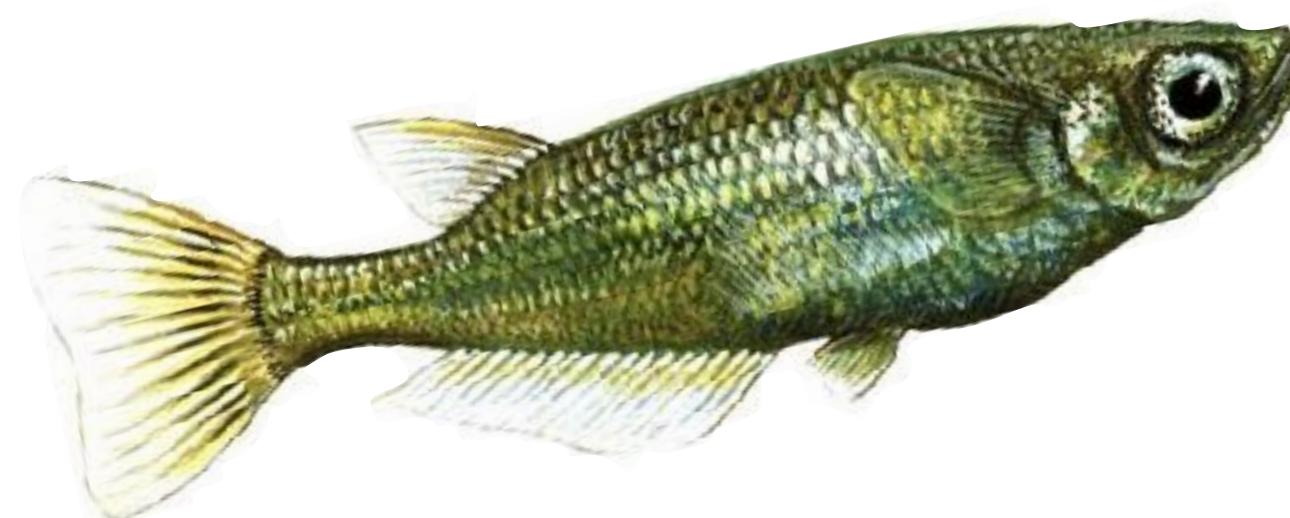


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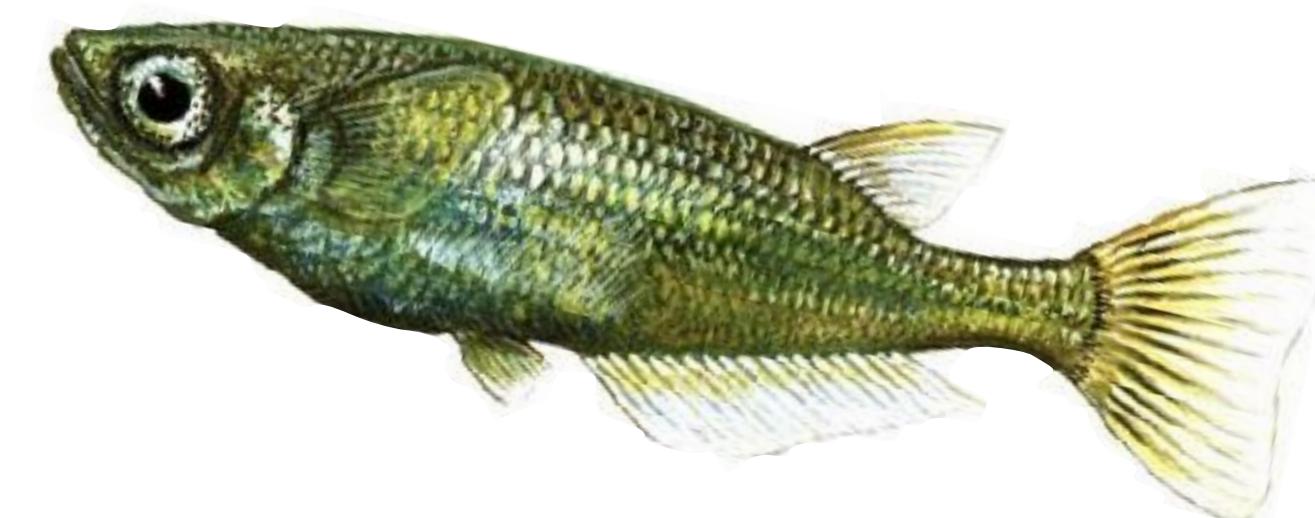
BSc (Hons) In Applied Biology
Oral Presentation

