Review Article
Emerging roles of long non-coding RNAs in osteonecrosis of the femoral head

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Abstract: Osteonecrosis of the femoral head (ONFH) is a potentially disabling orthopedic condition that, in most late-stage cases, requires total hip arthroplasty. Although direct trauma to the hip (e.g. femoral neck fracture, hip dislocation) that leads to vascular interruption is a strong risk factor for ONFH, there are many non-traumatic risk factors (e.g. use of corticosteroid, alcohol abuse) which molecular mechanisms in ONFH still remain obscured. Long non-coding RNAs (lncRNAs) is a class of regulatory RNAs that play crucial roles in various cellular functions, including cell proliferation, invasion, metabolism, apoptosis and stem cell differentiation. Recent studies also suggested their participation in bone development and regeneration, and a direct involvement in the pathogenesis of numerous of orthopaedic conditions, such as ONFH. LncRNAs are differentially expressed in ONFH tissues as well as bone marrow-mesenchymal stem cells and bone microvascular endothelial cells isolated from ONFH patients. Functional studies further established their critical roles in regulating biological processes, such as osteoblast survival and osteogenic differentiation of bone marrow-mesenchymal stem cells, which are closely related to ONFH. The current review aims at summarizing the recent advancement in this field and discussing the potential diagnostic, prognostic and therapeutic utilities of lncRNAs in the clinical management of ONFH.

Keywords: LncRNA, avascular necrosis, RNA sequencing, steroid

Introduction
Osteonecrosis of the femoral head (ONFH) occurs as a result of decreased blood flow to the bone tissue, leading to ischemia and osteocyte necrosis followed by a complicated repair process with bone resorption, which is predominated over bone formation. ONFH is a multifactorial disease involving both traumatic (e.g. femoral neck fracture, hip dislocation) and non-traumatic (e.g. use of corticosteroid, alcohol abuse, clotting disturbances) risk factors [1]. Patients harbouring certain polymorphisms in genes that related to the coagulation pathway, steroid metabolism, immunity, and the regulation of bone formation are also susceptible to the development of ONFH [2]. The exact mechanism about how most non-traumatic risk factors are linked to ONFH is still largely unknown, in spite of their frequent convergence on pathways, which causes decreased blood flow to the femoral head (e.g. intravascular obstruction due to thrombotic occlusion or fat embolism; intraosseous extravascular compression by lipid deposition and adipocyte hypertrophy in the marrow space) or impaired bone repair (e.g. reduced osteogenesis) [1]. Although efforts such as weight-bearing restriction, utilization of bisphosphonates and statins, core decompression and bone grafting have been adopted to treat or restrict disease progression in early-stage ONFH patients, total hip arthroplasty is frequently unavoidable for those who have progressed to the advanced stage [3].
Long non-coding RNAs (lncRNAs) are a recently identified class of non-protein-coding, regulatory RNAs that are longer than 200 nucleotides in length. Although many lncRNAs are reported to mediate their biological functions through sponging microRNAs (Another class of regulatory RNAs inducing degradation and/or inhibit translation of target mRNAs), some specific lncRNAs were proved to regulate gene expression via other mechanisms, including modulating DNA methylation, recruiting transcriptional factors, and controlling mRNA stability and splicing [4]. Accumulating evidences now support that lncRNAs play essential roles in a variety of physiological and pathological processes, including cell proliferation [5], cell death [6], stem cell pluripotency and lineage commitment [7], metabolic control [8] aging and degeneration [9], as well as inflammation and immunity [10]. Emerging studies also suggested that lncRNAs can regulate bone development and regeneration [11] and contribute to the pathogenesis of different orthopaedic conditions, e.g. osteoporosis [12], osteoarthritis [13], osteosarcoma [14] and intervertebral disc degeneration [15]. Recently, lncRNAs were also revealed to be involved in development of OFNH.

In this review, we summarized dysregulated lncRNAs as well as their functional roles in ONFH. We also discussed their potential possibility as druggable targets and diagnostic markers for ONFH treatment.

LncRNA expression profiling in ONFH

In ONFH, differentially expressed lncRNAs have been identified at the genome-wide level by RNA sequencing or microarray in osteonecrotic tissues, mesenchymal stem cells (MSCs) and bone microvascular endothelial cells (BMECs). Dysregulation of some sort of lncRNAs have also been substantiated by reverse transcription-quantitative PCR (RT-qPCR).

