Let-7 microRNAs and opioid tolerance

Ying He and Zaijie Jim Wang*

Department of Biopharmaceutical Sciences, Cancer Center, University of Illinois, Chicago, IL, USA

INTRODUCTION

Since first isolated from opium poppy in the early nineteenth century, morphine and related opioids remain as the most powerful analgesics to treat many forms of acute and chronic pain. However, repeated and prolonged use of opioids leads to tolerance, of which, the analgesic tolerance is the most problematic that can lead to therapeutic failure (i.e., inadequate pain control; McQuay, 1999). In some cases, even the highest tolerable dose of an opioid cannot achieve the desirable analgesic effect in patients (Wang and Wang, 2006; Harden, 2008). Development of opioid tolerance not only limits the analgesic efficacy, but the increased doses of opioids in order to counter tolerance exacerbate another problem, namely opioid addiction that is a significant medical and public health problem. Extensive efforts have been made to elucidate the mechanisms underlying opioid tolerance and drug addiction (Kieffer and Evans, 2002). Increasing evidence implicates the contribution of transcriptional and epigenetic regulation in opioid tolerance and drug addiction, such as activation and inhibition of transcription factors (Carlezon et al., 2005; Zachariou et al., 2006), modification of chromatin and DNA structure (Renthal et al., 2008; Guo et al., 2011), and induction of non-coding RNAs including microRNAs (Pietrzynkowski, 2011; Robison and Nestler, 2011).

MicroRNAs (miRs) are small non-coding RNA molecules that repress target gene expression through base-pairing with partially complementary sequences in the 3′-untranslated region (3′-UTR) of target mRNAs. Owing to recent cloning, sequencing, and computational efforts, the numbers of known miRs has been rapidly increasing, and to date, there are a total of 21,643 mature miRs found across 103 species, of which 1921 miRs are found in humans (miRBase Release 18.0, November 20111). With the emerging identification of miRs from humans to viruses, which have provided a crucial and pervasive layer of post-transcriptional gene regulation. The nervous system is a rich source of miR expression, with a diversity of miR functions in fundamental neurobiological processes including neuronal development, plasticity, metabolism, and apoptosis. Recently, the let-7 family of miRs is found to be a critical regulator of MOR function in opioid tolerance. Let-7 is the first identified human miR. Its family members are highly conserved across species in sequence and function. In the review, we will present a brief review of the opioid receptors, their regulation, and opioid tolerance as well as an overview of miRs and a perspective how miRs may interact with MOR and serve as a regulator of opioid tolerance.

Keywords: opioid, miR, epigenetics, addiction, pain

1http://microrna.sanger.ac.uk/sequences
opioids’ analgesia and antinociceptive tolerance (Matthes et al., 1996; Sora et al., 1997). Opioid tolerance may be result from opioid receptor desensitization and trafficking, which include opioid receptor down-regulation, internalization, and uncoupling from G-proteins due to chronic exposure to opioid agonists (Bailey and Connor, 2005; Martini and Whistler, 2007; Koch and Holtt, 2008; Lopez-Gimenez and Milligan, 2010). Receptor down-regulation as one of mechanisms contributing to opioid tolerance has been previously proposed (Davis et al., 1979; Tao et al., 1987; Bhargava and Gulati, 1990; Bernstein and Welch, 1998; Diaz et al., 2000). Chronic morphine treatment produced a marked decrease in brain MOR density (Davis et al., 1979; Tempel et al., 1988). Down-regulation of the high-affinity MOR site in rats has also been reported following continuous infusion of morphine (i.t.; Wong et al., 1996) or etorphine (s.c.; Tao et al., 1987). Morphine-induced MOR down-regulation was also observed in SH-SY5Y cells, with or without differentiation (Zadina et al., 1993). In addition to receptor down-regulation, chronic treatment with morphine has been shown to significantly reduce MOR-signaling in sensory neurons and brainstem nuclei (Sim et al., 1996; Johnson et al., 2006), which are in agreement with the findings that reduced receptor number and resultant reduced MOR-signaling contribute to opioid tolerance. On the other hand, there have been reports that MOR expression was not altered (Dum et al., 1979) or even up-regulated (Lewis et al., 1984) in the brain by various opioids. Some of the discrepancies may be caused by uncontrolled variables (e.g., different opioids, doses, methods of opioid treatments, anatomical regions, and times samples were taken, integrality of tissue samples before assays, and opioid receptor subtypes studied), as well as detection methods. It has been suggested that MOR down-regulation is agonist selective and depends on the agonist’s intrinsic efficacy (Nishino et al., 1990b; Chakrabarti et al., 1997; Chan et al., 1997; Koch and Holtt, 2008). The purity and selectivity of radiolabeled ligands – a problem not only for some early studies, but also in more recent reports employing questionable materials – used in studies are the other potential culprits for conflicting findings.