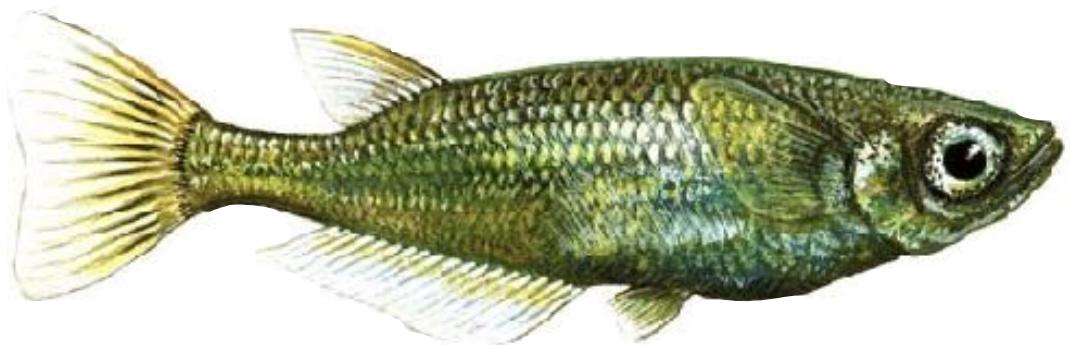
**Identification and characterization of hypoxia-responsive
microRNAs and target genes potentially associated with
regulation of reproductive functions in male marine medaka**



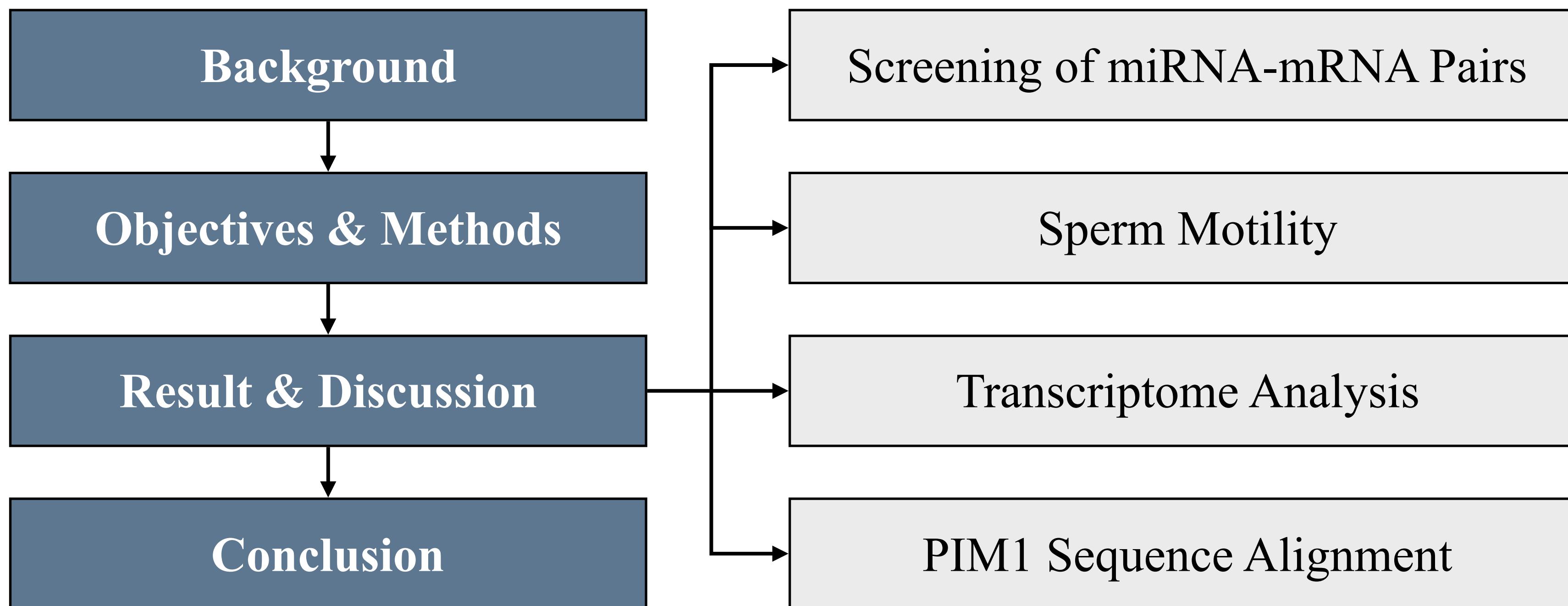
by
Chan Ka Lung
53574734

March 2017





Outline



Background

Hypoxia-responsive miRNAs alter testicular functions

- A study suggested the possible role of **hypoxia-responsive miRNAs** in targeting epigenetic changes that regulate **testicular functions** (Tse et al., 2016), and apoptosis and steroidogenesis in the ovary (Tse et al., 2015; Lai et al., 2016) using small RNA sequencing.

