon is important because each gene within the core amplicon may be a driver oncogene targeted by the amplification. Thus, every gene inside a core amplicon is a candidate driver gene and must be analyzed for oncogenic characteristics. In the study by Kendall *et al.* (7), the 14q13.3 core

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[2] The abbreviations used are: AD, adenocarcinoma; aCGH, array-based comparative genomic hybridization; NSCLC, non-small cell lung cancer; SQCC, squamous cell carcinoma; T-ALL, T-cell acute lymphoblastic leukemia; miRNA, microRNA.

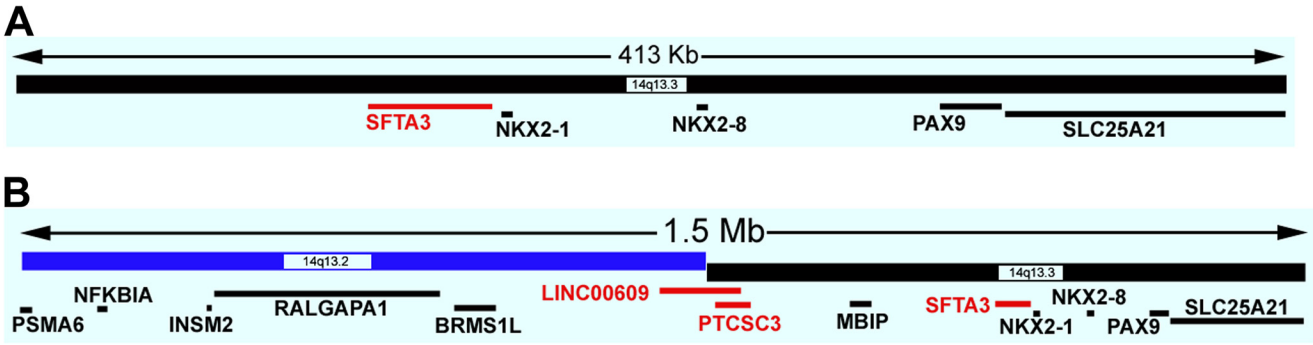

FIGURE 1. **Genes in the current UCSC Genome Browser annotation of the 14q13.3 core amplicons.** *A*, the core amplicon of 413 kb per Kendall *et al.* (7). The human genome coordinate shown is from GRCh37/hg19: chr14: 36,828,082–37,241,292. *SLC25A21* is not fully contained in the core amplicon. *B*, the core amplicon of 1.5 Mb per Kwei *et al.* (8). *SLC25A21* and *PSMA6* are not fully contained in the core amplicon. The human genome coordinate shown is from GRCh37/hg19: chr14: 35,778,082–37,311,292. Only the RefSeq-annotated genes are shown (95). Genes shown in *red* were not known previously to be part of the core amplicons. *RALGAPA1* is also known as *GARNL1*.

amplicon seems to contain functionally cooperating driver genes, as coexpression of the three individual coamplified genes in a pairwise manner enhanced the growth potential of the immortalized premalignant lung epithelial cells (BEAS-2B). A subsequent study, in which a gene expression signature-based strategy was used, suggests that the co-activation of the biological pathways of *NKX2-1* and *NKX2-8* is correlated with poor prognosis for patients with lung ADs (21), reminiscent of the *in vitro* functional cooperation between the coamplified 14q13.3 genes (7). However, transgenic mice with targeted lung overexpression of *Nkx2-1*/*Nkx2-8* or *Nkx2-1*/*Pax9* did not develop lung tumors, and overexpression of *Nkx2-1* alone in the murine lung failed to initiate tumor formation (22). Considering that normal mammalian lung epithelia are notorious for their transformation to full malignancies (23), other genetic lesions would most likely be needed to manifest these coamplified genes. For the two-gene core amplicon detected by Weir *et al.* (10), only the anti-*NKX2-1* RNAi decreased the colony-forming ability of NKX2-1$^+$ lung cancer cells with or without the 14q13.3 amplicon, but not that of NKX2-1$^-$ lung cancer cells, consistent with *NKX2-1* being the driver gene of the 14q13.3 amplification. Like Tanaka *et al.* (9), Weir *et al.* did not detect somatic exon mutations of *NKX2-1* in 384 lung adenocarcinoma DNA samples. Clearly, gene amplification is the main mechanism activating *NKX2-1* in lung cancer. However, as I will discuss below, point mutations and gene rearrangements are invoked to activate the *NKX2-1* oncogene in malignancies outside lung cancer.

