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

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Running title: NKX2-1 with Pro- and Anti-Cancer Activities

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SUMMARY: After the original identification of the 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 been concentrating on elucidating the roles of NKX2-1 in the development of the 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 review 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 back in 2006 (1). This was a logical conjecture based on the frequent NKX2-1 overexpression in lung adenocarcinomas (ADs) (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), implicating 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), WTI (15), REST (16), and others. In this review, I succinctly retrace and contrast the studies from the original discovery of NKX2-1 gene amplification to the multi-functionalities 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 2005, 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 the cancer cell lines with the 14q13.3 amplicon contains nine known genes: PSMA6, NFKBI1A, INSM2, BRMS1, MBIP, NKX2-1, NKX2-8, PAX9 and SLC25A21. However, the target gene of amplification in this

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amplicon was not pursued by functional analyses at that time. Within 2 years of this 2005 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 terminal respiratory unit type of lung ADs (19), Tanaka et al. investigated the potential involvement of NKX2-1 in the pathogenesis of lung ADs (9). After obtaining the evidence that RNA interference (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 adenocarcinoma specimens. No somatic point mutation was uncovered; nevertheless, 2.3% of lung ADs contained a 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 than that at 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 (19).

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. While 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 as to 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 (QPCR) to refine the amplicon boundaries, whereas Weir et al. used a statistical method (GISTIC (20)) to score amplicons. Nevertheless, NKX2-1 was found 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., the 14q13.3 core amplicon seems to contain functionally cooperating driver genes, as coexpression of the three individual coamplified genes in a pair-wise manner enhanced the growth potential of the immortalized premalignant lung epithelial cells (BEAS-2B). A subsequent study, using gene expression signature-based strategy, implicates that the co-activation of the biological pathways of NKX2-1 and NKX2-8 is correlated with poor prognosis of 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 (22); and overexpression of Nkx2-1 alone in the murine lung failed to initiate tumor formation (22). Considering that normal mammalian lung epithelia are notorious to be transformed 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., only the anti-NKX2-1 RNAi decreased the colony formation ability of NKX2-1⁺ lung cancer cells with or without the 14q13.3 amplicon, but not that of NKX2-1⁺ lung cancer cells (10), consistent with NKX2-1 being the driver gene of the 14q13.3 amplification. As in 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 later, point mutations and gene rearrangements are invoked to activate the NKX2-1 oncogene in malignancies outside lung cancer.

Kwei et al. also detected the 14q13.3 cytoband amplification as the most frequent focal lung cancer genomic amplification not associated with a known lung oncogene (8). 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 of NKX2-1 amplification and lung cancer in light of the fact that NKX2-1 is a critical lung
developmental factor (24-26) and a histological marker of lung ADs (27). Transfection of small interfering (si) RNAs 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 a putative functional importance of the amplification-driven NKX2-1 upregulation.

The 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. reveals that there is a new RefSeq gene termed Surfactant Associated 3 (SFTA3), which is less than 3 Kb away from NKX2-1 (Fig 1A) and was not known to be residing at 14q13.3 in 2007. Apparently, this gene is part of the core 14q13.3 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 if this gene constitutes a target of the 14q13.3 amplification.

NKX2-1 Amplification in Squamous Cell Carcinomas (SQCCs) of the Lung

Nonsmall cell lung cancer (NSCLC) accounts for nearly 80–85% of all lung cancer cases (29). Within NSCLC, ADs and 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 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 implicates that the NKX2-1 immunoreactivity may have been due to the type of antibody used and/or the selection of the SQCC cases containing either glandular differentiation or an adenocarcinoma 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). On the contrary, 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 (39,40). In particular, Tang et al. reported that NKX2-1 is amplified in 20.2% of the 99 lung SQCCs examined by fluorescence in situ hybridization, but NKX2-1 protein is not expressed in any SQCC (40). Obviously, NKX2-1 is unlikely to drive the 14q13.3 amplification 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. reported two NKX2-1-amplified lung ADs exhibiting NKX2-1 expression only in the cytoplasm, not in the nucleus (41). 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 per the UCSC Genome Browser (42), contains two more RefSeq non-protein coding genes than previously realized: LINC00609 and PTSC3 (Fig 1B). While there is nothing known about these two genes in lung cancer, there is a study implicating PTSC3 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-function al passenger genetic alteration in SQCCs. An 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). While 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. detected an allelic loss at 14q13 in lung tumors (47). 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, TCGA’s genomic analysis of lung SQCCs did not score a significant deletion at 14q13 (38). Perhaps, the 14q13 deletion occurs at a frequency below the threshold of the TCGA’s data analysis algorithm.

