lincRNA-RoR and miR-145 Regulate Invasion in Triple-Negative Breast Cancer via Targeting ARF6

Gabriel Eades, Benjamin Wolfson, Yongshu Zhang, Qinglin Li, Yuan Yao, and Qun Zhou

Abstract

Triple-negative (ER–, HER2–, PR–) breast cancer (TNBC) is an aggressive disease with a poor prognosis with no available molecularly targeted therapy. Silencing of microRNA-145 (miR-145) may be a defining marker of TNBC based on molecular profiling and deep sequencing. Therefore, the molecular mechanism behind miR-145 downregulation in TNBC was examined. Overexpression of the long intergenic noncoding RNA regulator of reprogramming (lincRNA-RoR), functions as a competitive endogenous RNA sponge in TNBC. Interestingly, lincRNA-RoR is dramatically upregulated in TNBC and in metastatic disease and knockdown restores miR-145 expression. Previous reports suggest that miR-145 has growth-suppressive activity in some breast cancers; however, these data in TNBC indicate that miR-145 does not affect proliferation or apoptosis but instead, miR-145 regulates tumor cell invasion. Investigation of miR-145-regulated pathways involved in tumor invasion revealed a novel target, the small GTPase ADP-ribosylation factor 6 (Arf6). Subsequent analysis demonstrated that Arf6, a known regulator of breast tumor cell invasion, is dramatically upregulated in TNBC and in breast tumor metastasis. Mechanistically, Arf6 regulates E-cadherin localization and affects cell-cell adhesion. These results reveal a lincRNA-RoR/miR-145/ARF6 pathway that regulates invasion in TNBCs.

Implications: The lincRNA-RoR/miR-145/ARF6 pathway is critical to TNBC metastasis and could serve as biomarkers or therapeutic targets for improving survival. Mol Cancer Res; 13(2): 330–8. ©2014 AACR.

Introduction

Breast cancer is the second leading cause of cancer-related deaths among women (1). Obstacles to improving clinical outcomes include better understanding of disease recurrence, overcoming drug resistance, and preventing metastasis. Improvements in breast cancer clinical treatment have come from rationally designed molecularly targeted therapeutics. For patients with estrogen receptor (ER)–positive disease, antiestrogen treatments, including selective ER modulators and aromatase inhibitors, have been a major success story. Furthermore, treatment of HER2/neu-overexpressing breast cancers with the recombinant humanized anti-HER2 monoclonal antibody trastuzumab has dramatically improved prognosis for these patients. For patients with triple-negative breast cancer (TNBC), those lacking ER, PR, and HER2 expression, there are currently no available molecularly targeted therapeutics (2). TNBC accounts for around 20% of cases of breast cancer in the United States where it is frequently observed in younger women and African American women (3). TNBC is frequently aggressive and fast growing but it does respond to chemotherapy. Nevertheless, understanding the molecular mechanisms driving TNBC will allow rational target selection and new drug development.

Dysregulation of microRNAs (miR) is emerging as a major contributor to tumorigenesis in breast cancers. In a recent study, Volinia and colleagues (4) examined breast tumor deep sequencing data in an attempt to identify miRs linked with breast tumor invasiveness. When comparing miR dysregulation in different molecular subtypes, they found that miR-145 was among the most significantly repressed miRs in TNBC. miR-145 is a reported growth suppressor downregulated in many cancer, including lung (5), prostate (6), breast (7), colon (8), and bladder cancers (9).

Recently, a role for miR-145 in the regulation of embryonic stem cell (ESC) renewal was reported (10). Levels of miR-145 in ESCs remain low, whereas upon forced differentiation, miR-145 levels increase dramatically and levels of pluripotency factors OCT4, SOX2, and KLF4 decrease. OCT4, SOX2, and KLF4 were all confirmed to be direct targets of miR-145 in ES cells and embryoid bodies. In addition to regulating ESC renewal, miR-145 has also been shown to be a regulator of adult stem cell renewal. miR-145 was found to regulate mesenchymal stem cell differentiation by targeting SOX9 (11), a master regulator of chondrocyte maturation that has also been implicated as an important regulator of the mammary stem cell state (12).