Kwei *et al.* (8) also detected the 14q13.3 cytoband amplification as the most frequent focal lung cancer genomic amplification not associated with a known lung oncogene. Following the initial cDNA-based aCGH profiling, they used a custom oligonucleotide tiling array consisting of probes covering 14q13.2~q13.3 at 300-bp intervals to fine map the core amplicon boundaries. In this case, the core amplicon covered eight genes (*NFKB1A*, *INSM2*, *GARNL1*, *BRMS1L*, *MBIP*, *NKX2-1*, *NKX2-8*, and *PAX9*). The authors chose to concentrate on a potential functional connection between *NKX2-1* amplification and lung cancer in light of the fact that *NKX2-1* is a critical lung developmental factor (24–26) and a histologic marker of lung ADs (27). Transfection of siRNAs against *NKX2-1* into amplified human lung adenocarcinoma cell lines diminished cell proliferation, which was attributed to both decreased cell cycle progression and increased apoptosis. Interestingly, human lung adenocarcinoma cell lines without the *NKX2-1* amplicon, albeit with detectable NKX2-1 expression, did not exhibit a similar behavior toward anti-*NKX2-1* siRNAs, suggesting the putative functional importance of the amplification-driven *NKX2-1* up-regulation.

Dynamic Nature of the Known Gene Content of a Core Amplicon

It is important to recall that the human genome is dynamic in that new functional elements continue to be discovered (28). Therefore, the known gene content of a core amplicon may change with time. Reanalysis of the 413-kb core amplicon of Kendall *et al.* (7) revealed that there is a new RefSeq gene termed surfactant-associated 3 (*SFTA3*), which is <3 kb away from *NKX2-1* (Fig. 1*A*) and which was not known to be residing at 14q13.3 in 2007. Apparently, this gene is part of the 14q13.3 core amplicon of varying sizes and boundaries discovered by multiple groups. Without further expression and functional studies of *SFTA3*, it would not be possible to determine whether this gene constitutes a target of 14q13.3 amplification.

NKX2-1 Amplification in Squamous Cell Carcinomas of the Lung

Non-small cell lung cancer (NSCLC) accounts for nearly 80~85% of all lung cancer cases (29). Within NSCLC, ADs and squamous cell carcinomas (SQCCs) are the two most frequent histologic subtypes, representing 50 and 30% of NSCLC, respectively (30). Although the histologic distinction between pulmonary ADs and SQCCs is obvious when these tumors are well differentiated, it can be difficult when they are poorly differentiated, especially in biopsies and cytology specimens. A well accepted immunostaining outcome of SQCCs is the expression of p63 and its N-terminal truncated form (ΔNp63) and the absence of NKX2-1 expression, *i.e.* ΔNp63$^+$/NKX2-1$^-$ (27, 31, 32). This strategy is related to the notion that NKX2-1 is rarely expressed in SQCCs but is frequently expressed in ADs. Although there are studies reporting NKX2-1 expression in SQCCs (33–35), a thorough review of all published studies on positive NKX2-1 expression in SQCCs suggests that the NKX2-1 immunoreactivity may have been due to the type of antibody used and/or the selection of the SQCC cases contain-

