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The Inescapable Influence of Noncoding RNAs in Cancer:

A meeting summary of the Keystone Symposium on Molecular and Cellular Biology, 'MicroRNAs and Noncoding RNAs in Cancer', Keystone, Colorado, USA, 7–12 July 2015

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Abstract

This report summarizes information presented at the 2015 Keystone Symposium on “*MicroRNAs and Noncoding RNAs in Cancer*”. Nearly two decades after the discovery of the first microRNA (miRNA), the role of noncoding RNAs in developmental processes and the mechanisms behind their dysregulation in cancer has been steadily elucidated. Excitingly, miRNAs have begun making their way into the clinic to combat disease such as hepatitis C, and various forms of cancer.

Therefore, at this Keystone meeting novel findings were presented that enhance our view on how small and long noncoding RNAs control developmental timing and oncogenic processes.

Recurring themes included, 1) how miRNAs can be differentially processed, degraded, and regulated by ribonucleoprotein (RNP) complexes, 2) how particular miRNA genetic networks that control developmental process, when disrupted, can result in cancer disease, 3) the technologies available to therapeutically deliver RNA to combat diseases such as cancer, and 4) the elucidation of the mechanism of actions for long noncoding RNAs, currently a poorly understood class of noncoding RNA. During the meeting there was an emphasis on presenting unpublished findings, and the breadth of topics covered reflected how inescapable the influence of noncoding RNAs are in development and cancer.

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In June 2015, a conference entitled “*MicroRNAs and Noncoding RNAs in Cancer*” sponsored by the Keystone Symposium on Molecular and Cellular Biology was held in Keystone, Colorado. The meeting was organized by Dr. Manel Esteller (Belvitge Biomedical Research Institute, Barcelona, Spain), Dr. Lin He (University of California, Berkeley, CA), and Dr. Frank J. Slack (Beth Israel Deaconess Medical Center, Boston, MA), all experts in the fields of noncoding RNAs (ncRNA) and cancer. Brian D. Adams (Yale University, New Haven, CT) was the conference assistant.

MiRNAs and other ncRNAs have recently emerged as key regulators of gene expression during development and are frequently aberrantly expressed in human disease states, in particular cancer. MiRNAs act to promote or repress cell proliferation, migration and apoptosis during development, all processes that go awry in cancer. Thus, miRNAs have the ability to behave like oncogenes or tumor suppressors. Their small size and molecular properties make miRNAs amenable as targets and therapeutics in cancer treatment. MiRNAs thus represent a paradigm shift in thinking about how gene regulation during development and disease can provide the oncologist with a potentially powerful new battery of agents to diagnose and treat cancer. Fast emerging as another class of equally important molecules, long non-coding RNAs (lncRNAs) are showing some of the same promise. However, while hundreds of human miRNAs and ncRNAs are known, we have a relatively poor understanding of their roles and targets, and there is still a limited literature on using these molecules in the clinic. Therefore, the objective of this conference was to focus on the exciting biology of miRNAs and ncRNAs in controlling developmental and cancer processes such as cell proliferation, differentiation, cell cycle, apoptosis and metastasis. To that end the meeting was divided into eight plenary sessions covering topics that highlighted the importance of small and long ncRNAs in development and cancer. Over 250 scientists from around the world participated in the meeting, and here we summarize some of the major themes that ran through the plenary sessions.

Dr. Joan A. Steitz (Yale University, New Haven, CT) gave the opening keynote address and presented elegant work regarding how viral ncRNAs use EBER2 to base pair with nascent RNA to guide an interacting transcription factor (PAX5) to its DNA target site, a previously undescribed function for a trans-acting ncRNA(1). Specifically, EBER2 is present at the terminal repeats (TRs) of the latent EBV genome, overlapping previously identified binding sites for the B cell transcription factor PAX5. EBER2 knockdown can phenocopy PAX5 depletion in upregulating the expression of genes nearest the TRs, LMP2A/B and LMP1. The EBER2-PAX5 interaction is evolutionarily conserved in the related primate Herpesvirus CeHV15 despite great sequence divergence. Dr. Steitz also discussed new unpublished work, in collaboration with Dan DiMaio’s lab (Yale University, New Haven, CT) that implicates the host integrator complex in the 3’-end processing of Herpesvirus saimiri miRNAs, which greatly expands on our knowledge of how miRNAs can be generated outside the canonical miRNA biogenesis pathway.