**DIRECT INTERACTION BETWEEN let-7 FAMILY miRs AND MOR**

The long 3′-UTR of MOR mRNA (Ide et al., 2005; Han et al., 2006) suggests that this region may contain physiologically relevant elements for regulating receptor expression by mechanisms such as miR targeting. Indeed, early research on 3′-UTR of human MOR mRNA suggested that MOR expression was increased after a 712-bp segment, immediately downstream of the stop codon, was removed (Zollner et al., 2000). Comparative bioinformatics predicted potential miRs that may interact with the human and mouse MOR (Table 1). Let-7 family of miRs was identified as a top candidate according to the number of putative target sites and alignment pattern. Our group experimentally validated the in silico prediction that members of the let-7 miR family can interact with the 3′-UTR of MOR mRNA at the predicted positions (He et al., 2010). Furthermore, downregulating let-7 with specific LNA-modified antisense oligodeoxynucleotides (LNA-let-7-AS) was found to increase MOR expression in SH-SY5Y cells, a human neuroblastoma cell line, suggesting that (1) MOR is a target of let-7; (2) expression of MOR is under constitutive suppression by let-7.

**Table 1 | MicroRNA targets predicted by miRanda**

(Continued)
In order to elucidate a physiological role of let-7 in regulating MOR and opioid tolerance, we further examined the in vivo relevance of let-7 miRs in cellular and animal models of opioid tolerance. For the former, SH-SY5Y cells were treated with morphine, while the expression level of MOR was reduced as determined by the western blotting method. Interestingly, up-regulation of let-7 occurred in MOR-expressing cells, but not in MOR-negative cells in mice brain cortex region as determined by in situ hybridization (He et al., 2010). This was in agreement with the aforementioned finding that MOR is a direct target of let-7. In order to further examine a causative role of let-7 in leading to opioid tolerance, we directly targeted let-7 in the mouse model of opioid tolerance. Treatment with the let-7 inhibitor (i.c.t.) decreased brain let-7 levels and partially attenuated opioid antinociceptive tolerance (He et al., 2010).

Previous reports from a number of different cell lines or animal models (e.g., Brodsky et al., 1995; Johnson et al., 2006) indicated that MOR mRNA was not changed upon treatment with morphine. We also found that the total MOR transcripts were unchanged by morphine. For example, MOR mRNA remained the same in SH-SY5Y cells following chronic morphine treatment. It raised a question as to how let-7 miRs repress MOR in opioid tolerance. We confirmed that let-7 did not affect MOR mRNA stability; however, polysome-bound MOR transcript was significantly decreased. Where is MOR mRNA hiding? It turns out that it can be accumulated in P-bodies. So these intriguing data led us to propose the following regulatory mechanism where upon let-7 can regulate opioid tolerance: let-7 recruits and sequesters MOR mRNA to P-bodies that are deprived of translational machinery, effectively reducing polysome-bound MOR mRNA and leading to translation repression. A similar pathway was observed in HEK293 and HeLa cells (Pillai et al., 2005), thus translation repression may serve as a general mechanism by let-7 to regulate its target gene expression (Figure 1). Keep in mind that let-7 activity is dampened in opioid tolerance by up-regulation of their transcripts. Nature works in a wonder to ensure the activity of let-7, ultimately dampening the activity of opioids upon persistent activation.

