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# Up-Regulation of microRNA-210 is Associated with Spermatogenesis by Targeting IGF2 in Male Infertility

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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**Background:** MicroRNAs (miRNAs) play pivotal roles in spermatogenesis. MicroRNA-210 (miR-210) expression was up-regulated in the testes of sterile men with non-obstructive azoospermia (NOA). However, the underlying mechanisms of miR-210 involved in the spermatogenesis in patients with NOA are unknown.

**Material/Methods:** Expression of miR-210 and insulin-like growth factor II (IGF2) in the testes of NOA cases (only including maturation arrest and hypospermatogenesis) were detected in this study. We carried out *in vitro* experiments to determine if IGF2 was directly targeted by miR-210 in NT2 cells.

**Results:** Compared with obstructive azoospermia (OA) as normal control, our results suggest that miR-210 was significantly up-regulated in testis of patients with NOA ($P<0.05$), and IGF2 was down-regulated, but without a significant difference. The results also indicated that IGF2 was directly targeted by miR-210 in NT2 cells.

**Conclusions:** The results showed that miR-210 was involved in spermatogenesis by targeting IGF2 in male infertility.

**MeSH Keywords:** Insulin-Like Growth Factor II • MicroRNAs • Spermatogenesis

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## Background

World-wide, infertility affects 10–15% of couples who are trying to conceive, and about 15% of these cases are caused by male factors, which affect 1 out of 20 men in the general population [1,2]. Most cases of male infertility are idiopathic, apart from several etiologies, such as obstruction of deferent duct, varicocele, sexual dysfunction, and cryptorchidism [3]. Although assisted reproductive technology (ART) has helped many sterile couples to conceive, non-obstructive azoospermia (NOA), which accounts for a considerable proportion of male infertility, has a dramatically lower rate of sperm retrieval and clinical pregnancy [4]. The etiological mechanism of NOA is unknown, but factors such as oxidative stress were considered to have effects on spermatogenesis, and some antioxidants have been effective in protecting spermatogenesis [5,6]. Therefore, it is helpful to explore the underlying pathogenesis of NOA in these patients.

MicroRNAs are a class of small RNAs that do not code amino acid sequences, but they play fundamental roles in regulating gene expression after transcription [7]. Lian et al. found 154 down-regulated miRNAs and 19 up-regulated miRNAs in testes of NOA patients compared to fertile males, by using microarray technologies [8]. Furthermore, some of the miRNAs have been shown to affect the proliferation, apoptosis, and DNA damage in germ cells [9–11]. MiR-210 is one of the 19 up-regulated miRNAs in testes of NOA patients, located within the genomic *loci* of transcript AK123483. It can be induced by hypoxia, and plays an essential role in cell adaptation to hypoxia [8,12]. miR-210 also affects regulation of diverse physiological processes, such as angiogenesis, cell survival, proliferation, cell cycle arrest, protein modification, and DNA damage repair [13,14].

Although miR-210 has been shown to be involved in regulation of physiological processes in various diseases and to be an up-regulated miRNA in testes of NOA patients, it remains unknown how miR-210 affects spermatogenesis. Hence, the aim of this study was to investigate the underlying mechanisms by which miR-210 is involved in the pathogenesis of spermatogenesis.

## Material and Methods

### Human testicular samples

We enrolled 25 patients (aged 18–41 years) with azoospermia (proven by 3 semen analyses from testicular biopsies from the First Affiliated Hospital of Anhui Medical University). Pathological examinations were performed on each testicular specimen. Combined with clinical features, 4 patients were diagnosed as having Sertoli-cell-only syndrome (SCOS), 7 patients were diagnosed as having maturation arrest (MA), 8 patients were diagnosed as having hypospermatogenesis, and the other 6 patients were diagnosed as having obstructive azoospermia (OA). All patients provided informed consent before their participation in this study. Our local Medical Ethics Committee approved this study before it began.

### Immunohistochemistry Staining

To examine the location of insulin-like growth factor II (IGF2) in human testicular tissues, we performed immunohistochemistry staining to detect the IGF2 expression. Tissues were cut into sections for immunoperoxidase staining after being treated with 4% PFA and paraffin wax. After the specific treatment with standard-procedure immunohistochemistry staining as described as Lian et al. [10], sections were incubated with IGF2 antibody (Abcam) overnight at 4°C and biotinylated secondary antibody (Abcam) for 2 h at room temperature.