Osteonecrotic tissues

Recently, Luo et al. reported distinct expression profiles of mRNA and IncRNA in three human steroid-associated ONFH samples and three human femoral head fracture samples using third-generation IncRNA microarrays [16]. A total of 1,657 mRNAs (1,092 upregulated and 565 downregulated) and 4,393 IncRNAs (1,179 upregulated and 3,214 downregulated) were found to be differentially expressed in the ONFH group. RT-qPCR was performed to validate selected IncRNAs and confirmed the upregulation of NR 027293 and downregulation of ENST00000565178, NR 038891, and T318-776 in the osteonecrotic tissues [16].

Moreover, Huang and colleagues compared the transcriptome difference between ONFH and femoral head fracture samples using RNA sequencing technology [17], revealing a total of 2,965 differentially expressed genes including 1,395 upregulated and 1,570 downregulated genes in the ONFH tissues, among which there were 575 upregulated IncRNAs and 27 downregulated IncRNAs. Co-expression analysis further pointed out a significant correlation of 144 mRNAs with the differentially expressed IncRNAs. These correlated genes were found to be closely related to serine-type endopeptidase, peptidase, and endopeptidase activity. Downregulation of IncRNA FAM201A was confirmed by RT-qPCR [17].

Bone marrow-mesenchymal stem cells

Increased adipogenic and/or decreased osteogenic differentiation of bone marrow-MSCs have been implicated in the pathogenesis of alcohol- and steroid-induced ONFH [18-20]. Wang et al. isolated MSCs from the bone marrow of patients with steroid-associated ONFH and compared their mRNA and IncRNA expression profiles to those with femoral neck fracture using microarray [21]. Resultingly, 2,775 mRNAs and 3,720 IncRNAs were identified to be differentially expressed, among which 838 mRNAs and 1,878 IncRNAs were upregulated, whereas 1,937 mRNAs and 1,842 IncRNAs were downregulated in the steroid-associated ONFH group. The top 10 differentially expressed mRNAs and IncRNAs were successfully verified by RT-qPCR, confirming the validity of their microarray results. Reconstruction of the coding-non-coding gene co-expression (CNC) network revealed the critical roles of two IncRNAs, HOTAIR and RP1-193H18.2, in regulating the osteogenic and adipogenic differentiation of bone marrow-MSCs [21].

Similarly, Xiang et al. examined the different expression of IncRNAs in bone marrow-MSCs isolated from patients with steroid-associated ONFH and those from patients with developmental dysplasia of the hip or femoral neck fracture through RNA sequencing [22]. Con-
sequently, they discovered a total of 3,114 differentially expressed mRNAs (1,979 upregulated and 1,135 downregulated) and 572 lncRNAs (181 upregulated and 391 downregulated). The hub function of the lncRNA RP11-154D6 was further predicted by reconstruction of the lncRNA-microRNA-mRNA network [22].

Bone microvascular endothelial cells

Genetic variations of angiogenesis-related genes were associated with altered risks for ONFH [23], whereas stimulation of angiogenesis with vascular endothelial growth factor (VEGF) showed therapeutic effects in a canine model of cryosurgically-induced ONFH [24]. Non-traumatic ONFH is also known to be associated with endothelial cell activation [25]. Therefore, it is of interest to delineate the association between the transcriptomic profile of bone endothelial cells and disease status in ONFH. For such purpose, Yu et al. harvested BMECs from patients who were undergone routine total hip replacement and exposed cells to hydrocortisone (0.1 mg/ml) for 24 h before microarray-based mRNA, microRNA and lncRNA profiling [26, 27]. Among 26,646 lncRNAs that were expressed above background in BMECs, 239 lncRNAs (73 upregulated and 166 downregulated), including NAV2-IT1, NAV2-AS5, NAV2-AS1, NTM-IT2, and ARHGEF19-AS1, showed significant dysregulation upon hydrocortisone exposure. RT-qPCR was applied to confirmed the upregulation of ENSG00000259007.1 and downregulation of XLOC_011117. Co-expression analysis of non-coding RNAs and their correlated mRNAs further revealed FoxO signaling as a potential compensatory response of BMECs to hydrocortisone [27]. In this connection, FoxO transcription factors was showed to be closely involved in the regulation of vessel formation in the adult [28].

LncRNA profiling studies on ONFH are summarized as Table 1. It is noteworthy that the top lncRNAs identified by different studies did not overlap. It might occur as a result of the use of different tissues or cell types for expression profiling and the clinical heterogeneity of ONFH.