### Table 1 | Continued

| miRNA       | Query target sites | Alignment score | PhastCons score |
|-------------|--------------------|-----------------|-----------------|
| hsa-miR-302d | 170                | 140             | 0.619627        |
| hsa-miR-504  | 1                  | 140             | 0.690845        |
| hsa-miR-521  | 89                 | 140             | 0.619627        |
| hsa-miR-9    | 234                | 140             | 0.619627        |
| hsa-miR-942  | 333                | 140             | 0.666615        |

**MOUSE OPRM1 3'-UTR**

- **mmu-miR-540-5p**: 278 (168.0) 0.62498
- **mmu-miR-540-3p**: 395 (159.0) 0.584342
- **mmu-miR-134**: 403 (158.0) 0.584342
- **mmu-miR-302d**: 165 (158.0) 0.586054
- **mmu-miR-139-5p**: 363 (149.0) 0.62498
- **mmu-miR-19a-3p**: 193 (148.0) 0.586054
- **mmu-miR-19b**: 193 (148.0) 0.586054
- **mmu-miR-496**: 241 (148.0) 0.586054
- **mmu-miR-532-3p**: 63 (148.0) 0.586054
- **mmu-miR-7a**: 388 (147.0) 0.62498
- **mmu-miR-7f**: 388 (147.0) 0.62498
- **mmu-miR-7c**: 386 (146.0) 0.62498
- **mmu-miR-486**: 214 (146.0) 0.586054
- **mmu-miR-741**: 512 (146.0) 0.567623
- **mmu-miR-383**: 93 (144.0) 0.586054
- **mmu-miR-695**: 404 (144.0) 0.584342
- **mmu-miR-7b**: 388 (143.0) 0.62498
- **mmu-miR-7e**: 388 (143.0) 0.62498
- **mmu-miR-188-3p**: 64 (143.0) 0.586054
- **mmu-miR-196b**: 386 (143.0) 0.62498
- **mmu-miR-302b**: 165 (143.0) 0.586054
- **mmu-miR-323-5p**: 233 (143.0) 0.586054
- **mmu-miR-466-5p**: 138 (143.0) 0.586054
- **mmu-miR-98**: 387 (143.0) 0.62498
- **mmu-miR-298**: 391 (142.0) 0.584342
- **mmu-miR-339-3p**: 71 (142.0) 0.586054
- **mmu-miR-381**: 528 (142.0) 0.590822
- **mmu-miR-682**: 186 (142.0) 0.586054
- **mmu-miR-7d**: 388 (141.0) 0.62498
- **mmu-miR-504**: 57 (141.0) 0.586054
- **mmu-miR-154**: 321 (140.0) 0.62498
- **mmu-miR-196a**: 387 (140.0) 0.62498
- **mmu-miR-340-5p**: 527 (140.0) 0.590822
- **mmu-miR-34b-3p**: 367 (140.0) 0.62498

(Ha et al., 2010) and the receptor radioligand binding using 3H-DAMGO (He and Wang, unpublished data). Therefore, chronic morphine treatment caused the increase of let-7 and decrease of MOR expression during the development of opioid tolerance in SH-SY5Y. Furthermore, the regulation of let-7 expression by morphine occurred in a mouse model of opioid tolerance. Mice were implanted s.c. with a 75-mg morphine pellet (75 mg morphine pellet/mouse, s.c.). Brain expression of let-7 increased gradually over time after morphine treatment, temporally correlating with the development of antinociceptive tolerance to morphine. Interestingly, up-regulation of let-7 occurred in MOR-expressing cells, but not in MOR-negative cells in mice brain cortex region as determined by in situ hybridization (He et al., 2010). This was in agreement with the aforementioned finding that MOR is a direct target of let-7. In order to further examine a causative role of let-7 in leading to opioid tolerance, we directly targeted let-7 in the mouse model of opioid tolerance. Treatment with the let-7 inhibitor (i.c.t.) decreased brain let-7 levels and partially attenuated opioid antinociceptive tolerance (He et al., 2010).