### RNA extraction and real-time PCR

To detect expression of miR-210, quantitative real-time PCR was carried out. RNAs were extracted from NT-2 cells or tissues and subjected to real-time PCR as described as Lian et al. [10]. Briefly, RNA extraction was performed following a standard Trizol protocol, real-time PCR was carried out with the ABI Step One System (Applied Biosystems), and the SYBR Premix Ex Taq II kit (TaKaRa Bio, Inc.) was used. U6 was taken as normalization to detect the expression levels of mature miR-210. Primers for Q-RT PCR were as follows:
miR-210
Forward primer: 5'-CAATAACTGTGCGTGTGACAGC-3'
Reverse primer: 5'-TATGGTTTTGACGACTGTGTGAT-3'
U6
Forward primer: 5'-CAGCACATATACTAAAATTGGAACG-3'
Reverse primer: 5'-ACGAATTTGCGTGTCATCC-3'

### Western blotting

Western blot analysis was carried out to detect protein expression of IGF2 in the human testicular tissues in the 3 groups and in NT2 cells. The experiments were carried out as previously described [10]. Anti-IGF2 (Abcam) was used for Western blot analysis, and we used β-Actin as a loading control to detect expression of IGF2. Protein levels were quantified by use of ImageJ system (USA) software.

### Cell lines/cell culture and transfection

The NT-2 cells are derived from human embryonal carcinomas. Dulbecco's modified Eagle's medium was used to culture the cells. We supplemented the medium with 10% fetal bovine





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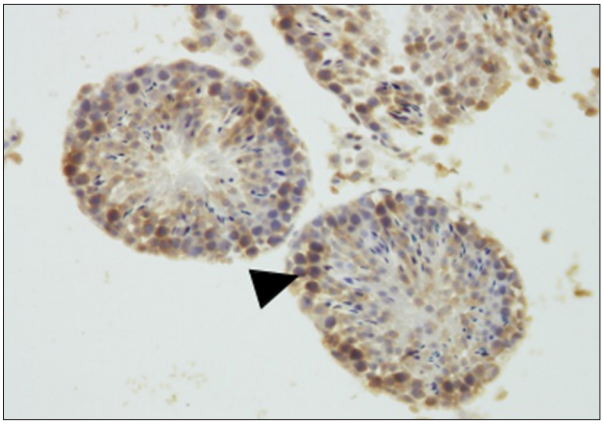

**Figure 1.** IGF2 foci exist in spermatocytes in the testis of humans.

serum (Life Technology Inc.), 1% antibiotics (100 Units/ml penicillin, and 100 ug/ml streptomycin, Life Technology Inc.). Cells were incubated at 37°C in a humidified incubator with 5% $CO_2$. To transfect oligonucleotides and plasmids into NT-2 cells, Lipofectamine RNAiMAX (Invitrogen) and Fugene HD (Roche) were used. All processes were performed in accordance with the protocols supplied by manufacturers.

### Statistical analysis

In this study, all the experiments were performed independently at least 3 times. Data are shown as mean ±SD and were analyzed using SPSS version 19.0. One-way ANOVA was used for comparisons of the data. P-values <0.05 were considered to be statistically significant.

## Results

### IGF2 located in spermatocytes in the testis of patients with OA

The IGF2 gene is part of a cluster of imprinted genes expressing the single polypeptide as IGF2, which is only produced from the paternal allele. The maternal allele is transcriptionally silent [15,16]. To clarify the location of IGF2 in human testicular tissues, immunohistochemistry staining was carried out to detect the expression of IGF2. We found IGF2 located in spermatocytes in the testes of patients with OA (Figure 1).

### Down-regulation of IGF2 in testes of MA and hypospermatogenesis patients

Because IGF2 is located in spermatocytes of the testis, we detected the expression of IGF2 in cases with MA, hypospermatogenesis, and OA, but not in the SCOS patients. We found that IGF2 was down-regulated in patients with MA and hypospermatogenesis compared to OA patients, which was considered as the control group with normal spermatogenesis, although without a significant difference (Figures 2, 3), possibly because there were fewer samples and longer preservation times of some samples.

### Up-regulation of miR-210 in testis of MA and hypospermatogenesis patients

Quantitative real-time PCR was performed to examined miR-210 expression in the testis of patients with MA, hypospermatogenesis, and OA. We found that miR-210 was significantly up-regulated in the testis of MA and hypospermatogenesis patients compared to OA patients (Figure 4). However, due to errors in the RNA extraction in the preliminary experiment, 3 testis samples (1 each) from MA, hypospermatogenesis, and OA patients were damaged and were not tested.

### IGF2 was targeted directly by miR-210

In the TargetScan database, because the 3'UTR of the IGF2-mRNA has a putative miR-210-binding site, IGF2 was predicted to be a potential target of miR-210. To identify whether the IGF2 gene was targeted by miR-210 directly, Renilla luciferase reporters, which include the wild-type full-length 3'UTR forms of miR-210 seeding sites, were used. Figure 5 shows that there was a 60% decrease in luciferase activity after cotransfection of the miR-210 mimic and the Renilla luciferase reporters into NT2 cells, and inhibiting miR-210 expression increased activity of the reporter Renilla luciferase. Expression of IGF2 protein was also significantly lower in the NT2 cells transfected with miR-210 mimics than in control cells, and knockdown of miR-210 with miR-210 inhibitor increased protein expression of IGF2 (Figures 6). The results demonstrated that miR-210 directly down-regulated IGF2 by decreasing mRNA stability.