Long noncoding RNAs (IncRNA) are noncoding RNA molecules greater than 200 nucleotides in size that are often critical regulators of gene expression. A majority of IncRNAs are intergenic [long intergenic ncRNA (lincRNA); ref. 13]. They are transcribed by RNA pol II, polyadenylated, spliced, and 5’ capped (14). IncRNAs are functionally diverse and can act as guides, tethers, decoys, and scaffolds (15). A new function of IncRNA has also been proposed, that of competitive endogenous RNA (ceRNA) for miRs or naturally occurring miR sponges. Such
lncRNA-RoR and miR-145 Regulate Invasion in Triple-Negative Breast Cancer

Materials and Methods

Cell culture
HEK293T, MCF-7, HS578T, and MDA-MB-231 cells were maintained in DMEM with 5% FBS and 1% glutamine (Invitrogen). MCF10A were grown in DMEM/F-12 medium supplemented with 10 μg/mL insulin, 100 ng/mL cholera toxin, 0.5 μg/mL hydrocortisone (Sigma), 20 ng/mL EGF, and 5% horse serum (Invitrogen). Cells were grown at 37°C in an atmosphere containing 5% CO2.

Human tissue array
Immunostaining of paraffin-embedded breast tumor tissue microarray (TMA; BR932; US Biomax) was performed to detect ARF6 protein expression. Additional paraffin-embedded ductal carcinoma in situ (DCIS) samples were obtained from the University of Maryland Pathology Biorepository and Research Core. Sections were deparaffinized and rehydrated using xylene and gradient ethanol. Antigens were retrieved by boiling in sodium citrate (10 mmol/L, pH 6.0), which was proceeded by blocking in 10%, goat serum in PBS for 1 hour. This was followed by overnight incubation (at 4°C) with mouse anti-ARF6, 1:200 in blocking buffer (Santa Cruz Biotechnology; sc7971) followed by biotin goat anti-mouse secondary antibody (1:200). The Avidin-Biotin Peroxidase Substrate Kit (Vector Laboratories) was used to develop brown precipitate. Hematoxylin was used for nuclei staining. Using light microscopy, cores were scored on a 0 to 3 scale (none, light, moderate, and intense) for staining intensity of ARF6.

Western blotting and proliferation assay
Western blotting was performed as previously described (25) using ARF6 (Santa Cruz Biotechnology 3A-1), E-cadherin (BD Transduction), or β-actin (Sigma). For proliferation assays, 1 × 10^4 MDA-MB-231 cells (control and treated) were plated in 96-well plates. After 3 days, MITT solution was added to wells [final (0.5 mg/mL)]. Cells were incubated at 37°C. Media were removed and MITT formazan crystals were solubilized in DMSO. Absorbance was measured at 560 nm in a microplate reader (Bio-Rad).

Plasmids, transfections, and luciferase assay
pCMV-miR-145 expression vector and pCMV-MIR control vectors were obtained from Origene. pBabe-lncRNA-RoR was obtained from Addgene (plasmid 45763). shRNA for lncRNA-RoR and scramble control shRNA was purchased from Origene using pGFP-C-shLenti backbone and the following target sequences: GGAAGCCTCAGAGTGGCAGCGG R: TCAGGAAGTGTCTGGCTGTCG, pre-miR-145: F: AGGGCCAGCAGCGG R: TCAGGAAAGTGTCTGGCTGTCG, pre-miR-145: F: GTTCACATTTCAGAGTCAGGTTGGC. ARF6: F: ATGGGGAAGGTGCTATCCAAAATC R: GCAGGACTGTGGATAGAG. Constitutively active ARF6 (Q67L) expression vector was obtained from Addgene (plasmid 10835). ARF6 3′-untranslated region (UTR) was amplified using the following forward and reverse primers: F: GAGGTGAGGCTGATGATGGACTGATCTGCAGAGTGGCAGCGG, R: TCAGGAAATGTCTCTGGCTGTG. ARF6 F: CTCACTGTTGGGAAGAGACTCCAG, R: AGGAAGCCTGAGAGTGCTGCTGCTG. The following primers were used for qRT-PCR.