**POTENTIAL TARGET GENES OF let-7 IN OPIOID TOLERANCE**

Let-7 was the first identified human miR. Its family members are highly conserved across species in sequence and function (Pasquinelli et al., 2000). Major roles of let-7 include the regulation of stem-cell differentiation, neuromuscular development, and cell proliferation & differentiation (Reinhart et al., 2000; Mansfield et al., 2004; Roush and Slack, 2008). Let-7 was initially identified as a heterochronic gene (Pasquinelli et al., 2000). In mammals, let-7 levels increase during embryogenesis and during brain development (Schulman et al., 2005; Wulczyn et al., 2007). Let-7 is undetectable in human and mouse embryonic stem cells, and the level of let-7 increases upon differentiation (Thomson et al., 2004, 2006; Wulczyn et al., 2007). This high expression of let-7 is then maintained in various adult tissues (Sempere et al., 2004; Thomson et al., 2004). Furthermore, let-7 is widely viewed...
as a tumor suppressor miR (Boyerinas et al., 2010). There is a clear link between loss of let-7 expression and the development of poorly differentiated, aggressive cancers (Takamizawa et al., 2004; Dahiya et al., 2008; Childs et al., 2009). Using the computational algorithm TargetScan 6.0\textsuperscript{2} to screen human 3'—UTR sequences containing let-7 family miRs complementary sites, 886 conserved targets, with a total of 989 conserved sites and 111 poorly conserved sites were predicted. High-mobility group AT-hook 2 (HMGA2), which is the top-scoring candidate gene on the list (Table 2), was confirmed as a direct let-7 target both in vitro and in vivo (Lee et al., 2006; Mayr et al., 2007; Shell et al., 2007). HMGA2 is a chromatin-associated non-histone protein capable of modulating chromatin architecture and thus affecting transcription. In addition to abundant studies on HMGA2 in embryogenesis (Zhou et al., 1995; Sun et al., 2009) and tumorigenesis (Peng et al., 2008; Qian et al., 2009), it would be interesting to investigate its possible involvement in opioid tolerance as a functional target gene of let-7.

Seed pairing rules are widely used to predict functional miR target sites. MiR-mRNA recognition requires the perfect complementarity of 6-nucleotide mRNA seed sites with the 5' terminal of miRs (positions 2–7; Bartel, 2009). However, when predictions based on such short complementary sequences are compared to experimental results from proteomic studies, the false-positive and false-negative rates appear to be above 50% (Easow et al., 2007; Baek et al., 2008; Mourelatos, 2008; Selbach et al., 2008). Several previous findings strongly indicated that a sizeable fraction of miR targets do not obey the "canonical" seed rule (Ha et al., 1996; Diddiano and Hobert, 2006; Tay et al., 2008; Stefani and Slack, 2012). For let-7, biological studies clearly demonstrated that genetically verified let-7 targets in Caenorhabditis elegans contain only imperfect binding sites, with bulges or G·U wobble pairs in the seed region (Vella et al., 2004). A recent study identified a new class of miR target site nucleation bulges and an alternative mode of miR target recognition by a pivot-pairing rule (Chi et al., 2012). They proposed a transitional nucleation model in which a transitional nucleation state determines the binding of miRs to nucleation bulge mRNAs. Therefore, the identification of non-canonical let-7—mRNA interactions may lead to important breakthroughs in discovering new let-7 targets and further decipher the functional role of let-7 in opioid tolerance.

With respect to let-7 target gene identification, another important issue need to be addressed is the redundancy of let-7 family members. There are 14 and 13 different let-7 family members in mouse and human, respectively (Roush and Slack, 2008). In human, these different members, let-7a-1, 7a-2, 7a-3, 7b, 7c, 7d, 7e, 7f-1, 7f-2, 7g, 7i, miR-98, and miR-202 are located on nine different chromosomes (Ruby et al., 2006). While let-7 was initially viewed as one single activity, emerging data suggest that the let-7 family contains miRs with different activities (i.e., targets). For example, it has been reported that let-7b\textsuperscript{*} was highly expressed but let-7e\textsuperscript{*} was drastically reduced in malignant mesothelioma (Guled et al., 2009). So the question remains as whether individual let-7 family member with its own expression pattern exerts specialized function during opioid tolerance development.

