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Chapter 4

ROLE OF *LET-7* NETWORKS IN STEM CELL MAINTENANCE AND DIFFERENTIATION

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1. ABSTRACT

Let-7 was identified in a genetic screen in *C. elegans*. *Let-7* regulates a number of fundamental activities in the cells such as cell division, proliferation and differentiation. *Let-7* is involved in a regulatory feed back loop with LIN28, which is one of the factors required for pluripotency of cells suggesting role of *Let-7* in stem cell differentiation and maintenance. In addition to the *Let-7*/LIN28 feedback loop which is important in cancer stem cell, other *Let-7* targets such as RAS and HMGA2 are essential for self renewal of cancer stem cells and maintenance of the undifferentiated state. *Let-7* promotes differentiation by suppressing self renewal pathways in the cells. The ability of *Let-7* members to regulate multiple pathways plays an important in fine tuning of cellular processes of self-renewal and differentiation.

2. INTRODUCTION TO *LET-7* FAMILIES AND NETWORKS

1) *Let-7* Family Is the First Identified Mirna Family

Let-7 was initially identified as a heterochronic gene by forward genetics in *Caenorhabditis elegans* (Reinhart et al., 2000), after lin-4. Although it was the second to be discovered, its high functional conservation from worms to humans has provided the guidelines for the discovery of many other miRNAs and also has laid the foundations of miRNA based regulation of gene expression (Pasquinelli et al., 2000). The *Let-7* family

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comprises of other miRNAs that share similar seed sequences as the *Let-7*. In *C. elgans* the *Let-7* family comprises of 9 members (*Let-7*, miR-48, miR-84, miR-241, miR-265, miR-793, miR-794, miR-795 and miR-1821). In humans, the *Let-7* family comprises of 13 members (*Let-7*a-1, *Let-7*a-2, *Let-7*a-3, *Let-7*b, *Let-7*c, *Let-7*d, *Let-7*e, *Let-7*f-1, *Let-7*f-2, *Let-7*g, *Let-7*i, miR-98 and miR-202) located on nine different chromosomes.

In *C. elegans Let-7* is known to regulate the larval to adult transition. The hypodermal skin cells known as the seam cells undergo asymmetrical divisions at each larval stage. The seam cells divide in a stem cell like manner, with one daughter cell self-renewing and the other daughter cell differentiating. At the larval 4 to the adult transition, these self-renewing daughter cells stop dividing, terminally differentiate, and secrete alae. In case of loss-of-function *Let-7* mutants, these seam cells continue to divide, fail to exit the cell cycle and fail to differentiate, causing no alae formation. This leads to bursting of the vulva leading to an ultimate death of the worm. Therefore the *Let-7* gene derives its name (lethal-7) from the lethality of its absence or mutation. It has a major role in larval 4 to the adult stage transition, and is detected at the larval 3 stage reaching its maximum level at the larval 4 stage. Expression of the *Let-7* at the larval stage 2, could cause skipping of the cell-cycle leading to differentiation and premature adult development at the larval 4 stage (Abbott et al., 2005).

The other members of the *Let-7* family such as miR-48, miR-84 and miR-241 also participate in regulation of the temporal patterning at the larval 2 to larval 3 stage transition, and serve as heterechronic genes (Caygill et al., 2008). This was demonstrated by the single, double and triple mutants for these miRNAs. The single mutants displayed weak defects of extra moulting at the adult stage, which was enhanced in miR-48/miR-84, miR-48/miR-241 double mutants. The double mutants showed incomplete alae formation and lethality. These defects occurred with higher penetrance in miR-48/miR-84/miR-241 triple mutants, clearly indicating functional cooperation among them (Abbott et al., 2005). The targets of these miRNAs are additional members of the heterochronic pathway, some of which are conserved in other organisms. The Ras GTPase-family member let-60 is one such example, which is also a target for the human *Let-7*.