RNA quantification
Total RNA was extracted with TRIzol reagent (Invitrogen). Small RNA was converted to complimentary DNA using the poly-A polymerase-based First-Strand Synthesis Kit (SA Bioscience). Subsequent miR analysis was performed by real-time PCR with miR-145 primer assays (SA Biosciences) normalizing to control U6 snRNA levels. Total RNA was converted to cDNA by first treating with DNase I to remove genomic DNA and then using M-MLV reverse transcriptase (Invitrogen) and oligo(dT)12-18 or random hexamer primers. The following primers were used for qRT-PCR: prim-145: F: GAAGATGGGTG-GTGAGTTGGC, R: TCAGGAAATGTCTCTGGCTGTCG, pre-miR-145: F: AGGGCCAGCAGCGG R: TCAGGAAAGTGTCTGGCTGTCG, pre-miR-145: F: GTTCACATTTCAGAGTCAGGTTGGC. ARF6: F: ATGGGGAAGGTGCTATCCAAAATC R: GCAGGACTGTGGATAGAG. Western blotting was performed as previously described (25) using ARF6 (Santa Cruz Biotechnology 3A-1), E-cadherin (BD Transduction), or β-actin (Sigma). For proliferation assays, 1 × 10^4 MDA-MB-231 cells (control and treated) were plated in 96-well plates. After 3 days, MITT solution was added to wells [final (0.5 mg/mL)]. Cells were incubated at 37°C. Media were removed and MITT formazan crystals were solubilized in DMSO. Absorbance was measured at 560 nm in a microplate reader (Bio-Rad).
primes: F: CAGTACGCTAGCAGCTTCGCTCCAGTCCGATG R: CAGTACGCTAGCAGCTTCGCTCCAGTCCGATG. PCR product was cloned downstream luciferase ORF into Nhe1 and Xho1 sites in pSGG-5’ubc vector. Phage (Promega) normalizing to Renilla luciferase activity (26).

previously described using the dual luciferase assay system (Promega) normalizing to Renilla luciferase activity (26).

Transwell invasion assay

Invasion assays were carried out using Transwell migration chambers (8-μm pore size; Costar) coated with 0.5 mg/mL Matrigel (BD Biosciences) on top of the membrane. A total of 0.5 × 10^5 cells/mL were seeded in the upper chamber in serum-free medium. The lower chamber contained 15% FBS. The cells were allowed to migrate toward the 15% FBS gradient overnight. Nonmigrated cells on the top of the membrane were removed with cotton swabs. The migrated cells were stained with 1% crystal violet in methanol/PBS and counted using a light microscope.

The Cancer Genome Atlas

Data from The Cancer Genome Atlas (TCGA) were analyzed using the UCSC Cancer Genome Browser (http://genome-cancer. soe.ucsc.edu/). The publicly available dataset analyzed was the TCGA breast–invasive carcinoma exon expression by RNAseq (IlluminaHiSeq) N = 1,160. ER, PR, and HER2 status were analyzed using UCSC Cancer Browser tools.

Statistical analysis

Statistical analysis was performed using the Student t test and P values of <0.05 were considered significant. Data were represented as mean ± SE. GraphPad Prism 4.0 software was used for all data analysis.

Results

miR-145 is downregulated in TNBC in which miR-145 regulates breast tumor cell invasion

The molecular underpinnings of TNBC are poorly understood and as such, there are no available molecularly targeted therapies. To gain a better understanding of the pathways driving tumorigenesis in TNBC, we began by examining the unique miR signatures of this breast cancer subtype. Analysis of breast tumor cell invasion miR-145 is downregulated in TNBC in which miR-145 regulates breast tumor cell invasion by RNAseq data that had previously revealed that miR-145 is a hallmark of TNBC (4). We began our study by using publicly available databases to verify previous observations regarding miR-145 expression. We examined RNAseq data from TCGA, which contained data from 1,106 breast cancer patient samples. As shown in Fig. 1A, miR-145 is downregulated in nearly every breast tumor sample compared with normal breast tissue (fold Δ and P value shown in Supplementary Fig. S1; ref. 28). Furthermore, we examined ER, PR, and HER2 status in patient data from TCGA and found that patients with TNBC clustered with the tumors having the lowest miR-145 expression. We also examined miR-145 expression in several samples of invasive ductal carcinoma (IDC) and matched normal tissue and again identified miR-145 loss in all samples of breast tumor tissue (Fig. 1B). Next, we examined TNBC cell models MDA-MB-231 and HS578T compared with the enontumorogenic mammary epithelial cell line MCF10A and the ER-positive breast cancer cell line MCF-7. We observed the strongest repression of miR-145 in TNBC models compared with...
nontumorigenic mammary epithelial cells or ER-positive breast cancer cells (Fig. 1C). Combined with previous studies, these data strongly indicate that miR-145 is dramatically silenced in TNBC.