### CONCLUSION AND FUTURE DIRECTIONS

Opioid tolerance, even to the analgesic actions of these drugs, is likely not caused by a single mechanism; rather an intricate neuronal circuitry involving multiple mechanisms may ultimately lead to the expression of tolerance seen in humans. Regulation of opioid tolerance, through MOR or other mechanisms, represents one of these mechanisms. What is not known is how many miRs are involved. Existing literature on miR-related mechanisms of opioid tolerance is sparse. A typical study in miR research field tends to survey transcript levels of miRs in a disease state (Zheng et al., 2010). For example, morphine-induced up-regulation of miR-13b in human monocyte-derived macrophages (Dave and Khalili, 2010), while miR-133b was decreased by morphine in zebrafish embryos (Sanchez-Simon et al., 2010). It is often extremely difficult to identify a casual relationship. Furthermore, moving from a cell line to in vivo relevance is another big hurdle to overcome.

In the case of let-7 miRs, it was identified as a critical regulator of both human and mouse MOR. Moreover, it was demonstrated to be relevant for both cellular opioid tolerance as well as animal models of opioid tolerance (He et al., 2010). Let-7 represents one of several miRs contributing to opioid tolerance. For example, it

\[\text{http://www.targetscan.org/}\]
| Target gene (1–50) | Total context score | Aggregate $P_{CT}$ | Target gene (51–100) | Total context score | Aggregate $P_{CT}$ |
|-------------------|---------------------|--------------------|----------------------|---------------------|--------------------|
| Hmga2             | −1.08               | >0.99              | Map4k3               | −0.46               | 0.96               |
| Fignl2            | −1.07               | >0.99              | Tmem211              | −0.46               | <0.1               |
| Lin28b            | −0.98               | >0.99              | Zfp583               | −0.46               | 0.96               |
| Trim71            | −0.98               | >0.99              | Dtx2                 | −0.46               | 0.99               |
| Zc3hav1l          | −0.85               | 0.86               | Diap2                | −0.46               | 0.98               |
| Fign              | −0.84               | >0.99              | Pgrmc1               | −0.46               | 0.9               |
| Nr6a1             | −0.8                | >0.99              | Pars2                | −0.46               | <0.1               |
| Z200002K05Rik     | −0.7                | >0.99              | Cd200r1              | −0.45               | 0.94               |
| Skil              | −0.67               | >0.99              | Abt1                 | −0.45               | <0.1               |
| Igdcc3            | −0.65               | >0.99              | Acot11               | −0.45               | 0.5                |
| Thrsp             | −0.62               | <0.1               | Kcnj11               | −0.45               | 0.81               |
| Prtg              | −0.61               | >0.99              | Zfp248               | −0.45               | <0.1               |
| Slt5a9            | −0.6                | 0.83               | Bcl213               | −0.44               | <0.1               |
| Tgfr1             | −0.58               | >0.99              | Gatm                 | −0.44               | 0.95               |
| Yod1              | −0.58               | >0.99              | Hic2                 | −0.44               | >0.99              |
| Smarcad1          | −0.57               | 0.96               | Ccnj                 | −0.44               | 0.97               |
| Gab2              | −0.57               | 0.6                | Arid3a               | −0.44               | >0.99              |
| Ngly1             | −0.54               | <0.1               | Hand1                | −0.44               | 0.98               |
| Kctd21            | −0.54               | 0.98               | Igf1r                | −0.44               | >0.99              |
| Dna2              | −0.53               | <0.1               | Rpsd3                | −0.43               | 0.59               |
| Ppp1r15b          | −0.53               | >0.99              | Pbx3                 | −0.43               | 0.99               |
| Nph3              | −0.53               | 0.94               | Zmat4                | −0.43               | 0.97               |
| Vezt              | −0.52               | <0.1               | Tmem164              | −0.43               | <0.1               |