In *Drosophila*, the *Let-7* family has just one member, which is the *Let-7* itself. The mature sequence of the *Let-7* is 100% identical to that of the *C. elegans.* Here too the *Let-7* functions as a heterochronic gene (Caygill et al., 2008; Sikol et al., 2008), being expressed at the end of the third larval stage and peaking at the pupal stage (Pasquinelli et al., 2000). The expression of *Let-7* coincides with the release of ecdysone and is responsible for the regulation of the neuromuscular junctions in the abdominal muscles. The *Let-7* mutants display defects in maturation of the neuromuscular junctions in the abdominal muscles and exhibit juvenile features in their neuromusculature, affecting their motility, flight and fertility (Sokol et al., 2008). The *Let-7* exerts this function by down-regulation of the abrupt gene.

In *Homo sapiens*, the *Let-7* family comprises of 10 mature miRNAs produced from 13 precursor sequences. The *Let-7* family is present in multiple locations in the genome and also has several isoforms. To distinguish between the isoforms, a letter is placed after the *Let-7* to indicate a *Let-7* with a slightly different sequence, and a number at the end denotes the same sequence present at different genomic locations. The mature *Let-7*a is produced from three separate precursors, namely: *Let-7*a-1, *Let-7*a-2, *Let-7*a-3 and the mature *Let-7*f is produced from two precursors at two different genomic locations, namely *Let-7*f-1, *Let-7*f-2 (Roush and Slack, 2008).

The level of *Let-7* rises during embryogenesis (Schulman et al., 2005). Moreover, reduced expression of *Let-7* family has been associated with cancers (Park et al., 2007) leading to a conclusion that like in *C. elegans*, the *Let-7* family is involved in promoting differentiation and function as mainly tumor suppressors in humans. *Let-7* expression is undetectable in human and mouse embryonic stem cells and its level rises upon differentiation (Thomson et al., 2006).

2) *Let-7* Members Act in Concert as a miRNA Network

The *Let-7* family in humans comprising of 13 members appear to be acting together as a network, as in many cancer types the expression of various *Let-7* genes is down-regulated. The first direct piece of evidence for a role of *Let-7* in cancer was the observation that most or all of the *Let-7* family members were reduced in a significant number of lung cancer cell lines and primary human lung cancer tissues (Takamizawa et al., 2004). It was shown for the first time that *Let-7* expression is frequently reduced in lung cancers and that alterations in their expression could have a prognostic impact on the survival of surgically treated lung cancer patients.

Using miRNA microarrays, several miRNAs aberrantly expressed in human ovarian cancer tissues and cell lines were studied, among which several members of the *Let-7* family were found regulated (Dahiya et al., 2008). It was shown that the absence of *Let-7* family could be used to determine tumor origin and proliferation state because tumor suppressor miRNAs were significantly down-regulated in primary effusion lymphoma (PEL) and in Kaposi sarcoma (KS), an endothelial cell tumor.

The production of the *Let-7* family of miRNAs is known to be negatively regulated by LIN28. The embryonic cells contain LIN28 that acts as a Drosha inhibitor, preventing the transcription of the *Let-7* family members. It was observed by a study carried out in the mouse embryonal carcinoma cell line P19 that in-spite of the levels of the primary transcripts being high, the levels of the processed precursor and mature forms were low. This was because of the binding of a Drosha inhibitor to a conserved region in the loop causing inhibition of the *Let-7* processing. Differentiation of P19 with retinoic acid leads to an induction in *Let-7* caused by decay in LIN28. The similar happens during the embryonic development, when Drosha freed of LIN28 inhibition processes *Let-7* efficiently leading to an increase in *Let-7* mature species (Newman and Thomson, 2008)

Therefore, it is clear that restoration of *Let-7* processing block is essential for reprogramming stem cells, whereas a combination of LIN28 with NANOG, SOX2 and OCT3/4 can convert a human somatic cell to a pluripotent stem cell. The major regulatory controls in *Let-7* production are at the Drosha step involving LIN28, at the Dicer processing step and even at the primary transcript processing step (Wulczyn et al., 2007; Chang et al., 2008).