Dr. Carlo M. Croce (Ohio State University, Columbus, OH) gave the closing keynote addresses discussing the history of the miRNA field, and how this biological equivalent of dark matter have important roles in regulating cellular function and dysfunction, most notably in cancer. Dr. Carlo Croce's seminal discoveries are well known to the international scientific community. During his talk, he reviewed the most important ascertainments that shed light into the complex molecular mechanisms of carcinogenesis, ranging from the classical IG/MYC t(8;14) translocations in Burkitt lymphoma (BL)(2), t(14;18) translocations involving BCL2 in follicular lymphoma (FL)(3), identification of TCL1 in T-cell malignancies(4), to how miRNAs are deregulated in cancer. Dr. Croce further detailed the role of miR-15 and miR-16 in CLL pathogenesis, which are either deleted or downregulated in patients with B-cell CLL(5).

One of the most recent findings from Croce's group is the co-deletion of miR-3676 and TP53 in CLL(6). Since TCL1 is a miR-3676 target, the loss of that miRNA may cause high levels of TCL1 expression, resulting in CLL progression. Finally, Dr. Croce elaborated how miR-21 is significantly overexpressed in many cancers could be used as an important biologic marker. He pointed out that lung tumor-secreted miR-21 in exosomes can bind TLR8 and TLR7, leading to an activation of these receptors in immune cells, such as macrophages(7). These findings highlight how miRNAs can function as agonists of TLRs and regulate tumor cell interactions within the tumor microenvironment. One of the major themes highlighted during the plenary sessions was the role of *MicroRNAs in Development*. Dr. Amy E. Pasquinelli (University of California, San Diego, CA) discussed how, unexpectedly, the *let-7* primary transcript (pri-*let-7*), which is processed into the mature *let-7* miRNA, is a major target of Argonaute (ALG-1) in *C. elegans*. Association of ALG-1 with pri-*let-7* promotes processing and this interaction is mediated by mature *let-7* miRNA through a conserved complementary site in its own primary transcript, thus creating a positive-feedback loop(8). Additionally, she presented data regarding the use of cross-linking immunopurification coupled with high-throughput sequencing (CLIP-seq) to identify target sites bound by the miRNA complex during *C. elegans* development. This is important, since mature miRNAs use imperfect base pairing to recognize sequences in target mRNA transcripts, and identifying biologically relevant target sites is an outstanding challenge. This talk was followed by Dr. Helge Grosshans (Friedrich Miescher Institute, Basel Switzerland) who is investigating the basis of lethality upon inactivation of the *let-7* miRNA in *C. elegans*. Contrary to the notion that miRNAs act by moderately 'tuning' the expression of a large number of targets, thus providing gene expression robustness, Dr. Grosshans showed that this phenotype was fully explained by regulation of a single target. Specifically, genome editing demonstrated that regulation of LIN-41/TRIM71 alone is necessary and sufficient to explain key aspects of *let-7* function in development(9). Dr. Grosshans also revealed that LIN-41, a conserved animal stem cell factor, acts as a posttranscriptional gene regulator that silences a small number of direct targets. Dr. George Q. Daley (Boston Children's Hospital, Boston, MA) then summarized the role of LIN28, an RNA binding protein originally identified in a screen for heterochronic mutants in *C. elegans*, and how it regulates protein expression through effects on the *let-7* family of tumor suppressor miRNAs as well as through direct interactions with mRNA(10–12). He also reviewed how LIN28 has an

important role in a wide array of biological phenotypes including reprogramming to pluripotency, oncogenesis, sexual maturation, growth, and metabolism.