Next, we began investigating what function miR-145 may play in TNBC. In ER-positive breast cancer, miR-145 was previously shown to regulate tumor cell proliferation (29). We examined the impact of miR-145 overexpression on the proliferation of MDA-MB-231 cells. We found that in TNBC cells miR-145 activation failed to significantly affect cell proliferation as examined via MTT assay (Fig. 1D). Furthermore, we also examined cell proliferation by performing Ki67 staining. We found that nearly 100% of control MDA-MB-231 cells positively stained for nuclear Ki67, indicating active proliferation (Supplementary Fig. S2A).

We next examined what impact miR-145 might have on tumor cell invasion in TNBC. We grew cells on Matrigel-coated Transwell inserts and tested the impact of miR-145 overexpression. We found that miR-145-overexpressing TNBC cells demonstrated a significant decrease in tumor cell invasion compared with control cells (Fig. 1E and F). These data suggest that miR-145 may regulate invasion-related gene expression in TNBC. We also confirmed these results in a separate TNBC cell line, HS578T, again finding that miR-145 affects invasion but not proliferation in TNBC cells (Supplementary Fig. S2B and S2C).

lincRNA-RoR is overexpressed in TNBC in which it serves as competitive endogenous RNA for miR-145

We next wanted to identify the molecular mechanisms responsible for miR-145 downregulation in TNBC. It was recently shown that in ESCs miR-145 is subjected to posttranscriptional regulation via competitive endogenous RNA (19). lincRNA-RoR was found to contain miR-145-binding elements and function as a competitive sponge for miR-145 binding. To test whether lincRNA-RoR might regulate miR-145 in TNBC, we began by examining the expression profile of lincRNA-RoR in normal breast tissue, early-stage tumors (DCIS), and
invasive breast tumors (IDC) using qRT-PCR. We found that lincRNA-RoR was significantly upregulated in DCIS and IDC tumor tissues, showing the highest expression in invasive tumor tissues (Fig. 2A). Next, we examined lincRNA-RoR in breast cancer cell lines. We found that lincRNA-RoR was significantly overexpressed in MDA-MB-231 and HS578T TNBC cells when compared with normal tissue (Fig. 2B). Subsequently, we wanted to examine cellular localization of lincRNA-RoR to probe its potential to interact with cytoplasmic miR-145. We performed in situ hybridization and were able to detect lincRNA-RoR in the cytoplasm and nucleus of breast cancer cells (Supplementary Fig. S3A). For further confirmation, we performed MS2-binding assays, in which we cloned 24 MS2-binding sites downstream of lincRNA-RoR, and tested the ability of MS2, there are foci of GFP–MS2 fusion protein with a nuclear localization signal to sequester lincRNA-RoR–MS2 in the cytoplasm. In the absence of lincRNA-RoR–MS2, all the GFP–MS2 is localized in the nucleus (Supplementary Fig. S3B). However, in the presence of lincRNA-RoR–MS2, there are foci of GFP–MS2 in the cytoplasm. These results also support the cytoplasmic localization of lincRNA-RoR in which it may interact with miR-145.