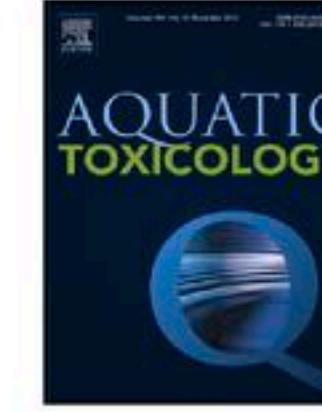
Aquatic Toxicology 180 (2016) 266–273

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Hypoxia alters testicular functions of marine medaka through microRNAs regulation

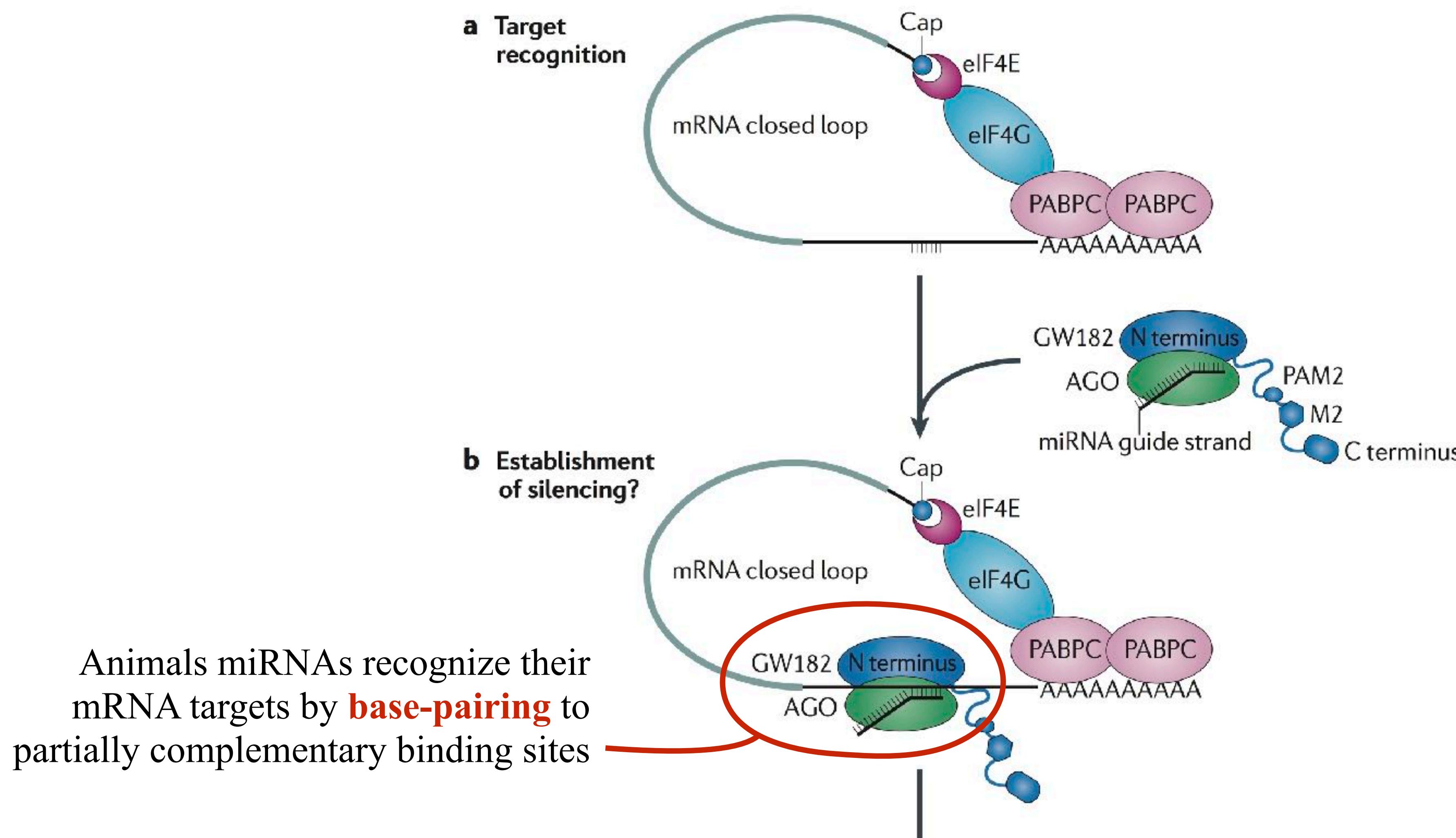
Anna Chung-Kwan Tse ^{a,1}, Jing-Woei Li ^{b,1}, Simon Yuan Wang ^a, Ting-Fung Chan ^b,
Keng Po Lai ^{c,*}, Rudolf Shiu-Sun Wu ^{d,e,**}