| Suv39h2           | −0.51               | 0.44               | Bcat1                | −0.43               | 0.99               |
| Gdf6              | −0.5                | 0.98               | Px1                  | −0.43               | <0.1               |
| Brwd1             | −0.5                | <0.1               | Als2cl               | −0.43               | <0.1               |
| Coil              | −0.49               | 0.97               | 9000012K11Rik        | −0.42               | 0.94               |
| Lrig3             | −0.49               | 0.89               | Atg10                | −0.42               | <0.1               |
| Hoxb1             | −0.49               | 0.98               | Limd2                | −0.42               | 0.94               |
| Zcchc9            | −0.48               | <0.1               | Zfp341               | −0.42               | <0.1               |
| B3gnt7            | −0.48               | 0.76               | Fam103a1             | −0.42               | 0.87               |
| Entpd7            | −0.48               | <0.1               | Med28                | −0.42               | 0.56               |
| Acvr1c            | −0.48               | 0.99               | Smug1                | −0.42               | <0.1               |
| Lrig2             | −0.48               | 0.99               | Trpm3                | −0.42               | <0.1               |
| Bzv1              | −0.48               | >0.99              | Fras1                | −0.42               | 0.96               |
| Gnptab            | −0.48               | 0.98               | Uhrf2                | −0.41               | 0.96               |
| Wdr41             | −0.48               | <0.1               | Cdc25a               | −0.41               | 0.8                |
| Cdk34             | −0.47               | 0.99               | Igf2bp1              | −0.41               | >0.99              |
| Slc35d2           | −0.47               | 0.98               | Cep110               | −0.41               | 0.94               |
| Dclre1b           | −0.47               | 0.12               | Mmps33               | −0.41               | <0.1               |
| Hif1an            | −0.47               | 0.61               | Tnfsf10              | −0.41               | <0.1               |
| Zfp322a           | −0.47               | 0.82               | Arhgap28             | −0.41               | 0.98               |
| Adrs2             | −0.47               | 0.98               | Apbb3                | −0.41               | 0.98               |
| Tmpress11f        | −0.47               | 0.92               | Fndc3a               | −0.41               | 0.97               |
| Ddx19b            | −0.47               | 0.98               | Col1a2               | −0.41               | 0.97               |
| Ddx19a            | −0.47               | 0.98               | Gemin7               | −0.41               | <0.1               |
| Galt1             | −0.47               | >0.99              | Wnt9a                | −0.41               | 0.99               |
| Greb11            | −0.47               | 0.99               | Igf2bp2              | −0.41               | 0.93               |
| Ercc6             | −0.47               | 0.98               | Tmem2                | −0.41               | 0.98               |
| Dab2             | −0.46               | 0.89               | Zfp879               | −0.41               | 0.35               |
was found miR-23b could interact with the MOR 3'-UTR via a K box motif (5'-UGUGAU-3') in SH-SYSY and mouse P19 cells (Wu et al., 2008, 2009).

While MOR is involved in the development of morphine tolerance, it is not the only target for the action of miRs in opioid tolerance (Matthes et al., 1996; Sora et al., 1997). With the increasing understanding of miRs in epigenetic regulation, further research is needed to identify other target genes of miRs (including let-7). A number of receptors, ion channels, and protein kinases, which have been implicated in opioid tolerance, such as the NMDA receptors, PKC, and CaMKII (Kieffer and Evans, 2002; Wang and Wang, 2006; Ueda and Ueda, 2009) may become potential targets for let-7.

In summary, miR-mediated mechanisms provide a novel direction toward unraveling the complex mechanisms involved in the development of opioid tolerance. These studies will enrich our knowledge on basic principles of neuronal and behavioral adaptation in opioid tolerance, and eventually lead to novel design and development of pharmacological treatments for opioid tolerance.

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