Several important oncogenes have been identified as the targets of *Let-7*, including RAS, MYC and HMGA2 (Chang et al., 2008; Johnson et al., 2005; Mayr et al., 2007). These target genes, making use of the RAS–MEK pathway contribute to Epithelial to Mesenchymal transition (EMT), though *Let-7* miRNA-mediated HMGA2 down-regulation had no effect on the prevention of the transformed phenotype in pancreatic cancer cells (Watanabe et al., 2008). Another *Let-7* miRNA-mediated tumor metastasis-regulating pathway was addressed

recently. Raf kinase inhibitory protein, which inhibits mitogen-activated protein kinase signaling cascades, can decrease transcription of LIN28 by MYC. Suppression of LIN28 enabled it to enhance *Let-7* processing in breast cancer cells, allowing the elevated *Let-7* expression to inhibit HMGA2 that activated pro-invasive and pro-metastatic genes. As *Let-7* targets RAS, the upstream activator of RAF1, a positive feedback loop emerged to control tumor invasion and metastasis (Dangi-Garimella et al., 2009). However, there are also cases, where only some specific members of the family members are down-regulated in cancers. Although a frequent down-regulation of the *Let-7* family members is observed, there are few studies showing upregulation of some *Let-7* family members in cancer (Lawrie et al., 2009).

Therefore, in-spite of the members having a vast overlapping set of targets its not very clear, as to whether all the members of the *Let-7* family have the same function or whether they have different functions. Moreover, their regulation is not just tissue specific but also cell specific. Members of the *Let-7* family can have differential regulation within the same cell (Guled et al., 2009). This may enable *Let-7* to have differential functions in stem cells and differentiated cells. It is unclear how this differential regulation mechanism works but the different isoforms of *Let-7* primary miRNAs, the coding region and the timing of expression of these miRNAs could have a role in this regulation.

3. CELLULAR FUNCTIONS REGULATED BY *LET-7* MIRNA NETWORKS

1) Role of *Let-7* in Cancer as a Tumor Suppressor

Let-7 is known to be targeting several oncogenes. The main target of *Let-7* is RAS oncogene that is found to be highly deregulated in human cancers. The 3'UTR of RAS contains several *Let-7* complementary sites (LCS) and it was found to have an inverse correlation to the *Let-7* family. In lung cancers the *Let-7* family members were found to be low as compared to RAS, which was found to be up-regulated. The role of *Let-7* in inhibiting RAS was also proved in non small cell lung cancer (NSCLC), where it was shown in a mouse model that *Let-7*g inhibited tumor growth by suppression of RAS (Kumar et al., 2008). The second major target is HMGA2 which is a chromatin-associated non-histone and a high mobility group protein having an oncogenic role in a variety of tumors including mesenchymal tumors and lung cancers. It is widely expressed in undifferentiated embryonal tissues but undetectable in adult normal tissues. It is also expressed in both benign and malignant tumors.

Ectopic expression of *Let-7* reduced HMGA2 levels and cell proliferation in lung cancer. The effect of *Let-7* on HMGA2 is dependent on multiple target sites present in its 3'UTR. It was also shown that the separation of the 3'UTR from the ORF by chromosomal translocations released the oncogene from repression. It was revealed by the study that there exists a reciprocal relation between the *Let-7* growth suppressor and the HMGA2 oncogene. In differentiated tissue there occurs an induction in *Let-7* with corresponding absence of HMGA2, similarly in lung cancers HMGA2 over expression occurs owing to reduction in *Let-7* expression or loss of the *Let-7* target sites (Mayr et al., 2007; Hebert et al., 2007; Wang et al., 2007). Around twelve oncogenes were identified to be *Let-7* regulated (Boyerinas et al.,

2010). These genes were picked out by overlap between computational prediction and experimental validation of being down-regulated when *Let-7* was up-regulated.