## Discussion

During recent decades several studies have focused on the effects of miRNAs on spermatogenesis in male infertility [9–11,17]. However, it was not understood how miR-210, which is one of the up-regulated miRNAs in testes of patients with NOA, was involved in spermatogenesis in male infertility.

The transformation of diploid spermatogonia into mature haploid cells in spermatogenesis is a complex biological process in the testes of males [18]. The insulin/IGF system takes part in the processes of cell proliferation, cell growth, differentiation, and survival, which affects nearly every organ in the body [19]. Also, insulin/IGF plays an important role in the proper function of the testis in males [20]. IGF2 binds to IGF1R and INSR-A with a high affinity and binds to INSR-A/IGF1R, INSR-B/IGF1R,





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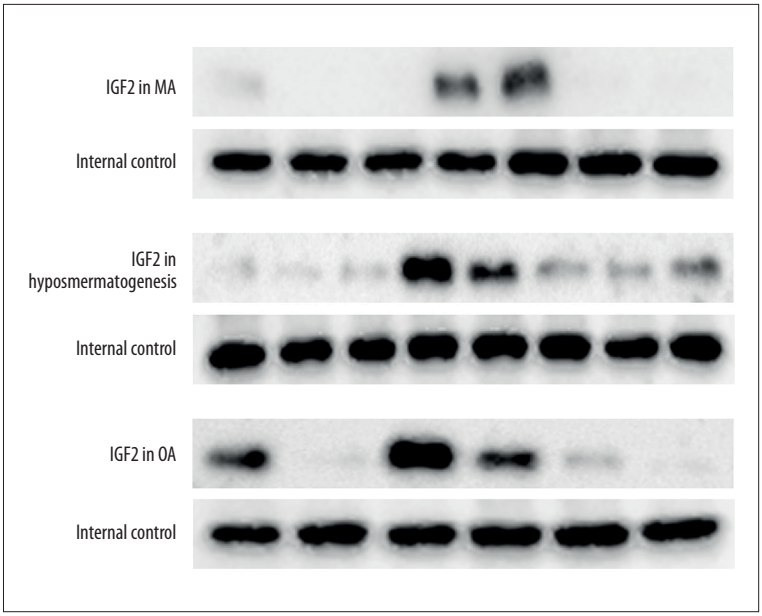


**Figure 2.** Expression of IGF2 in testes of patients with MA, hypospermatogenesis, and OA.

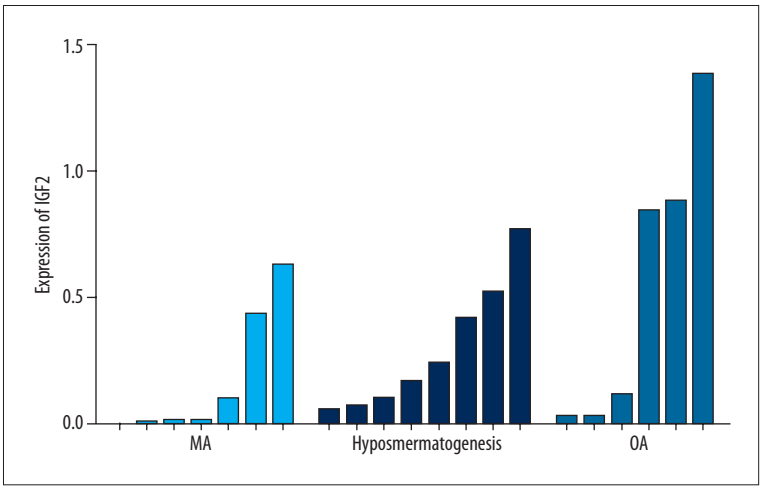


**Figure 3.** IGF2 was down-expressed in MA and hypospermatogenesis compared to OA patients, but without a significant difference (MA – maturation arrest, OA – obstructive azoospermia).