Functional roles of specific lncRNAs in ONFH

AWPPH

AWPPH (lncRNA associated with poor prognosis of hepatocellular carcinoma) is a newly discovered lncRNA whose expression was deregulated in a variety types of tumors, including liver cancer [29], osteosarcoma [30], and non-small cell lung cancer [31]. A recent study showed that AWPPH levels were reduced in both serum and mesenchymal stem cells (MSCs) isolated from non-traumatic ONFH patients as compared with those from healthy control [32]. Moreover, low levels of AWPPH in serum and MSCs could differentiate non-traumatic ONFH patients from healthy controls with the area under the curve of 0.8177 and 0.8259, respectively, showing a possibility to be served as a diagnostic marker. Clinicopathological correlation analysis further revealed that reduced AWPPH levels were associated with a shorter course of disease (<5 years) but irrelevant with patients’ age, gender, cigarette smoking or drinking habits. Bone morphogenic proteins (BMPs), for example BMP-2, are known to induce osteoblastic differentiation of MSCs through activating the transcription factor RUNX2 [33]. In this connection, BMP-2 was showed to be induced the expression of AWPPH in human bone marrow-MSCs. Importantly, elevated AWPPH level led to an increased RUNX2 expression, while it was reversed by knockdown of AWPPH. RUNX2 expression levels were also

Table 1. LncRNAs expression profiles in osteonecrosis of the femoral head

<table>
<thead>
<tr>
<th>Num</th>
<th>Method</th>
<th>sample</th>
<th>upregulated</th>
<th>downregulated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Microarray RT-PCR</td>
<td>ONFH patients</td>
<td>1,179 LncRNAs NR 027293</td>
<td>3,214 LncRNAs ENST00000565178 NR 038891 T318776</td>
<td>[16]</td>
</tr>
<tr>
<td>2</td>
<td>Microarray RT-PCR</td>
<td>ONFH tissues</td>
<td>575 LncRNAs</td>
<td>27 LncRNAs FAM201A</td>
<td>[17]</td>
</tr>
<tr>
<td>3</td>
<td>Microarray RT-PCR</td>
<td>mesenchymal stem cell from ONFH</td>
<td>1,878 IncRNAs HOTAIR RP1-193H18.2</td>
<td>1,842 IncRNAs</td>
<td>[21]</td>
</tr>
<tr>
<td>4</td>
<td>Microarray RT-PCR</td>
<td>mesenchymal stem cells from ONFH</td>
<td>181 IncRNAs</td>
<td>391 IncRNAs</td>
<td>[22]</td>
</tr>
<tr>
<td>5</td>
<td>Microarray RT-PCR</td>
<td>mesenchymal stem cells from ONFH</td>
<td>73 IncRNAs ENSG00000259007.1</td>
<td>166 IncRNAs XLOC_011117</td>
<td>[26, 27]</td>
</tr>
</tbody>
</table>
markedly decreased in MSCs, which were isolated from non-traumatic ONFH patients. Thus, these findings collectively suggested that the aberrant downregulation of AWPPH might participate in the progression of ONFH through dampening RUNX2 signaling in MSCs. The reduced circulating levels of AWPPH might also enable this IncRNA to act as a potential biomarker for diagnosing non-traumatic ONFH [32]. Nevertheless, further work is demanded to figure out whether this IncRNA could functionally repress osteoblastic differentiation of MSCs.

**RP11-154D6**

RP11-154D6 was reported as another differentially expressed IncRNAs in bone marrow-MSCs isolated from patients with steroid-induced ONFH [22]. Bioinformatic analysis further revealed an extensive interaction of RP11-154D6 with microRNAs. Moreover, RP11-154D6 expression was detected to be increased during osteogenic differentiation but decreased during adipogenic differentiation of bone marrow-MSCs. Functionally, enforced expression of RP11-154D6 by a lentiviral vector in bone marrow-MSCs promoted osteogenic differentiation, which was evidenced by increased expression of osteocalcin (OCN) and RUNX2, but diminished adipogenic differentiation and reduced expression of adipogenic markers, such as lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor γ (PPARγ). These data collectively suggested that the downregulation of RP11-154D6 tilts the balance of bone marrow-MSC differentiation from osteogenesis to adipogenesis [22]. Restoration of RP11-154D6 expression might thus represent a potential prophylactic or even therapeutic strategy for steroid-induced ONFH.