2) *Let-7* as a Master Regulator of Cell Proliferation

The above mentioned studies suggest that *Let-7* may control a variety of processes both during the development and maintenance of adult tissue homeostasis. In order to provide evidence for the crucial role of *Let-7* on cellular growth and proliferation in mammalian cells, the levels of *Let-7* were manipulated by using exogenously transfected pre-*Let-7* RNAs (to overexpress *Let-7*) and anti-*Let-7* oligonucleotides (to reduce the *Let-7* activity). A human lung cancer cell line (A549) and a human liver cancer cell line (HepG2) were first transfected by the synthetic miRNAs and monitored. A significant amount of proliferating cells were reduced in both the cell lines. In contrast to this, transfection of these cell lines with the antisense oligonucleotides against *Let-7* brought about a two-fold increase in proliferation. Here again, the inverse correlation between *Let-7* levels and cellular proliferation justifies the function of *Let-7* as regulator of cell division and cell survival (Johnson et al., 2007).

3) Gene Ontology (GO) Classes and Functions of the Targets of *Let-7*

In order to determine the cellular pathways regulated by *Let-7*, a microarray analysis of the A549 and HepG2 cell lines treated with *Let-7* miRNAs was carried out (Johnson et al., 2007). The differentially expressed genes were identified and were grouped by their associated biological functions using the Gene Ontology database. The primary GO classes associated with the differentially expressed genes were found to be linked to cell cycle. The GO categories were DNA replication, M phase of mitotic cell-cycle, mitotic cell cycle, cell cycle checkpoint, cell division, DNA replication initiation, mitotic check point and spindle organization and biogenesis.

It was thus concluded that the over-expression of *Let-7* causes human cancer cells to decrease cell cycle progression. *Let-7* is known to control several cell proliferation genes, which strongly suggests that *Let-7* is a key regulator of cell cycle progression. It was shown that *Let-7* directly regulates a few cell cycle proto-oncogenes like: RAS, CDC25A, CDK6 and cyclin D thus controlling cell proliferation by reducing the flux through the pathways promoting the G1 to S transition. Many of these genes are oncogenes and in cancer cells with poor levels of *Let-7*, these genes may get up-regulated which is likely to stimulate cell cycle, DNA synthesis and hence cell division.

4) Regulation of Dicer by *Let-7* through a Negative Feed Back Loop

Among the several genes known to be targets of *Let-7* family and that show an inverse correlation with its expression is the Dicer. A luciferase assay using a reporter in the 3'UTR of Dicer revealed that *Let-7* directly affects Dicer. Over expression of *Let-7* reduces the expression of Dicer, which eventually causes the reduction in the levels of other mature miRNAs along with own mature miRNA expression. Thus its involvement in a negative feed

back loop helps in maintaining the equilibrium state of Dicer and various miRNAs (Forman et al., 2008; Jakymiw et al., 2010; Tokumaru et al., 2008).

4. ROLE OF *LET-7* IN MAINTENANCE OF STEMNESS

As seen in the earlier sections, *Let-7* regulates a number of genes involved in cell division and proliferation. We now turn to another very important aspect of *Let-7* regulation in stem cells and its impact on stem cell differentiation. Stem cell research is a cutting edge research that has given an impetus to regenerative medicine and several disease treatments, and promises to revolutionize 21st century medicine. It is therefore, crucial to understand the biology of stem cells, the network of factors required to induce and maintain stemness as well as the mechanisms that govern differentiation into specific tissues.

Stem cells express a set of factors that are responsible of their "stemness". They have a unique transcriptional profile that has to be maintained, and which on differentiation shifts to an alternate profile. Both transcriptional regulation and epigenetic regulation play pivotal roles in maintaining the existing profile as well its plasticity and were identified by comparisons of the expression profiles of the embryonic stem cells and their differentiated derivatives (Ramalho-Santos et al., 2002).

1) Regulation by Transcription Factors

The transcription factors OCT4, NANOG, SOX2, were identified by chromatin immunoprecipitation (CHIP) coupled with microarrays and were found to regulate several genes involved in differentiation and development and maintain them at low levels in the ES cells. These three transcription factors have common set of targets and also regulate transcription of each other. In addition to OCT4, SOX2 and NANOG, many other factors required for pluripotency have been identified, including LIN28, SAL14, DAX1, ESSRB, TBX3, TCL1, RIF1, NAC1 and ZFP281. These pluripotency factors regulate each other form a complicated transcriptional regulatory network in embryonic stem (ES) cells (Wang et al., 2006).