ing either glandular differentiation or an AD component (36). Under this backdrop, one may anticipate that the *NKX2-1*-containing 14q13.3 amplicon would not occur in SQCCs as it does in ADs. Indeed, a single nucleotide polymorphism microarray-based analysis of 47 primary SQCC DNA samples did not score the 14q13.3 amplicon (37). In contrast, The Cancer Genome Atlas (TCGA) recently uncovered the 14q13.3 cytoband as one of the 32 significant amplification hubs in lung SQCCs; the core amplicon contains these eight genes: *RALGAPA1*, *BRMS1L*, *MBIP*, *SFTA3*, *NKX2-1*, *NKX2-8*, *PAX9*, and *SLC25A21* (38). The existence of the *NKX2-1* amplicon in lung SQCCs was also detected by others (8, 39, 40). In particular, Tang *et al.* (40) reported that *NKX2-1* is amplified in 20.2% of the 99 lung SQCCs examined by fluorescence *in situ* hybridization, but the NKX2-1 protein is not expressed in any SQCC. Obviously, *NKX2-1* is unlikely to drive the 14q13.3 amplicon in the amplified SQCCs lacking NKX2-1 expression. The possibility of *NKX2-1* being a passenger amplified gene may also play out in a small fraction of lung ADs, as Barletta *et al.* (41) reported two *NKX2-1*-amplified lung ADs exhibiting NKX2-1 expression only in the cytoplasm, not in the nucleus. To reconcile this issue, I offer these hypotheses. (*a*) Other coamplified genes near *NKX2-1* may functionally substitute for *NKX2-1*, as suggested by Kendall *et al.* (7). These coamplified genes should include the recently discovered gene *SFTA3*. Moreover, the larger core amplicon uncovered by Kwei *et al.* (8), in the current genome annotation according to the UCSC Genome Browser (42), contains two more RefSeq non-protein-coding genes than previously realized: *LINC00609* and *PTCSC3* (Fig. 1*B*). Although there is nothing known about these two genes in lung cancer, there is a study implicating *PTCSC3* as a tumor suppressor in thyroid cancer (43). In view of the demonstrated cancer roles of long noncoding RNAs (44, 45), it is not impossible that these two genes also transmit biological consequences of the *NKX2-1*-containing amplification. (*b*) The 14q13.3 amplicon is a non-functional passenger genetic alteration in SQCCs. Evidence against this hypothesis came from the amplified SQCC cell line (NCI-H2170), which became less tumorigenic when either *NKX2-8* or *PAX9* was knocked down by stable RNAi (7). *FOXA1* at 14q21.1, ~1.07 Mb away from *NKX2-1* at the telomeric side, has been proposed to be the driver of 14q DNA copy number gains in SQCCs (17, 46). Although this may be true for some larger scale chromosomal gains at 14q, it does not explain the focal 14q13.3 amplification over *NKX2-1* in SQCCs. Future functional studies of the 14q13 amplification in SQCCs are needed to shed more light on this puzzle.

To further complicate the matter, Harris *et al.* (47) detected an allelic loss at 14q13 in lung tumors. The allelic loss, *i.e.* loss of heterozygosity, occurs in ADs by gene amplification and in SQCCs/adenosquamous tumors by deletion (47). These observations suggest that the 14q13.3 cytoband can undergo DNA copy number alterations in both directions in lung cancer. Seven genes are affected by the 1.2-Mb core-deleted region found by Harris *et al.*: *MBIP*, *SFTA3*, *NKX2-1*, *NKX2-8*, *PAX9*, *SLC25A21*, and *MIPOL1*. The main target gene(s) of this core deletion is not defined. However, the report that *Nkx2-8* null mice developed spontaneous bronchial adenomas and squamous cancer suggests that *Nkx2-8* may be targeted by this DNA loss (48). Nevertheless, the genomic analysis of lung SQCCs by TCGA did not score a significant deletion at 14q13 (38). Perhaps, the 14q13 deletion occurs at a frequency below the threshold of the data analysis algorithm of TCGA.

Pro-oncogenic Activities of *NKX2-1*

The initial mindset following the discovery of *NKX2-1* amplification was that *NKX2-1* is an oncogene. Consistent with this perception, a reduction of endogenous NKX2-1 expression in NKX2-1$^+$ lung cancer cells compromised the cellular capacity to grow without attachment (10) and decreased cell proliferation with a concomitant higher level of apoptosis (8, 9). These evidences were taken as proof that NKX2-1$^+$ lung cancer cells are "addicted" to NKX2-1 functionally. Mechanistic studies identified receptor tyrosine kinase-like orphan receptor 1 (*ROR1*) as a direct transcriptional target of NKX2-1; it also mediates the NKX2-1-dependent survival signaling in lung ADs (49). Genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-seq) and mRNA profiling identified another direct transcriptional target of NKX2-1 (50), *LMO3*, which is a member of the LMO family of oncogenes that are translocated in T-cell acute lymphoblastic leukemia (T-ALL) (51, 52). Interestingly, LMO3 suppression in *NKX2-1*-amplified cell lines increased apoptosis but had no effect on cell proliferation (50), suggesting multiple downstream effectors of amplified *NKX2-1*. Many NKX2-1-binding sites are enriched with neighboring recognition sequences of other oncogenic transcription factors such as AP1 and FOXA1 (22, 50), indicating cooperative transcriptional regulation between NKX2-1 and other oncogenic transcription factors.