**MIAT**

The IncRNA MIAT (myocardial infarction associated transcript) exhibits dysregulation in various diseases, such as myocardial infarction, ischemic stroke and cancers, which mediates biological functions through sponging miR-22-3p, miR-24 and miR-150 [34]. A recent study revealed that MIAT levels in osteonecrotic tissues were significantly elevated than that in non-necrotic tissues from patients with steroid-associated ONFH [35]. Expression levels of MIAT were found to be gradually decreased during osteogenic differentiation of rat MSC. Silencing of MIAT with small interfering RNAs (siRNAs) facilitated the expression of osteogenic markers ALP, RUNX2 and OCN. Alizarin Red staining further revealed the increased formation of mineralized nodules in MIAT-knockdown rat MSCs. The traditional Chinese medicine HXTL (Huo Xue Tong Luo) used for osteonecrosis treatment promoted histone H3 lysine 27 trimethylation (H3K27me3; a repressive histone mark) and inhibited H3K4me3 (an active histone mark) in the promoter of MIAT to repress its expression [35]. These data suggested that the aberrant overexpression of MIAT might inhibit MSC osteogenic differentiation to lead to steroid-associated ONFH, whereas HXTL might protect against ONFH development through epigenetic silencing of MIAT.

**EPIC1**

EPIC1 (epigenetically-induced IncRNA1) is a novel Myc-interacting IncRNA showing dysregulation in multiple cancer types [36-38]. A higher expression level of EPIC1 in necrotic femoral head tissues than that in normal tissues surgically isolated from patients with steroid-associated ONFH was ever reported [39]. In this regard, endogenous expression of EPIC1 was proved to protect OB-6 osteoblastic cells and primary human osteoblasts from dexamethasone-induced apoptosis, which was characterized by mitochondria depolarization and cytochrome c release. On the contrary, knockdown of EPIC1 resulted in the opposite effect. Importantly, Myc-knockout OB-6 cells were more susceptible to dexamethasone-induced apoptosis, whereas EPIC1 overexpression protected the wild-type but not the Myc-knockout OB-6 cells from dexamethasone, suggesting that Myc was a target gene of EPIC1 in osteoblasts [39]. Thus, these findings indicated that upregulation of EPIC1 in osteonecrotic tissues might represent a compensatory mechanism to sustain osteoblast survival in response to glucocorticoid treatment (Table 2; Figure 1).

**HOTAIR**

HOTAIR (HOX antisense intergenic RNA) is a recently discovered IncRNA that plays a crucial role in regulating phenotypes pertinent to cancer formation, such as proliferation, survival, migration, and genomic stability [40]. Wei et al. reported that HOTAIR expression in MSCs of patients with non-traumatic ONFH was higher than those from healthy donors or patients with osteoarthritis [41]. Endogenous expression of
Table 2. Functional characterization of the lncRNAs in ONFH

<table>
<thead>
<tr>
<th>IncRNAs</th>
<th>Expression</th>
<th>Functional role</th>
<th>Related gene or drugs</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWPPH</td>
<td>Down</td>
<td>mesenchymal stem cell differentiation</td>
<td>BMP-2</td>
<td>protect</td>
<td>[32]</td>
</tr>
<tr>
<td>RP11-154D6</td>
<td>Down</td>
<td>mesenchymal stem cell differentiation</td>
<td></td>
<td>protect</td>
<td>[22]</td>
</tr>
<tr>
<td>LncRNA-Miat</td>
<td>Up</td>
<td>mesenchymal stem cell differentiation</td>
<td>HXTL capsule</td>
<td>Damage</td>
<td>[35]</td>
</tr>
<tr>
<td>Lnc-EPIC1</td>
<td>Up</td>
<td>mesenchymal stem cell differentiation</td>
<td>Dex</td>
<td>Damage</td>
<td>[39]</td>
</tr>
<tr>
<td>HOTAIR</td>
<td>Up</td>
<td>mesenchymal stem cell differentiation</td>
<td>miR-17-5p SMAD7</td>
<td>Damage</td>
<td>[41]</td>
</tr>
<tr>
<td>MALAT1</td>
<td>down</td>
<td>mesenchymal stem cell differentiation</td>
<td>Dex Ppm1e AMPK miR-214</td>
<td></td>
<td>[43]</td>
</tr>
</tbody>
</table>

HOTAIR decreased the level of osteogenic differentiation markers, such as RUNX2, COL1A1 and ALP, in human bone marrow-MSCs, whereas such impact was reversed by knockdown of HOTAIR. Mechanistically, HOTAIR was proved to suppress the osteogenic differentiation markers through the miR-17-5p-SMAD7 (A negative regulator of BMP and transforming growth factor-β signalling) pathway, in which transfection with miR-17-5p mimic or knockdown of SMAD7 nullified the effect of HOTAIR on osteogenesis [41]. These findings indicated that aberrant overexpression of HOTAIR in MSCs contributes to steroid-associated ONFH through sponging miR-17-5p and subsequent derepression of SMAD7 and attenuation of osteogenic BMP signalling. Consistently, by microarray-based profiling of lncRNA expression in BMSCs and validation with RT-qPCR, Wang et al. reported a significant upregulation of HOTAIR in patients with steroid-associated ONFH [21].