2) Regulation by Epigenetic Factors

Besides, transcription factors, a number of epigenetic factors are also essential for maintaining the pluripotency of the ES and the somatic stem cells. As the substrate of transcription, chromatin is subjected to various forms of epigenetic regulation like chromatin remodeling, histone modifications, histone variants and DNA methylation. For example, trimethylation of lysine 9 and lysine 27 of histone 3 (H3K9 and H3K27) correlate within active regions of chromatin, whereas H3K4 trimethylation and acetylation of H3 and H4 are associated with active transcription (Jenuwein and Allis, 2001), and DNA methylation generally represses gene expression (Santos and Dean, 2004). For maintaining pluripotency, those genes whose up-regulation leads to differentiation should be inactive. Poly-comb group

proteins (PcG) are functional in silencing these developmental regulators. The pluripotency factors such as OCT4, NANOG and SOX2 form a significant fraction of PcG. The genes regulated by the PcG proteins are co-occupied by nucleosomes with trimethylated H3K27. These genes are downregulated in the ES and are activated when differentiation is induced.

3) Crosstalk between Transcriptional and Epigenetic Regulation

Both the transcriptional and epigenetic pathways cross-talk with one another to maintain pluripotency. Pluripotency factors regulate genes encoding epigenetic control factors. It has been shown that OCT4, SOX2 and NANOG co-regulate certain genes encoding components of chromatin remodeling and histone modifying complexes, such as SMARCAD1, MYS3 and SET (Boyer et al., 2005). Moreover, pluripotency factors also interact with histone modifying enzymes and chromatin remodeling complexes (Wang et al., 2006). Finally, the genes of pluripotency factors are themselves subjected to epigenetic regulation (Loh et al., 2007).

Thus the interplay of the two help in activating the genes required for pluripotency and suppresses those genes that bring about development and differentiation (Chen and Daley, 2008). But in-spite of the genes controlling differentiation being transcriptionally inactive, they are maintained in a potent state for transcriptional activation. Also, the hyperdynamic chromatin structure in ES cells contributes to a rapidly plastic transcriptional profile that underlines the multi-lineage differentiation potential of ES cells. Upon differentiation, a diminution of pluripotency factors occurs that leads to dramatic changes in the transcriptional profile, where just the reverse begins to occur; the genes controlling differentiation get active, the pluripotency causing genes become suppressed and the chromatin structure gets compact. Post transcriptional regulation of factors in both transcriptional and epigenetic regulatory networks adds another layer of regulation towards this process. Such complex layers of regulation are necessary to achieve the precise temporal and spatial gene expression necessary for the stem cells to differentiate. MiRNAs are important post transcriptional regulators and could play a role in regulating the transcriptional and epigenetic networks in stem cells.

4) Evidence of the Role of *Let-7* in Regulation of Pluripotency

Additionally, microRNAs have emerged as key regulators of pluripotency (Gunaratne, 2009; Mallanna et al., 2010). Yu et al., (2007) demonstrated that self renewal and maintenance of the undifferentiated state required reduced *Let-7* miRNA levels. They showed this in cancer stem cells isolated from breast tumor xenografts. This requirement appears to be mediated through HMGA2 and RAS proteins. RAS targeting by *Let-7* miRNAs appeared to be essential for self renewal of cancer stem cells whereas HMGA2 regulation seemed to be important for maintenance of the undifferentiated state. This mechanism of HMGA2 mediated regulation of stemness maintenance in cancer stem cells appears to be similar to *Let-7*/HMGA2 mechanism in embryonic stem cells. Although Yu et al. (2010) have shown that antisense targeting of *Let-7* in cancer stem cells triggered de-differentiation of a breast tumor cell line, this study provides no direct evidence that lack of *Let-7* miRNAs is essential for maintenance of stemness. However the data from this study provide very strong circumstantial evidence in favor of this hypothesis.