NKX2-1 itself is translocated with different partner genes in T-ALL (53). In these T-ALL patients, the *NKX2-1* gene product is ectopically overproduced, and cell-based assays implicate it as oncogenic (53). The *NKX2-1* translocation in T-ALL harbors a route to investigate the oncogenic mechanism of *NKX2-1* in reference to the recent finding that the TLX1 homeodomain oncoprotein mediates T-cell maturation arrest in T-ALL via interaction with ETS1 and suppression of T-cell receptor-α gene expression (54). The parallel may be drawn between *NKX2-1* and *TLX1* because some of the *TLX1*-translocated T-ALLs cocluster with *NKX2-1*-translocated cases by gene expression profiling (53). Ectopic expression of oncogenic *NKX2-1* need not be driven by genetic alterations. Recently, it was documented that *NKX2-1* expression is switched on in diffuse large B-cell lymphomas by epigenetic modifications (55). Through proteomic analyses of sera derived from a number of mouse models of lung cancer, Taguchi *et al.* (56) detected the presence of multiple proteins whose genes are known transcriptional targets of NKX2-1. These results manifest an NKX2-1-driven serum protein signature in lung cancer and suggest that NKX2-1 may be a master regulator supervising the lung secretome.

Finally, although point mutations of *NKX2-1* do not seem to occur in lung cancer, a germ-line mutation (A339V, 1016C→T) of *NKX2-1* was found in patients with multinodular goiter and papillary thyroid cancer (57). Overexpression of this NKX2-1 mutant in normal thyroid cells enhanced cell proliferation (57). If indeed the oncogenic activity of *NKX2-1* can be

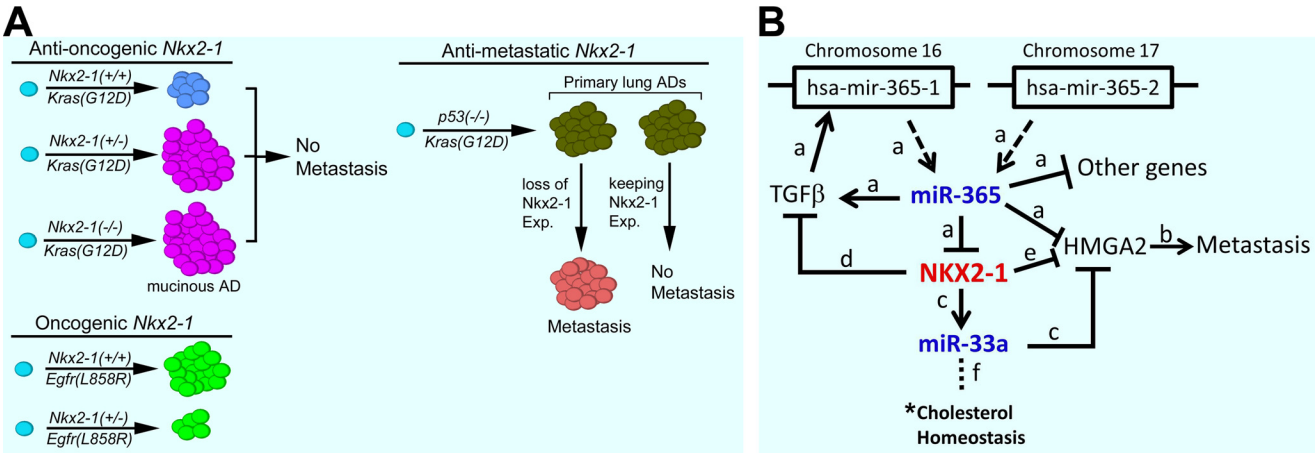

FIGURE 2. **Schematics of *NKX2-1* functions and its interactions with miRNAs.** *A*, the graphical presentation summarizes the opposing functional roles of Nkx2-1 in mouse models (11, 22, 64). *Exp*, expression. *B*, *NKX2-1* is imbedded in a miRNA-based signaling network, with miRNAs acting both up- and downstream. The relevant studies establishing individual interactions are indicated as follows: *a*, Ref. 82; *b*, Refs. 11 and 96; *c*, Ref. 84; *d*, Ref. 68; *e*, Ref. 11; and *f*, Refs. 87–90. *, a direct impact on cholesterol homeostasis by NKX2-1 remains to be demonstrated. This figure was modified from Qi *et al.* (82) with permission.

activated via a point mutation, the mystery is then why such a mechanism is never utilized in lung cancer. By the same token, a cohort of 216 primary and metastatic thyroid tumors were analyzed and found to be negative for *NKX2-1* gene amplification (39). In light of the importance of *NKX2-1* to thyroid biology (58–60) and the identification of 14q13.3 as a risk locus for thyroid cancer by a genome-wide association study (61), the lack of *NKX2-1* gene amplification in thyroid malignancies remains a mystery.