We next wanted to confirm the interaction of miR-145 with the sites predicted by Miranda (30) targeting algorithms (Fig. 2C). We began by testing the impact of lincRNA-RoR overexpression on miR-145 levels in HEK-293T cells (which lack lincRNA-RoR expression). We found that lincRNA-RoR overexpression resulted in a significant decrease in miR-145 levels (Fig. 2D). Next, we examined the impact of cotransfection of miR-145 and lincRNA-RoR and found that cotransfection overcame the negative repression of endogenous miR-145 and resulted in decreasing lincRNA-RoR levels. This suggests a tug of war between these two molecules with some threshold where either the miR or the lincRNA gains the upper hand and silences its partner. We also cloned lincRNA-RoR sequence into a luciferase miR reporter construct to examine the ability of miR-145 to bind sequences in lincRNA-RoR. We found that miR-145 overexpression resulted in decreased luciferase activity compared with control cells (Fig. 2F), indicating miR-145 binding to sites in lincRNA-RoR. Next, we examined this interaction in breast cancer cell lines. We found that lincRNA-RoR overexpression resulted in decreasing mature, but not primary or precursor miR-145 levels in MDA-MB-231 TNBC cells (Fig. 2G). Finally, using lentiviral shRNA, we knocked down lincRNA-RoR in MDA-MB-231 cells and found that this lead to an increase in miR-145 expression. Taken together, these results suggest that in TNBC cells lincRNA-RoR can function as a sponge and repress miR-145 expression.

Finally, we examined whether lincRNA-RoR regulation of miR-145 could affect breast cancer cell invasion. We transfected MCF7

![Figure 2](image_url)

**Figure 2.**
cells with tetON–lincRNA-RoR + rtTA and after 24 hours induced lincRNA-RoR expression with 100 ng/mL doxycycline. Induced cells were grown atop Matrigel-coated Transwell inserts and cells were allowed to migrate for 24 hours. Following 24 hours, we observed a significant increase in invasive cells, whereas control MCF7 cells showed little to no invasive activity (Supplementary Fig. S4A).

miR-145 directly targets the 3′UTR of ARF6 mRNA

In addition to understanding the molecular mechanisms underlying the regulation of miR-145, we wanted to probe what invasive pathways miR-145 might regulate in TNBC cells. We examined computationally predicted targets of miR-145 using TargetScan algorithm (31). Among the highest scoring predicted mRNA targets of miR-145 was ADP-ribosylation factor 6 (ARF6). ARF6 is a small GTPase that has been previously implicated as a critical regulator of tumor cell invasion in metastatic breast cancer (21). A previous proteomics study revealed that miR143/145 modulation altered ARF6 protein levels in colon cancer cells, suggesting that ARF6 might be a direct target for miR-145 (32). ARF6 mRNA 3′UTR contains an impressive five predicted miR-145-binding elements (Fig. 3A). We began by examining ARF6 expression in TNBC. We found that ARF6 demonstrated inverse expression relative to miR-145. ARF6 was dramatically overexpressed in TNBC cells compared with nontumorigenic mammary epithelial cells (Fig. 3B and C). To test the predicted binding of miR-145 to ARF6 mRNA, we cloned the ARF6 3′UTR downstream a luciferase ORF and performed luciferase reporter assays for ARF6 3′UTR. We found that overexpression of miR-145 resulted in a significant decrease in luciferase activity compared with control cells (Fig. 3D). Next, we examined ARF6 expression in MDA-MB-231 cells following miR-145 overexpression. We detected a small but significant decrease in ARF6 mRNA levels following miR-145 overexpression (Fig. 3E). However, following miR-145 overexpression, we detected a dramatic decrease in ARF6 protein levels (Fig. 3F). These data suggest that miR-145 inhibition of ARF6 is mostly occurring by interfering with ARF6 translation. Finally, to test whether our previously
identified interaction between lincRNA-RoR and miR-145 affect the targeting of ARF6 mRNA by miR-145, we examined ARF6 protein levels following knockdown of lincRNA-RoR by shRNA. We found that knockdown of lincRNA-RoR in MDA-MB-231 cells resulted in decrease in ARF6 protein (Supplementary Fig. S4B). These results support a lincRNA-RoR/miR-145/ARF6 pathway in TNBC cells.

It was previously reported that ARF6 might contribute to breast cancer invasion via regulating invadopodia or by regulating cell–cell adhesion through controlling E-cadherin localization (33, 34). This group found that in the presence of EGF ligand (activating EGFR signaling) ARF6 was able to repress E-cadherin at the protein level. We examined the impact of overexpression of constitutively active ARF6 on the invasive activities of nonmetastatic MCF-7 breast cancer cells. As confirmation of the earlier observations, we found that in the presence of EGF, MCF-7 cells overexpressing ARF6 showed a decrease in E-cadherin protein levels (Fig. 3G). Furthermore, MCF-7 cells overexpressing ARF6 were capable of invading Matrigel in Transwell invasion assays as evidenced by the staining of invasive protrusions on the bottom of Transwell inserts, whereas control MCF-7 cells demonstrated no invasive capabilities in this assay (Fig. 3H).