Additional sessions covered the role of *MicroRNAs in Cell Differentiation and Mammalian Physiology*. Dr. Silvia Monticelli (Institute for Research in Biomedicine, Bellinzona, Switzerland) discussed how miRNAs play an important role in affecting the ability of naïve CD4 T cells to differentiate into a number of different memory and effector subsets. Specifically, she showed that miR-181a was selectively induced in both human and mouse naïve T cells differentiating into the T_H17 subset(13). In T_H17 cells, miR-181a also regulated responses to cognate antigens through modulation of ERK phosphorylation. These results have important implications for both autoimmune diseases as well as some types of tumor-related responses. Next was Dr. Lin He, who discovered that loss of all miR-34a family members results in postnatal mortality due to defective mucociliary clearance(14). Deficiency of this entire miRNA family causes impaired motile ciliogenesis in respiratory epithelia, primarily due to aberrant basal body docking to the apical membrane of multiciliated cells. These findings clearly indicated the functional importance and functional robustness of homologous miRNAs in mammalian development. Her presented work also highlighted the role of miR-34 miRNAs in cell fate plasticity in pluripotent stem cells. Finally, two talks underscored how dysfunctional miRNA networks can contribute to inappropriate responses following pathological stress that can then promote several disease conditions such as diabetes. First, Dr. Eran Hornstein (Weizmann Institute of Science, Rehovot, Israel) presented data on how miRNAs, such as miR-375, can alter the quiescent/cycling states of pancreatic β cells(15,16). He also discussed how certain miRNA could control pancreatic β cells differentiation as well as transcription factors that regulate insulin production. Second, Dr. Markus Stoffel (ETH Zürich, Zürich, Switzerland) discussed how miR-7 is a negative regulator of glucose-stimulated insulin secretion in β cells by directly regulating genes that control late stages of insulin granule fusion with the plasma membrane(17). He also discussed how the miR-200 family is strongly induced in islets of diabetic mice and that β cell-specific overexpression of miR-200 in mice is sufficient to induce β cell apoptosis and lethal type 2 diabetes(18).

Another theme emerging in the miRNA field, that of *Regulation of microRNA Biogenesis*, was covered in two talks highlighting certain RNA binding proteins important in this process during differentiation and tumorigenesis. Dr. Richard Gregory (Harvard Medical School, Boston, MA) presented novel findings regarding how the primary pri-miR-17~92 transcript was dynamically regulated during embryonic stem cell (ESC) differentiation. Specifically, pri-miR-17~92 is processed to a biogenesis intermediate, termed 'progenitor-miRNA' (pro-miRNA), which is an efficient substrate for microprocessor and is required to selectively license production of pre-miR-17, -18a, -19a, 20a, and -19b from this cluster. The endonuclease CPSF3, and the spliceosome-associated ISY1, are responsible for pro-miRNA biogenesis and expression of all miRNAs within the cluster except miR-92. Thus, pro-miRNA processing is key step controlling miRNA expression and explains the posttranscriptional control of miR-17~92 expression in development. Dr. Elsa Flores (University of Texas MD Anderson Cancer Center, Houston, TX) then presented her lab's work on manipulating the p53 family members, p63 and p73, which interact with p53 extensively to suppress tumorigenesis(19). Specifically, her group found several small

molecules able to reduce p63 protein stability, and a specific set of miRNAs essential for the effectiveness of the drugs, both *in vitro* and *in vivo*.

This meeting also highlighted research concerning the role of *Long noncoding RNA in Development and Physiology*. Dr. Michael G. Rosenfield (University of California, San Diego, CA) discussed how histone methyltransferases and demethylases could regulate heat-shock proteins in the nucleus. Specifically, that a highly conserved arginine residue, R469, in HSP70, was monomethylated by co-activator-associated arginine methyltransferase 1 (CARM1) and demethylated by jumonji-domain-containing 6 (JMJD6)(20). Ultimately, this modification alters the ability of HSP70 to directly regulate retinoid acid-induced retinoid acid receptor $\beta 2$ (RAR $\beta 2$) gene transcription by controlling RAR $\beta 2$ binding to chromatin. These findings expand the repertoire of non-histone substrates targeted by PRMT4 and JMJD6, and reveal a new function of HSP70 in gene transcription at the chromatin level. Dr. Anton Wutz (ETH Zürich, Zürich, Switzerland) went to describe a genetic screening strategy aimed at the identification of silencing factors important in *Xist*-mediated X chromosome inactivation. Using haploid mouse embryonic stem (ES) cells carrying an inducible *Xist* allele and a gene trap retrovirus mutagen(21), his group was able to test in a genome-wide manner which genes contributed to the silencing mechanism of *Xist*. Given loss of *Xist* has been observed in cancer development, elucidating the mechanism of *Xist* function will contribute to the understanding of its role in tumorigenesis and development. Dr. Mitchel Guttman (California Institute of Technology, Pasadena, CA) presented findings addressing the long-standing question regarding how the lncRNA *Xist* silences genes on the X chromosome. Using his novel RNA antisense purification (RAP) methodology to map RNA-chromatin interactions(22), his group identified novel proteins required for *Xist*-mediated transcriptional silencing, such as SHARP, SAF-A, and LBR(23). SHARP, which interacts with the SMRT co-repressor and activates HDAC3, is also required for the exclusion of RNA polymerase II from the inactive X, as well as the *Xist*-mediated recruitment of the polycomb repressive complex 2 (PRC2) across the X chromosome. Dr. Irene Bozzoni (University La Sapienza, Rome, Italy) then discussed the characterization of a muscle-specific lncRNA, linc-MD1, which is strongly downregulated in dystrophic Duchenne myoblasts(24,25). Acting as a sponge RNA through its ability to bind miR-133 and miR-135, linc-MD1 impacts the activity of the corresponding mRNA targets, and controls crucial processes in muscle differentiation.