^a School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, China
^b School of Life Sciences, State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong SAR, China
^c Department of Biology and Chemistry, City University of Hong Kong, Hong Kong SAR, China
^d Department of Science and Environmental Studies, The Education University of Hong Kong, Hong Kong SAR, China
^e State Key Laboratory in Marine Pollution, Hong Kong

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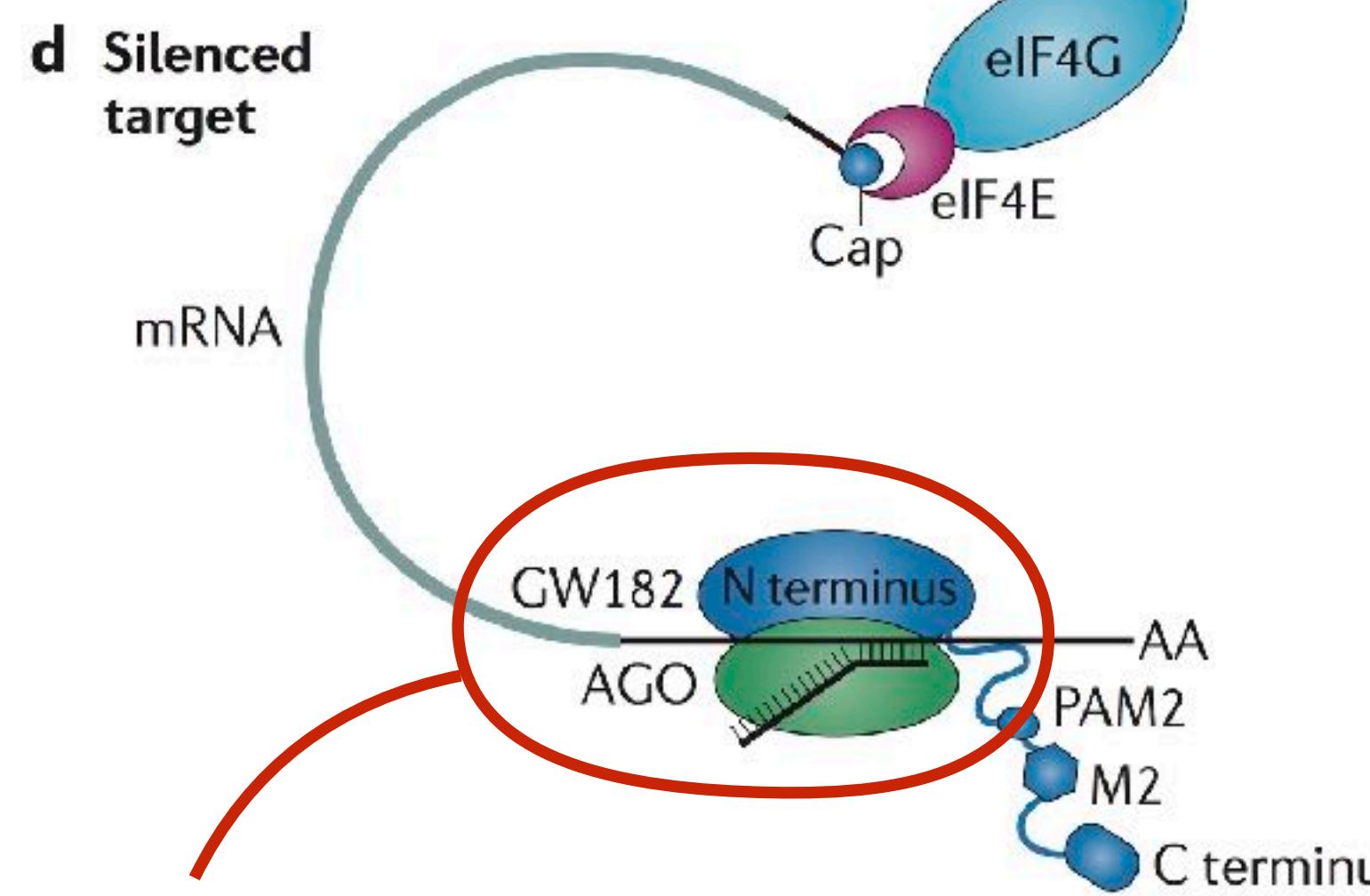
Mechanisms of miRNA-mediated Gene Silencing in Animals