Anti-oncogenic Activities of *NKX2-1*

In a 2004 study by Kang *et al.* (62), a progressive decrease in Nkx2-1 expression was detected from the wild-type lung adenomas to ADs in a TGF$\beta 1^{+/-}$ mouse model treated with the carcinogenic ethyl carbamate. In a separate study that conditionally knocked out *Nkx2-1* in the adult murine thyroid, a genotoxic carcinogen induced a higher incidence of adenomas in *Nkx2-1* null mice relative to wild-type or *Nkx2-1*$^{+/-}$ mice (63). Although these data suggest an anti-cancer function of Nkx2-1, the pervasive thinking in the field was shaped by the discovery of *NKX2-1* amplification and concentrated on unraveling the oncogenic mechanism of *NKX2-1*, largely ignoring a possible role of NKX2-1 as a tumor suppressor. However, in 2011, an unexpected finding by Winslow *et al.* (11) brought the anti-tumorigenic side of *NKX2-1* into the spotlight. They administered lentiviral vectors expressing the Cre recombinase intratracheally into transgenic mice (*Kras*$^{G12D/+}$;*p53*$^{flox/flox}$), which later developed multifocal lung ADs. Some of the primary lung tumors eventually led to macroscopic metastases. The stable lentiviral integration sites allowed primary tumors to be unambiguously linked to their related metastases. Gene expression profiling of two types of primary lung tumors (non-metastatic and metastatic) indicated that *Nkx2-1* was consistently and significantly depressed in the metastatic primary tumors with clonally related metastases. These observations implicate Nkx2-1 as an anti-metastatic factor in lung ADs. Winslow *et al.* went on to show that Nkx2-1 prevents primary tumors from metastasizing by suppressing Hmga2. Upon loss of Nkx2-1 expression, derepressed Hmga2 would then facilitate the progression to metastases. It is important to note that pathologists routinely encounter NKX2-1^{+} metastatic tumors derived from primary human lung ADs, testifying to the complexity of human lung cancers.

Two subsequent thorough animal studies proved that Nkx2-1 is anti-oncogenic and capable of blunting tumor initiation in specific genetic contexts (Fig. 2*A*) (22, 64). Maeda *et al.* (22) created transgenic mice harboring a constitutive loss of an *Nkx2-1* allele with a conditional activation of the *Kras*^{G12D} oncogene in the murine respiratory epithelium. Interestingly, the lung tumor number and volume of *Kras*^{G12D};*Nkx2-1*$^{+/-}$ mice increased compared with those of *Kras*^{G12D};*Nkx2-1*$^{+/+}$ and control mice. The tumor histology of *Kras*^{G12D};*Nkx2-1*$^{+/-}$ mice, unlike that of *Kras*^{G12D};*Nkx2-1*$^{+/+}$ mice, is reminiscent of human mucinous AD of the lung. To complement their loss-of-function approach, Maeda *et al.* made triple transgenic mice (*Scgb1a1-rtTA*;[*tetO*]-FLAG-*Nkx2-1*;[*tetO*]-*Kras4b*^{G12D}) such that the expression of Nkx2-1 and Kras^{G12D} was turned on simultaneously in the pulmonary epithelium. The result indicates that coexpression of Nkx2-1 reduces the number and volume of Kras-induced lung tumors, showcasing the anti-oncogenic activity of Nkx2-1 in retarding mutant Kras-induced tumor initiation and progression. Because *EGFR* is a critical lung oncogene not associated with lung mucinous ADs (32), Maeda *et al.* (22) created recombinant mice with a conditional *Egfr*^{L858R} allele in the background of *Nkx2-1* haploinsufficiency (*Egfr*^{L858R};*Nkx2-1*$^{+/-}$). In contrast to the Kras-initiated tumors, the tumor number and volume were significantly decreased in the *Egfr*^{L858R};*Nkx2-1*$^{+/-}$ mice compared with the *Egfr*^{L858R};*Nkx2-1*$^{+/+}$ mice, indicating that *Nkx2-1* enhances *Egfr*-mediated tumorigenesis. The manifestation of the anti- or pro-oncogenic activities of *Nkx2-1* in transgenic mice harboring different mutant oncogenes (*Kras* or *Egfr*) demonstrates the importance of the signaling network to the Nkx2-1 function. None of the *Egfr*-driven mouse lung ADs (*Egfr*^{L858R};*Nkx2-1*$^{+/+}$ or *Egfr*^{L858R};*Nkx2-1*$^{+/-}$) was of the mucinous subtype, in contrast to those seen in the *Kras*^{G12D};*Nkx2-1*$^{+/-}$ model. An interpretation of these data is that the wild-type level of Nkx2-1