MALAT1

Apart from HOTAIR, MALAT1 (metastasis associated lung adenocarcinoma transcript 1) as another highly dysregulated lncRNA in steroid-associated ONFH was identified by Wang et al., which levels were significantly reduced in patients’ BMSCs [21]. Consistently, Fan and colleagues confirmed that MALAT1 levels were substantially lowered in necrotic femoral head tissues than the surrounding normal femoral head tissues from patients with steroid-associated ONFH [42]. The downregulation of MALAT1 could be recapitulated when OB-6 and hFOB1.19 osteoblastic cells and primary human osteoblasts were treated with dexamethasone. Functionally, expression of MALAT1 protected human osteoblasts from dexamethasone-induced cell death, whereas MALAT1 knockdown aggravated the cytotoxicity. The
cytoprotective effect of MALAT1 in osteoblasts was associated with the activation of 5' AMP-activated protein kinase (AMPK) signalling through downregulating PPM1E and the subsequent attenuation of oxidative stress through increasing NRF2 activity [42]. Another recent study showed that dexamethasone impaired osteogenic differentiation of BMSCs, which was paralleled by the downregulation of MALAT1 [43]. Functionally, MALAT1 expression attenuated the inhibitory effect of dexamethasone on osteogenic differentiation, which was mediated through sponging of miR-214 and derepression of its target ATF4 (an osteogenic transcription factor) [43]. These findings suggested that corticosteroid-mediated downregulation of MALAT1 contributes to ONFH through inducing osteoblast cell death and inhibiting osteogenic differentiation of MSCs via regulating the PPM1E-AMPK-NRF2-oxidative stress and miR-214-ATF4 axes, respectively. Restoration of MALAT1 expression or activating its downstream signaling, including AMPK and ATF4, might thus potentially prevent steroid-induced ONFH.

Conclusions and future perspectives

ONFH is a potentially disabling diseases with poorly defined etiology and pathophysiology, which hindered the development of mechanism-driven prophylactic and therapeutic strategies [44]. LncRNA dysregulation (e.g. upregulation of MIAT and HOTAIR and downregulation of AWPPH, RP11-154D6 and MALAT1) was found to play crucial pathogenic roles in steroid-associated ONFH through interacting with signalling pathways pertinent to osteoblast survival and differentiation of bone marrow-MSCs (Figure 1). In this regard, agents that promote osteoblast survival or osteogenic differentiation of MSCs have been shown to protect against disease development in animal models of steroid-induced ONFH [45-48]. Studies scrutinized in this review have also hinted at the potentially therapeutic roles of IncRNAs in steroid-associated ONFH. Nevertheless, in some studies where osteonecrosis tissues were used for IncRNA profiling, the cellular source of the deregulated IncRNAs remains poorly defined as the tissues contained trabecular bone, bone marrow, blood vessels and cartilage. It is also worthwhile to note that a single IncRNA could have divergent functions in different cell types. Future studies involving enrichment of specific cell types before profiling or single-cell transcriptomics could tackle this issue [49].

Pertinent to clinical practice, upregulated IncRNA might be targeted by different approaches, such as genetic ablation by CRISPR/Cas9-mediated, knockdown by antisense oligonucleotides or siRNAs or steric blockade of IncRNA-protein interactions by small molecules [50]. Nevertheless, cell type-specific delivery of IncRNA-directed therapeutics remains a technical challenge. To this end, cell type-specific delivery of siRNAs with aptamer-siRNA chimeras might be hopeful [50], but whether it can be achieved in human still remains to be further demonstrated. From the perspectives of biomarker development, some circulating IncRNAs might differentiate steroid-associated ONFH patients from healthy subjects. Nevertheless, it would be more appropriate to include patients with the same underlying medical conditions receiving corticosteroid treatment but without the development of ONFH as the control group [51]. Importantly, longitudinal profiling of circulating IncRNAs in corticosteroid-treated patients may be helpful to identify IncRNAs the precede the onset of ONFH. Large-cohort, multi-centre validation of the identified IncRNA markers is also required for effective clinical development. Despite these challenges, it is still hopeful that, with more translational studies, the use of IncRNAs as biomarkers and therapeutic targets in ONFH can be realized in the future.

Disclosure of conflict of interest

None.

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References

LncRNAs in ONFH