More recently Melton et al (2010) demonstrated that *Let-7* overexpression led to differentiation of embryonic stem cells in the absence of DGCR8 (a gene essential for miRNA maturation). However this effect was not seen in the wild type embryonic stem cells with normal levels of DGCR8. The introduction of the ES cell cycle regulating (ESCC) miRNAs into DGCR8 -/- cells rescued the cell cycle defects and promoted the self-renewal tendency. On the other hand, the introduction of the *Let-7* miRNAs suppressed self renewal in these cells. In wild type cells, however the ESCC miRNAs inhibit the capacity of *Let-7* to silence self-renewal.

These findings suggested that the "functional antagonism" between *Let-7* and ESCC miRNA families causes them to have opposing effects on ESC self-renewal. In order to find out the mechanism for the opposing roles; a pathway analysis on the miRNA regulated transcripts was carried out. Overlaps between the miRNA regulated genes and CHIP detected pluripotency transcription factors. Around 88 transcription factors down-regulated by *Let-7* were found to be up-regulated by ESCC miRNAs. These transcription factors included pluripotency genes such as LIN28, SALl4, n-MYC and c-MYC. qRT-PCR, western analysis and reporter assays also confirmed the opposing effects of *Let-7* and ESCC on the levels of LIN28, SALl4, n-MYC and c-MYC.

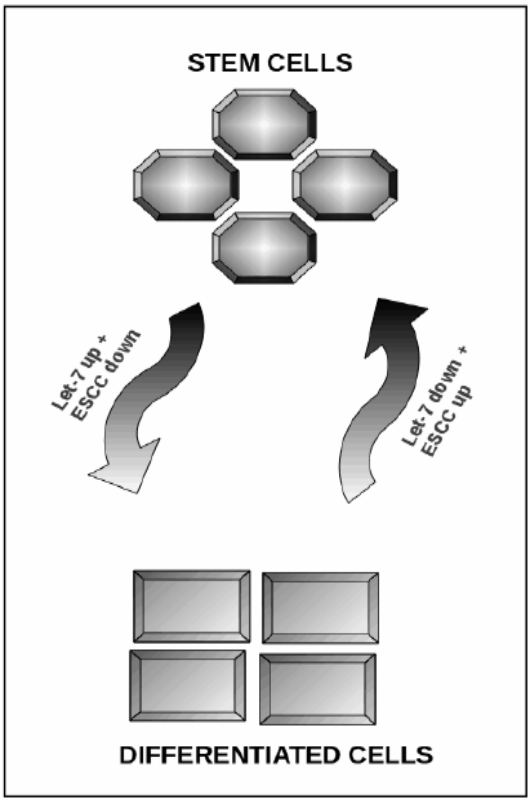

Figure 1. *Let-7* levels determine stemness and differentiation status of embryonic stem cells. *Let-7* and ESCC miRNAs act in an antagonistic manner to trigger differentiation or maintain stemness of ES cells.

Let-7 promotes differentiation by suppressing the self-renewal pathway, whereas the ESCC miRNAs promote the self-renewal pathway. Inhibition of *Let-7* promotes de-differentiation and can reprogram the somatic cells to induced pluripotent cells (iPS cells) (Figure 1). This is a good example of two different miRNA networks regulating the same gene network in an antagonistic manner.

Both of them act in self-reinforcing loops to maintain the self renewing versus the differentiated states. In the self-renewing state, the ESCC miRNAs increase the expression of LIN28 and c-MYC. LIN28 blocks *Let-7* expression and lowers it levels. The upregulated c-MYC along with the transcription factors OCT4, SOX2, NANOG activate the ESCC miRNAs further and promote the undifferentiated state. As the ES cells differentiate, OCT4, SOX2, NANOG are downregulated, resulting in loss of the ESCC miRNAs and LIN28. With the loss of LIN28, levels of *Let-7* rise rapidly. The increase in *Let-7* levels brings about down-regulation of its own inhibitor LIN28 as well as of MYC. Furthermore *Let-7* inhibits the downstream targets of OCT4, NANOG, SOX2, TCF3 to maintain the differentiated state.