expression guards against $Kras^{G12D}$ from steering the tumor differentiation state toward the mucinous type. It is known that raising oncogenic Ras expression in thyroid cells down-regulates Nkx2-1, which in turn inhibits thyroid differentiation (65, 66). Clearly, the differentiation state of lung tumor cells is also dictated by Nkx2-1 expression status and the coexisting oncogenic events.

Independently, Snyder *et al.* (64) modeled the *Nkx2-1* deletion in a variety of transgenic mice. They first generated a strain of mice that relied on the Cre recombinase to simultaneously activate a mutant $Kras^{G12D}$ allele and become $Nkx2\text{-}1^{-/-}$ (64). In doing so, the tumor burden increased by 2∼3-fold in the $Kras^{LSL\text{-}G12D}$;$Nkx2\text{-}1^{F/F}$ mice in comparison with the $Kras^{LSL\text{-}G12D}$;$Nkx2\text{-}1^{F/+}$ mice, and the tumors of the $Kras^{LSL\text{-}G12D}$;$Nkx2\text{-}1^{F/F}$ mice resembled mucinous ADs, in keeping with the findings of Maeda *et al.* (22). Lung-specific conditional knock-out of *Nkx2-1* never gave rise to macroscopic tumors, despite alteration of the differentiation state in the adult lung (64). The investigators then temporally separated the activation of the $Kras^{G12D}$ allele from *Nkx2-1* deletion by placing the two alleles under the control of two different recombinational events. Mice with an activated $Kras^{G12D}$ allele were allowed to go for 2∼7 months, followed by *Nkx2-1* deletion using tamoxifen/Flp recombinase. The *Nkx2-1* deletion conferred a significant increase in tumor cell proliferation without an effect on apoptosis. Six weeks after *Nkx2-1* deletion, the tumor burden was 4-fold higher in the *Nkx2-1*-deleted mice relative to control mice, documenting that the absence of Nkx2-1 enhances the initiation and progress of *Kras*-driven tumorigenesis in the lung. Snyder *et al.* (64) also noted that Nkx2-1 appears to be involved in repressing a gastric differentiation state. The loss of *Nkx2-1* may unleash this phenotype in a specific and conducive lung cell type, inducing the mucinous ADs, which are positive for gastric markers (GKN1 and CTSE) repressed by Nkx2-1. The investigators further characterized the genome-wide binding events of Nkx2-1 and analyzed the data in the context of other transcription factors, revealing functionally important links of Nkx2-1 to Foxa1, Foxa2, and Hnf4α.

Other molecules that may mediate the anti-oncogenic/anti-metastatic activities of NKX2-1 have been identified by either profiling or candidate gene approaches. Hosono *et al.* (67) found myosin-binding protein H (*MYBPH*) to be under the positive and direct transcriptional regulation of NKX2-1 through gene expression profiling of immortalized lung epithelial cells stably expressing an *NKX2-1* transgene. MYBPH reduces single cell motility via negative regulation of actomyosin organization, forming a basis of the anti-metastatic function of NKX2-1. Saito *et al.* (68) identified that NKX2-1 transactivates E-cadherin and counters the epithelial-mesenchymal transition of lung cancer cells conferred by TGFβ. In fact, TGFβ is known to be antagonistic to NKX2-1-directed gene transcription (69). In view of the significant roles of epithelial-mesenchymal transition in cancer metastasis (70, 71), this finding could also explain the anti-metastatic role of NKX2-1. Finally, Runkle *et al.* (72) reported that NKX2-1 directly transactivates two molecules, occludin (*OCLN*) and claudin-1 (*CLDN1*), at the lung epithelial tight junction. Niimi *et al.* (73) found that *claudin-18* is a transcriptional target of *Nkx2-1*. *NKX2-1* knockdown conferred human lung cancer cell resistance to anoikis, and expression of occludin restored cellular sensitivity to anoikis; overexpression of occludin impeded migration and induced anoikis in lung carcinoma cells. These data point to a putative involvement of tight junction proteins in helping NKX2-1 suppress lung cancer progression (74). A working model derived from these data is that the anti-oncogenic/anti-metastatic activities of NKX2-1 are mediated by a variety of molecules across multiple cellular pathways.