It is becoming increasingly clear that long noncoding RNAs can also impact tumorigenic processes. Therefore, several talks highlighted the role of *Noncoding RNAs in Cancer*. Dr. Joshua T. Mendell (University of Texas Southwestern Medical Center, Dallas, TX) presented work on a lncRNA termed NORAD which, when deleted in human cells, results in chromosomal instability and aneuploidy. Dr. John L. Rinn (Harvard University, Boston, MA) discussed the unique roles of certain ncRNAs, and how they may relate to tumorigenic processes. He also unveiled a new technology termed CRISPR-Display, which uses Cas9 to ectopically target functional RNAs and RNP complexes to genomic loci(26). Dr. Sohail F. Tavazoie (Rockefeller University, New York, NY) presented findings regarding how tRNA fragments can bind certain RNA binding proteins and promote metastatic processes. Dr. Kevin V. Morris (University of New South Wales, Sydney, Australia), discussed how the protein encoded by an antisense transcript *MYCNOS*, originating from the opposite strand at

the MYCN locus(27), can explain the phenotypes associated with amplification of MYCN. *MYCNOS* transcripts function as a modulator of the *MYCN* locus, affecting *MYCN* promoter usage and recruiting various proteins, including the RAS GTPase-activating protein-binding protein G3BP1, to the upstream *MYCN* promoter. David L. Spector (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) presented his lab's findings regarding the involvement of certain ncRNAs in breast cancer pathogenesis, and how some could be promising therapeutic targets. Finally, Dr. Pier Paolo Pandolfi (Beth Israel Deaconess Medical Center, Boston, MA) discussed the competitive endogenous RNA (ceRNA) code and its involvement in cancer(28–30). Furthermore, he discussed how circular RNAs(31,32) could modulate tumorigenic processes, and that they function as super ceRNAs(33), in part, through the sequestration of miRNAs. His talk illustrates how RNA in all forms (circular, linear, as a pseudogene, miRNAs, and mRNAs) talk to one another to control essential cell processes, that when disrupted could result in cancer.

Finally, the discussion of *MicroRNAs and Cancer* occurred over the course of three plenary sessions. Dr. Manel Esteller began by highlighting the importance of an aberrant epigenetic setting in the dysregulation of ncRNAs in human cancer. He presented several illustrative examples of how numerous types of ncRNAs such as miRNAs, snoRNAs, piRNAs and transcribed-ultra conserved regions (T-UCRs) underwent promoter hypermethylation-associated silencing in cancer cells. He also demonstrated that there was crosstalk between different types of epigenetic marks and ncRNAs due to DNA methylation of antisense RNAs able to regulate the expression of sense coding RNAs, and that histone methyltransferase are able to bind to ncRNAs to regulate expression of host genes. Next, Dr. George A. Calin (University of Texas MD Anderson Cancer Center, Houston, TX) presented his findings that cellular and Kaposi's associated Herpesvirus (KSHV) miRNAs are differentially expressed in the plasma of sepsis and early postsurgical patients. In particular, virally encoded miR-K-10b and miR-K-12-12* expression was increased immediately following surgery, suggesting that the levels of the KSHV's miRNAs in plasma can be used as biomarkers of sepsis(34). He also discussed how a novel lncRNA, CCTA2, transcribed from the 8q24, promoted metastasis and chromosomal instability in colon cancer(35). Therefore, *CCTA2* has the potential to become a useful diagnostic and/or prognostic marker for colon cancer. Dr. Andrea Ventura (Memorial Sloan Kettering Cancer Center, New York, NY) presented an update of his labs work regarding building better mouse models of human cancer. Specifically, how CRISPR/Cas9 could be used to generate chromosomal rearrangements in mice, resulting in an Eml4-Alk-driven lung cancer mouse model(36). The resulting tumors express the Eml4-Alk fusion gene, display histopathologic and molecular features typical of ALK(+) human NSCLCs, and respond to ALK inhibitor treatment. He also presented data regarding characterization of genetically engineered mice harboring selective targeted deletions of individual components of the miR-17~92 cluster(37). Dr. Xiao-Fan Wang (Duke University Medical Center, Durham, NC) then discussed how in glioblastoma, hypoxia-induced miRNAs are critical for the response of glioma initiating cells to hypoxic stress. Interestingly, the biogenesis of several hypoxia-induced miRNAs was also regulated post-transcriptionally by hypoxia inducible factors (HIFs). Taken together, these findings reveal a direct role of HIF in regulating miRNA biogenesis, which is important for glioblastoma progression in the hypoxic tumor microenvironment.