5. ROLE OF *LET-7* IN ADULT STEM CELLS

Evidence for the role of *Let-7* family in maintenance of differentiation state also comes from the role of this miRNA family in adult stem/progenitor cells. *Let-7* family miRNAs seem to influence differentiation of adult stem cells through many RNA binding proteins. Kawahara et al. (2011) showed that Musashi1 in co-operation with LIN28 blocks the biogenesis of the entire *Let-7* miRNA family in Neuronal Stem/Progenitor cells. This regulation of *Let-7* biogenesis was found to be an important event in the differentiation of neuronal precursors from embryonic stem cells. Further this suppression of *Let-7* miRNA levels may also contribute to maintain the undifferentiated state of the neuronal stem/progenitor cells.

The LIN28/*Let-7* feedback loop has also shown to be important in cancer stem cells expression of high levels of Aldehyde Dehydrogenase (ALDH) (an enzyme that has been used as a functional stem cell marker in the hematopoietic system) (Yang et al., 2010). In this study inhibition of *Let-7* expression by LIN28 was found to be a pre-requisite for maintenance of stemness in ALDH+ cancer stem cells. Overexpression of *Let-7* led to decreased numbers of ALDH+ cancer stem cells. However it was unclear from this study if the cancer stem cells differentiated to a more mature phenotype. Since differentiation is one of the key end points of current cancer therapy, the *Let-7*/LIN28 mechanism could be used as a target for exhausting cancer stem cells within a tumor. This study also demonstrates that the same mechanism is active in differentiation of normal mammary gland progenitor cells.

Suppression of *Let-7* activity also leads to proliferation of progenitors and stem cells in the hematopoietic system. Recently Ikeda et al. (2011) have shown that deletion of HMGA2 binding site of *Let-7* miRNAs leads to increase in erythroid and myeloid progenitors as well as KSL stem cells. Absence of *Let-7* miRNA binding site also led to erythroid progenitors which were growth factor independent compared to wild type progenitors that needed erythropoietin for their survival. Thus absence of *Let-7* seems to favor a proliferative phase in these cells.

Our group has shown that unlike most other stem/progenitor cells, *Let-7* family members are highly expressed in Mesenchymal Stromal Cells (MSCs) (Koh et al., 2010). It is unclear why *Let-7* is highly expressed in MSCs when it is suppressed in all other stem cell types. This might reflect the more differentiated nature of MSCs (more progenitor and not a stem cell phenotype) or it is possible that *Let-7* acts via different mechanism to promote MSC self renewal.

The *Let-7* family miRNAs do not seem to play a role in osteochondrogenic or adipogenic differentiation of MSC. However, our study shows the ability of the *Let-7* miRNA network to regulate a gene network possibly involved in hepatic differentiation of MSC. The transcription factor HNF4A is at the hub of this network (Figure 2). Interestingly HNF4A is not predicted to be directly regulated by any miRNA, but the ability of the *Let-7* miRNA network to indirectly regulate the expression of HNF4A further supports the idea that *Let-7* miRNA network can act in concert to regulate cellular differentiation.

It is interesting to note that most genes in this network are suppressed when *Let-7* family miRNAs are over-expressed (MSC) and up-regulated when *Let-7* family gene expression goes down (HEPG2 cells).