Data Placing *NKX2-1* Firmly in a MicroRNA-based Signaling Network

In the lung, microRNAs (miRNAs) play critical roles in both development and tumorigenesis (75–79). Over 100 miRNAs are dynamically regulated during organogenesis of a normal murine lung (80). Considering that NKX2-1 is a vital controller of lung development and cancer, it was surprising that a direct interaction between *NKX2-1* and miRNAs was not discovered until 2012. Qi *et al.* (82) discovered the first miRNA (*i.e.* miR-365) that directly targets *NKX2-1* by screening the top 10 miRNAs predicted by the TargetScan algorithm (81) to bind directly to the 3′-UTR of *NKX2-1*. Expression profiling identified other putative target genes of miR-365 and miR-365*. Exploration of human lung cancer genomics data uncovered that *NKX2-1* gene amplification was significantly associated with DNA copy number loss at one of the two genomic loci encoding the precursor RNA of mature miR-365 (*i.e.* mir-365-1). This implies the putative existence of genetic selection pressure to lose the repressive miR-365 that would otherwise suppress amplified *NKX2-1*. Intriguingly, a signaling loop exists among TGFβ, miR-365, and NKX2-1, with TGFβ up-regulating miR-365 via the mir-365-1 precursor gene, which in turn represses *NKX2-1* (82). Moreover, miR-365 feeds back to the TGF signaling pathway by specifically up-regulating TGFβ2 (Fig. 2*B*) (82). The observation of miR-365 repressing *NKX2-1* was later reproduced in a separate study (83).

The first miRNAs that are directly regulated by NKX2-1 were uncovered by Rice *et al.* (84). Motivated by the finding of *Nkx2-1* repressing *Hmga2* by Winslow *et al.* (11), Rice *et al.* speculated that *NKX2-1* directly activates miRNAs to repress *HMGA2*. Using two complementary strategies, gain- and loss-of-expression of NKX2-1/Nkx2-1, they found a selection of miRNAs that are putatively directly controlled by NKX2-1. One such miRNA, miR-33a, was further characterized because there are three predicted binding sites for miR-33a in the 3-kb-long 3′-UTR of *HMGA2*. The experimental data confirmed that *HMGA2* is a genuine target of miR-33a and that NKX2-1 directly transactivates the host gene of miR-33a, *SREBF2*, to up-regulate miR-33a, which then keeps *HMGA2* expression in check (84). An intriguing implication from this study relates to the fact that both SREBP2 (the gene product of *SREBF2*) (85, 86) and miR-33a (87–90) are critical players in cholesterol homeostasis, suggesting that NKX2-1 may influence cholesterol metabolism in the lung and that cholesterol metabolism may be mechanistically involved in the lung cancer biology of NKX2-1 (Fig. 2*B*).

Conclusions

In recent years, we have witnessed a rapid unfolding of lung cancer biology in connection with the fundamental lung developmental transcription factor gene *NKX2-1*. The dual faces of NKX2-1 as both a pro- and an anti-cancer factor are complex but not unique in the NK2 family of transcription factors. The *NKX2-2* oncogene is translocated in T-ALL (53) and is an essential signaling mediator of the driver oncogene *EWS-FLI* in Ewing sarcoma (91). In contrast, *NKX2-2* suppresses self-renewal of glioma-initiating cells and is frequently down-regulated in human gliomas (92). Intriguingly, the disparate outcome of *NKX2-1* also extends to its correlation with clinical parameters of lung AD patients. Positive NKX2-1 expression is associated with good prognosis (41, 93, 94), but *NKX2-1* amplification is linked with poor prognosis (40, 41). The biochemical activity of NKX2-1 as a transcription factor precludes it from direct drug discovery. However, as more signaling partners of NKX2-1 are uncovered, there will be opportunities to initiate translational research to create and develop NKX2-1-dependent strategies as tools to manage NKX2-1-positive lung cancer.

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