**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 a 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 leukemias (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 API1 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 TCRα 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 needs 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. detected the presence of multiple proteins whose genes are known transcriptional targets of NKX2-1 (56). These results manifest a 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 germline mutation (A339V, 1016 C>T)) of NKX2-1 was found in patients with multinodular goiter and papillary thyroid cancer (57). Overexpression of this mutant NKX2-1 in normal thyroid cells
enhanced cell proliferation (57). If indeed the oncopgenic activity of \textit{NKX2-1} can be 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 \textit{NKX2-1} gene amplification (39). In light of the importance of \textit{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 \textit{NKX2-1} gene amplification in thyroid malignancies remains a mystery.

\textbf{Anti-Oncogenic Activities of \textit{NKX2-1}}

In a 2004 study by Kang et al. (62), a progressive decrease of Nkx2-1 expression was detected from the wild-type lung, adenomas, to ADs in a \textit{TGF}\textsubscript{β1}\textsuperscript{−/−} mouse model treated with carcinogenic ethyl carbamate. In a separate study which conditionally knocked out Nkx2-1 in the adult murine thyroid, a genotoxic carcinogen induced higher incidence of adenomas in the Nkx2-1 null mice relative to wild-type or Nkx2-1\textsuperscript{+/−} mice (63). Although these data implicate an anti-cancer function of Nkx2-1, the pervasive thinking in the field was shaped by the discovery of \textit{NKX2-1} amplification and concentrated on unraveling the oncogenic mechanism of \textit{NKX2-1}, largely ignoring a possible role of Nkx2-1 as a tumor suppressor. But in 2011, an unexpected finding by Winslow et al. brought the anti-tumor suppressor into the spotlight (11). They administered lentiviral vectors expressing the Cre-recombinase intratracheally into transgenic mice (\textit{KrasG12D/+}; \textit{p53flos/flox}) which later developed multi-focal 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 holds primary tumors from metastasizing by suppressing Hmga2. Upon losing Nkx2-1 expression, derepressed Hmga2 would then facilitate the progression to metastases. It is important to note that pathologists routinely encounter Nkx2-1\textsuperscript{+} 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 (22,64) (\textbf{Fig 2A}). Maeda et al. created transgenic mice harboring a constitutive loss of a Nkx2-1 allele with a conditional activation of \textit{Kras}\textsuperscript{G12D} oncogene in the murine respiratory epithelium (22). Interestingly, lung tumor number and volume of \textit{KrasG12D;Nkx2-1\textsuperscript{+/-}} mice increased compared with those of \textit{KrasG12D;Nkx2-1\textsuperscript{−/-}} and control mice. The tumor histology of \textit{KrasG12D;Nkx2-1\textsuperscript{−/-}} mice, unlike those of \textit{KrasG12D;Nkx2-1\textsuperscript{+/-}} mice, is reminiscent of human mucinous adenocarcinoma of the lung. To complement their loss-of-function approach, Maeda et al. made triple transgenic mice (\textit{Scgb1a1-rtTA;[tetO]-Flag–Nkx2-1;[tetO]-Kras4b\textsuperscript{G12D}}) such that the expression of Nkx2-1 and \textit{KrasG12D} 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. Since \textit{EGFR} is a critical lung oncogene not associated with lung mucinous ADs (32), Maeda et al. created recombinant mice with a conditional \textit{EGFR\textsuperscript{{L858R}}} allele in the background of Nkx2-1 haplinsufficiency (\textit{EGFR\textsuperscript{{L858R}};Nkx2-1\textsuperscript{−/-}}). In contrast to the Kras-initiated tumors, tumor number and volume were significantly decreased in the \textit{EGFR\textsuperscript{{L858R}};Nkx2-1\textsuperscript{−/-}} mice compared with the \textit{EGFR\textsuperscript{{L858R}};Nkx2-1\textsuperscript{+/-}} mice, indicating that Nkx2-1 enhances \textit{EGFR}-mediated tumorigenesis. The manifestation of the anti- or pro-oncogenic activities of Nkx2-1 in transgenic mice harboring different mutant oncogenes (\textit{Kras} or \textit{EGFR}) demonstrates the importance of the signaling network to the Nkx2-1 function. None of the \textit{EGFR}-driven mouse lung ADs (\textit{EGFR\textsuperscript{{L858R}};Nkx2-1\textsuperscript{−/-}} or \textit{EGFR\textsuperscript{{L858R}};Nkx2-1\textsuperscript{+/-}}) were of the mucinous subtype, in contrast to those seen in the \textit{KrasG12D;Nkx2-1\textsuperscript{−/-}} model. An interpretation of these data is that wild-type level of Nkx2-1 expression guards against \textit{KrasG12D} from steering the tumor differentiation state toward the
mucinous type. It is known in thyroid cells that raising oncogenic Ras expression would downregulate 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. modeled the Nkx2-1 deletion in a variety of transgenic mice (64). They first generated a strain of mice which relied on the Cre recombinase to simultaneously activate a mutant Kras\textsuperscript{G12D} allele and become Nkx2-1\textsuperscript{−/−} (64). In doing so, the tumor burden increased by 2–3-fold in the Kras\textsuperscript{LSL-G12D};Nkx2-1\textsuperscript{FF} mice in comparison to that of the Kras\textsuperscript{LSL-G12D};Nkx2-1\textsuperscript{FF} mice and, the tumors of the Kras\textsuperscript{LSL-G12D};Nkx2-1\textsuperscript{FF} mice resembled mucinous ADs, in keeping with the findings of Maeda et al. (22). Lung-specific conditional knockout of Nkx2-1 never gave rise to macroscopic tumors, in spite of alteration of differentiation state in the adult lung (64). The investigators then temporally separated the activation of the Kras\textsuperscript{G12D} allele from Nkx2-1 deletion by placing the two alleles under the control of two different recombinational events. Mice with activated Kras\textsuperscript{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 in apoptosis. Six weeks after Nkx2-1 deletion, the tumor burden was 4-fold higher in the Nkx2-1-deleted mice relative to controls, documenting that the absence of Nkx2-1 enhances the initiation and progress of Kras-driven tumorigenesis in the lung. Snyder et al. 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 which may mediate the anti-oncogenic/anti-metastatic activities of NKX2-1 have been identified by either profiling or candidate gene approaches. Hosono et al. found that MYBPH, myosin binding protein H, to be under positive and direct transcriptional regulation of NKX2-1 through gene expression profiling of an immortalized lung epithelial cell line stably expressing a NKX2-1 transgene (67). MYBPH reduces single cell motility via a negative regulation of actomyosin organization, forming a basis of the anti-metastatic function of NKX2-1. Saito et al. identified that NKX2-1 transactivates E-CADHERIN and counters the epithelial mesenchymal transition (EMT) of lung cancer cells conferred by TGFβ (68). In fact, TGFβ is known to be antagonistic to NKX2-1-dected gene transcription (69). In view of the significant roles of EMT in cancer metastasis (70,71), this finding could also explain the anti-metastatic role of NKX2-1. Finally, Runkle et al. reported that NKX2-1 directly transactivates two molecules, OCCLUDIN and CLAUDIN-1, at the lung epithelial tight junction (72); Niimi et al. found that Claudin-18 is a transcriptional target of Nkx2-1 (73). 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 tumorogenesis (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 2011. Qi et al. 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 (82). Expression profiling identified other putative target genes of miR-365 and miR-365*. Exploration of human lung cancer
genomics data uncovered that NNX2-1 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 NNX2-1. Intriguingly, a signaling loop exists among TGFβ, miR-365, and NNX2-1, with TGFβ upregulating miR-365 via the mir-365-1 precursor gene which in turn represses NNX2-1 (82). Moreover, miR-365 feedbacks to the TGF signaling pathway by specifically upregulating TGFβ2 (Fig 2B) (82). The observation of miR-365 repressing NNX2-1 was later reproduced by a separate study (83).