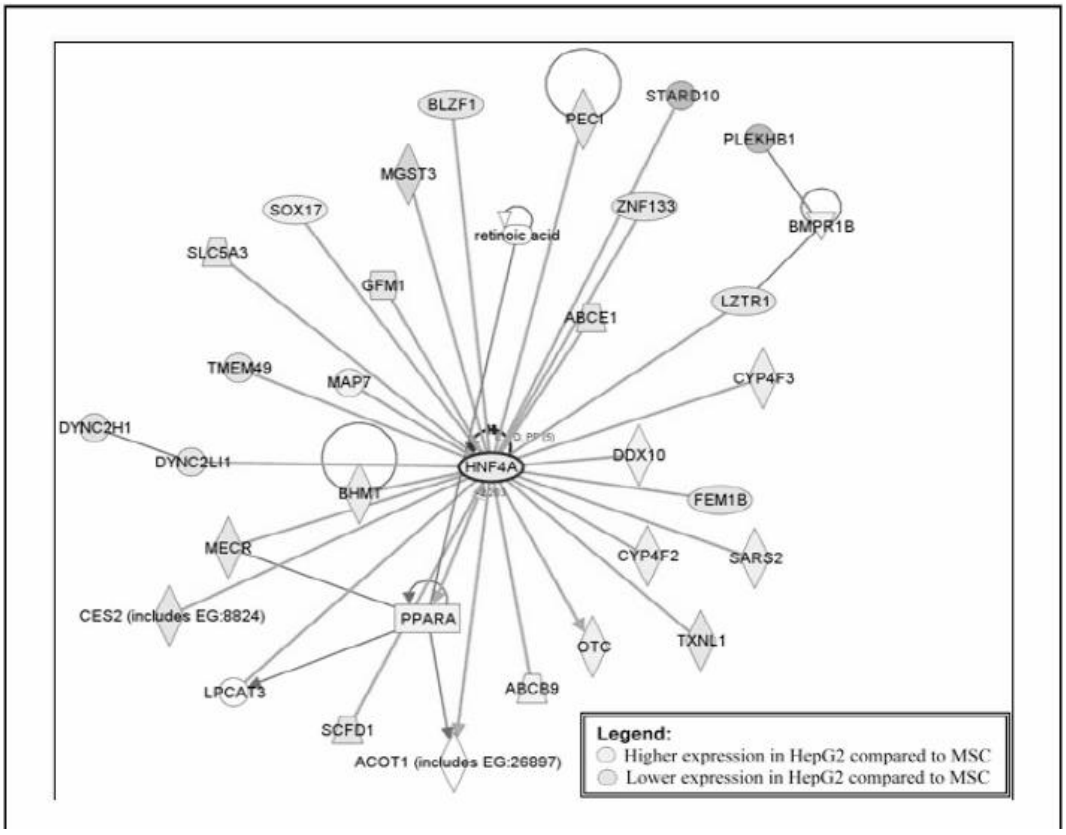

Figure 2. *Let-7* family miRNAs regulate a gene network with HNF4A as its hub.

CONCLUSION

Thus there is increasing evidence that the *Let-7* family of miRNAs regulate multiple gene networks critical in maintenance of the stem cell phenotype as well as differentiation of stem cells to desired lineages. It is unlikely that *in vivo* miRNAs act as single entities. Increasingly gene networks and pathways have been shown to be regulated by miRNAs belonging to a single family. The *Let-7* family members regulate critical genes involved in cellular proliferation and differentiation. This unique ability to regulate multiple gene networks simultaneously gives the *Let-7* family miRNAs the ability to regulate the cellular processes of self renewal and differentiation. It will be interesting to study whether *Let-7* family members use similar mechanisms for such regulation especially in different adult stem/progenitor cells and embryonic stem cells. Also *Let-7* family members might interact with other miRNA networks to achieve this regulation of stem cell maintenance. Understanding how these networks interact will provide new insights into miRNA regulation of stem cell differentiation.

It will be interesting to explore the role of *Let-7* miRNAs in self renewal and differentiation of stem/progenitor cells from different tissues. Although in embryonic stem cells levels of *Let-7* correlate inversely with the differentiation status of the cell, our data from MSC shows this may not be the case in other stem cells; especially adult stem cells. The role of *Let-7* family miRNAs in maintenance of stem cells from intestines, skin, muscle and other tissues remains to be explored. Exploring the role of *Let-7* regulation in these cells may lead to novel mechanisms of *Let-7* mediated regulation that are different from the HMGA2 or LIN28 mediated mechanisms.

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