ARF6 overexpression alters E-cadherin localization and disrupts cell–cell junctions

To further examine the potential importance of ARF6 overexpression in breast cancer, we performed gain-of-function studies in MCF10A nontumorigenic mammary epithelial cells. We performed three-dimensional (3D) cell culture experiments with MCF10A cells overexpressing ARF6 and examined E-cadherin localization using immunofluorescence. 3D cell culture can be used to recapitulate mammary organogenesis in which control MCF10A cells grow into hollow acinar structures with polarized luminal and basolateral surfaces. Control MCF10A cells formed hollow acini structures with E-cadherin localization to cell–cell junctions (Fig. 4A). In ARF6-overexpressing MCF10A cells, there was an obvious loss of E-cadherin expression and localization to cell–cell junctions. Furthermore, morphology of ARF6-overexpressing acini was altered with greater spacing between nuclei as evidenced by DAPI staining, suggesting a disruption in cell–cell junctions.

The miR-145 target ARF6 is overexpressed in lymph node metastasis

As there is previously no clinical data suggesting that ARF6 expression is associated with breast tumor invasiveness, we examined ARF6 expression using IHC in a TMA with core samples from matched normal breast, primary tumor, and lymph node metastasis (Fig. 4B). We detected higher levels of ARF6 in some samples of primary tumor (IDC); however, it did not account for a statistically significant difference with ARF6 levels detected in our normal breast tissue. On the other hand, we did detect a statistically significant increase in ARF6 expression in lymph node metastasis.
staining in lymph node metastasis cores (P < 0.03). These data offer support for the clinical relevance of ARF6s in breast cancer metastasis.

**Discussion**

Deep sequencing studies have previously shown that miR-145 downregulation is a hallmark of TNBC (4). Here, using Transwell invasion assays, we have found that miR-145 regulates tumor cell invasion in TNBC and not apoptosis or proliferation. We examined the molecular mechanism responsible for miR-145 downregulation in TNBC and found that the lincRNA-RoR regulates mature miR-145 by serving as a competitive endogenous RNA sponge. This is the first report of this ceRNA network in human cancer.

To better understand the function of miR-145 in TNBC, we examined the predicted targets of miR-145 and identified ARF6, a small GTPase known to regulate endocytic recycling and previously implicated in breast tumor invasion (21). ARF6 mRNA 3'UTR contains five predicted miR-145-binding sites. Using a 3'UTR luciferase reporter, qRT-PCR, and Western blotting, we validated miR-145 targeting of the 3'UTR of ARF6 mRNA. Next, we found that ARF6 overexpression in MCF-7 cells promoted a more invasive phenotype as evidenced by Transwell invasion assays. We examined ARF6 function via 3D cell culture and found that overexpression of ARF6 results in loss of E-cadherin localization and disruption of cell–cell junctions.

Finally, we examined ARF6 expression in a breast tumor tissue array and found that ARF6 levels were significantly higher in lymph node metastasis, suggesting a role of ARF6 in breast cancer metastasis. On the basis of our *in vitro* findings, it is important to next examine whether miR-145 and ARF6 can regulate TNBC metastasis in vivo.

Previously, miR-145 and lincRNA-RoR have been implicated in embryonic and adult stem cells (10). These molecules may also be critical regulators of cancer stem cell biology, as it was previously reported that miR-145 is silenced in breast cancer stem cells. There is mounting evidence of a powerful connection between epithelial–mesenchymal transition and breast cancer stem cell (12). Here, we have demonstrated a connection between miR-145 and invasion/metastasis that involves altered cell morphology and loss of epithelial adherens junction protein E-cadherin. In future studies, it will be interesting to test whether miR-145 and lincRNA-RoR play important roles in regulating the cancer stem cell phenotype in TNBC, which has previously been shown to play a critical role in drug resistance and metastasis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: G. Eades, Q. Zhou

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Wolfison, Y. Zhang, Y. Yao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, Y. Yao

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Li, Q. Zhou

Study supervision: Q. Zhou

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**References**


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