The first miRNAs that are directly regulated by NNX2-1 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 NNX2-1 directly activates miRNAs to repress HMGA2. Using two complementary strategies, gain- and loss-of-expression of NNX2-1/Nkx2-1, they found a selection of miRNAs which are putatively directly controlled by NNX2-1. One of such miRNAs, miR-33a, was taken for further characterization 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 NNX2-1 directly transactivates the host gene of miR-33a, SREBF2, to upregulate miR-33a which then holds HMGA2 expression in check (84). An intriguing implication with 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, implicating that NNX2-1 may influence cholesterol metabolism in the lung and that cholesterol metabolism may be mechanistically involved in the lung cancer biology of NNX2-1 (Fig 2B).

Conclusions

In the recent years, we have witnessed a rapid unfolding of lung cancer biology in connection with the fundamental lung developmental transcription factor gene, NNX2-1. The dual faces of NNX2-1 as both a pro- and an anti-cancer factor are complex but not unique in the NK2 family of transcription factors. The NNX2-2 oncogene is translocated in T-ALL (53) and is an essential signaling mediator of the driver oncogene EWS-FLI in Ewing’s sarcoma (91). On the other hand, NNX2-2 suppresses self-renewal of glioma-initiating cells and is frequently downregulated in human gliomas (92). Intriguingly, disparate outcome of NNX2-1 also extends to its correlation with clinical parameters of lung AD patients. Positive NNX2-1 expression is associated with good prognosis (41,93,94), but NNX2-1 amplification is linked with poor prognosis (40,41). The biochemical activity of NNX2-1 as a transcription factor precludes it for direct drug discovery. However, as more signaling partners of NNX2-1 are uncovered, there will be opportunities to initiate translational research to create and develop NNX2-1-dependent strategies as tools to manage NNX2-1-positive lung cancer.
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**FIGURE LEGENDS**

**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 in red were not known previously to be part of the core amplicons. *RALGAPA1* is also known as *GARNL1*.

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