Given much is known regarding the role of miRNAs in cancer, a large effort has been undertaken to use certain miRNAs as biomarkers, or to develop mimic and antisense therapeutic strategies to directly target the miRNA. Dr. Frank J. Slack presented his labs findings regarding the delivery of anti-miR-155 to a mouse model of diffuse B-cell lymphoma, using a novel technology termed pHLIP(38), which targets areas of the tumor where the pH is low. This is an exciting advancement given delivery of synthetic RNA can now be modified to circumvent bio-distribution to and subsequent clearance by the liver. Dr. Anita Seto (miRagen Therapeutics, Boulder, CO) described miRagen's strategy to leverage rare hematological cancers for a rapid path to first-inhuman studies for miRNA inhibitors. She described the pharmacological activity of a highly optimized miR-155 inhibitor, MRG-106, in cutaneous T-cell lymphoma (CTCL) cell lines. Since, MRG-106 resulted in reduction in cell proliferation, and activation of the programmed cell death pathway, miRagen will investigate the safety, tolerability and pharmacokinetics of MRG-106 in a phase 1 trial in CTCL patients. Dr. Eva M. Hernando (New York University Cancer Center, New York, NY) presented work identifying certain miRNAs often found to be lost in aggressive primary melanomas. Additionally, she has defined a miRNA expression-based signature from primary melanoma tissue that, in combination with existing histopathology-based prognostic measures, accurately predicted the development of brain metastasis(39–41). Dr. Aurora Esquela-Kerscher (Eastern Virginia Medical School, Norfolk, VA) also discussed that miR-888 was the most differentially expressed miRNA observed in human metastatic prostate cancer cells relative to non-invasive PC3-N cells(42). Further, using a novel miRNA-based biomarker source termed expressed prostatic secretions in urine (EPS urine) it was found that miR-888 levels were preferentially elevated in prostate cancer patients with high-grade disease. Therefore, this miRNA holds promise as a diagnostic tool using an innovative prostatic fluid source as well as a therapeutic target for aggressive prostate cancer.

The goal of the 2015 Keystone Symposia meeting on *MicroRNAs and Noncoding RNAs in Cancer* was to bring together investigators from many fields, RNA, bioinformatics, genomics, medicine, chemistry, bioengineering and cancer biology in order to facilitate new ideas regarding the means by which ncRNAs impact tumor initiation and progression and ways to harness them for medicinal uses. The work presented at this meeting underscores the notion that as more investigators study the mechanisms by which noncoding RNAs function in processes such as development and cancer, more questions arise. For instance, the concept that many pseudogenes, circular RNAs, and other noncoding RNAs can serve as sponges for miRNAs implies that there is a complex network of RNA communication within cells, which is tightly regulated for proper cell function. Additionally, the finding that new splicing factors and traditional cell cycle regulators such as p63 can control miRNA biogenesis and processing will inevitably result in a re-thinking of the canonical miRNA biogenesis model. The identification of new functionally relevant long noncoding RNAs such as NORAD, MYCNOS, and others, means that the thousands of uncharacterized long noncoding RNAs, while expressed at fairly low levels, can have significant biological consequences when dysregulated. Finally, given our knowledge regarding some of these noncoding RNAs such as miR-155, there will be a continued and rapid effort to develop these agents into diagnostic and therapeutic tools, which hopefully will result in

improvements in clinical practice. It will be exciting to see, as the mysteries of noncoding RNA biology are uncovered, how this will shape our current understanding of essential biological processes and impact cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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