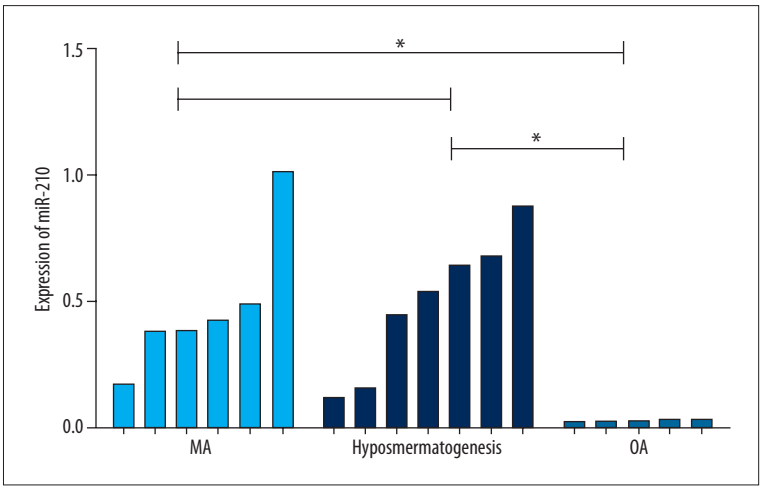


**Figure 4.** miR-210 was significantly up-regulated in testis of patients with MA and hypospermatogenesis compared to OA (MA – maturation arrest, OA – obstructive azoospermia; * P<0.05).





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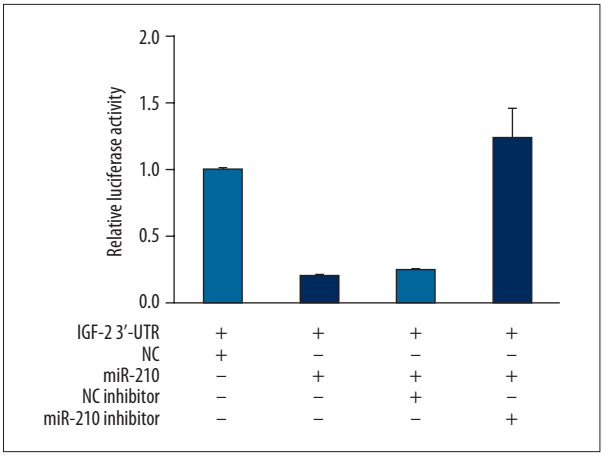


**Figure 5.** Cotransfection of the miR-210 mimic and the reporters into NT2 cells resulted in an 60% decrease in luciferase activity, whereas inhibition of miR-210 expression increased the reporter activity.

but with lower affinity [21]. Pitetti et al. found that in inactivated INSR and IGF1R, there was a 79% reduction in daily sperm production in adult mouse testes by a conditional KO approach [22]. Taken together, the aforementioned data suggest that IGF2 might be involved in the process of spermatogenesis.

To examine the specific mechanism by which miR-210 is associated with the process of spermatogenesis, quantitative real-time PCR was performed to detect miR-210 expression. We found that miR-210 was significantly up-regulated in the testes of subjects with MA and hypospermatogenesis patients compared to OA. These results agree with findings of Lian et al. using microarray technologies performed in NOA and normal controls [10]. Several studies have suggested that this miRNA could be mediated by hypoxia and participate in various types of regulation of angiogenesis, cell survival, proliferation, cell cycle arrest, and protein modification [12–14]. Furthermore, some researchers even found that miR-210 might be considered as one of the indicated markers in some diseases, such as clear cell renal cell carcinoma and acute myeloid leukemia [23,24]. In the present study we found that IGF2 was targeted by miR-210 directly in the *in vitro* experiment in NT2

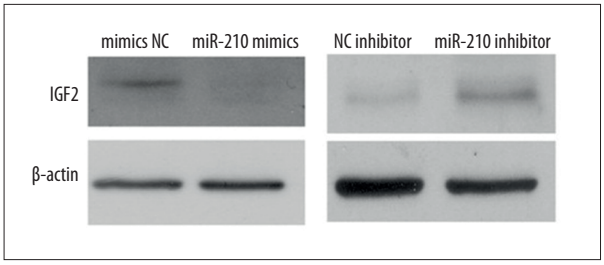


**Figure 6.** Expression of IGF2 was significantly decreased in transfected NT2 cells with miR-210 mimics compared to in control cells, and knockdown of miR-210 with miR-210 inhibitor increased protein expression of IGF2.

cells, and miR-210 might be associated with spermatogenesis by targeting IGF2 in male infertility.

Some limitations should be considered in this research. Firstly, as some errors occurred in the RNA extraction in the preliminary experiment, miR-210 of 3 testes samples were damaged and not detected in the subsequent quantitative real-time PCR experiment, which might have affected our results. Secondly, we did not investigate the functions of miR-210 and IGF2 *in vitro* or *in vivo*, and we plan to do this in future research.

## Conclusions

We demonstrated that miR-210 might be associated with spermatogenesis by targeting IGF2 in male infertility. Future mechanistic studies on the role of miR-210/IGF2 in the process of spermatogenesis in male infertility will provide new insights into the diagnosis and management of male infertility.

### Competing interests

There are no conflicts of interests to disclose.

### Acknowledgements

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