(Modified from Huntzinger & Izaurralde, 2011)

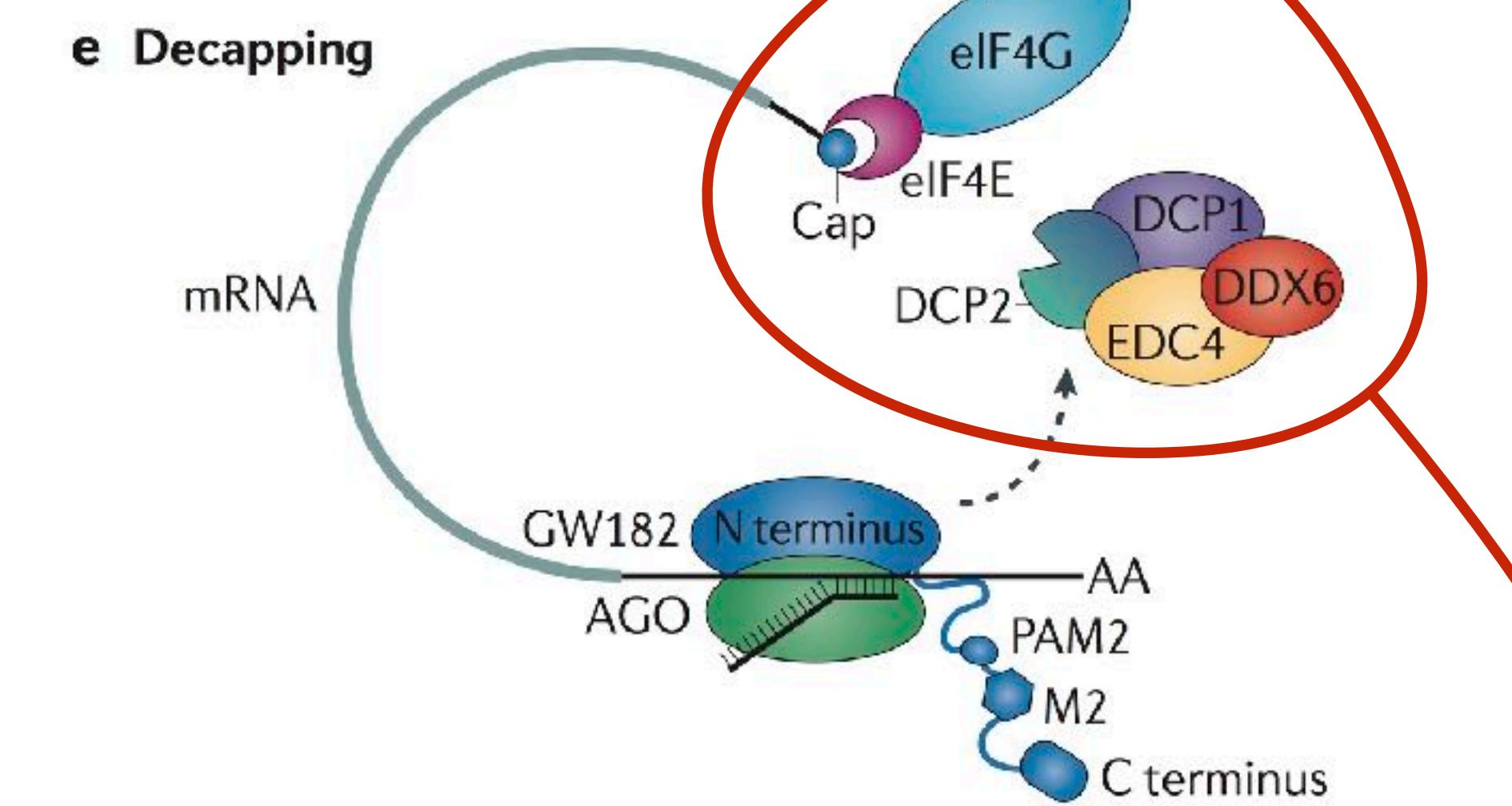




The AGO–GW182 complex directs the mRNA to **deadenylation**



Depending on the cell type and/or target, deadenylated mRNAs can be stored in a **translationally repressed state**



Alternatively, deadenylated mRNAs are decapped and rapidly **degraded** by exonuclease

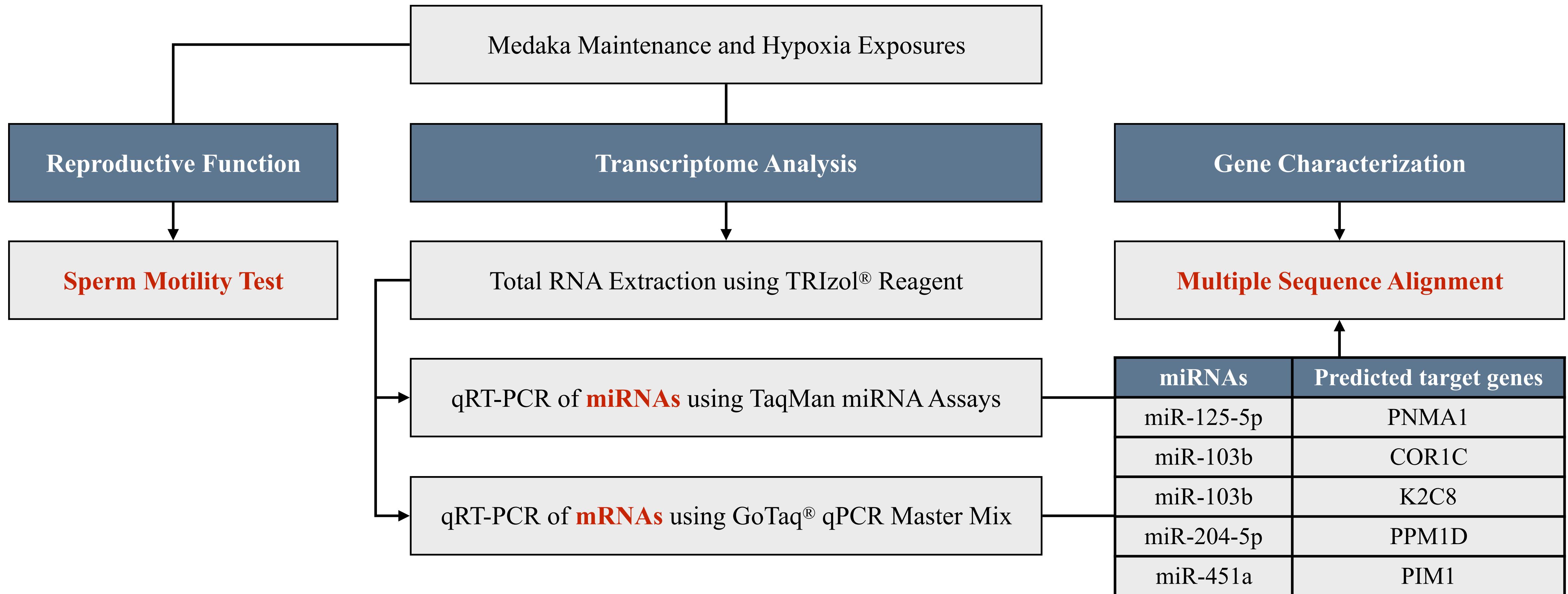
Objectives & Methods

Objectives

- To follow up on the earlier study, this project aims to **identify and characterize several hypoxia-responsive miRNAs** in marine medaka testes that may silence target genes that **control reproductive functions** in male fish.

Pairs	miRNAs with differential expression in hypoxic medaka testes	Gene Target Predicted by Bioinformatic Analysis
1	miR-125-5p	PNMA1
2	miR-103b	COR1C
3	miR-103b	K2C8
4	miR-204-5p	PPM1D
5	miR-451a	PIM1

Methods



Result & Discussion

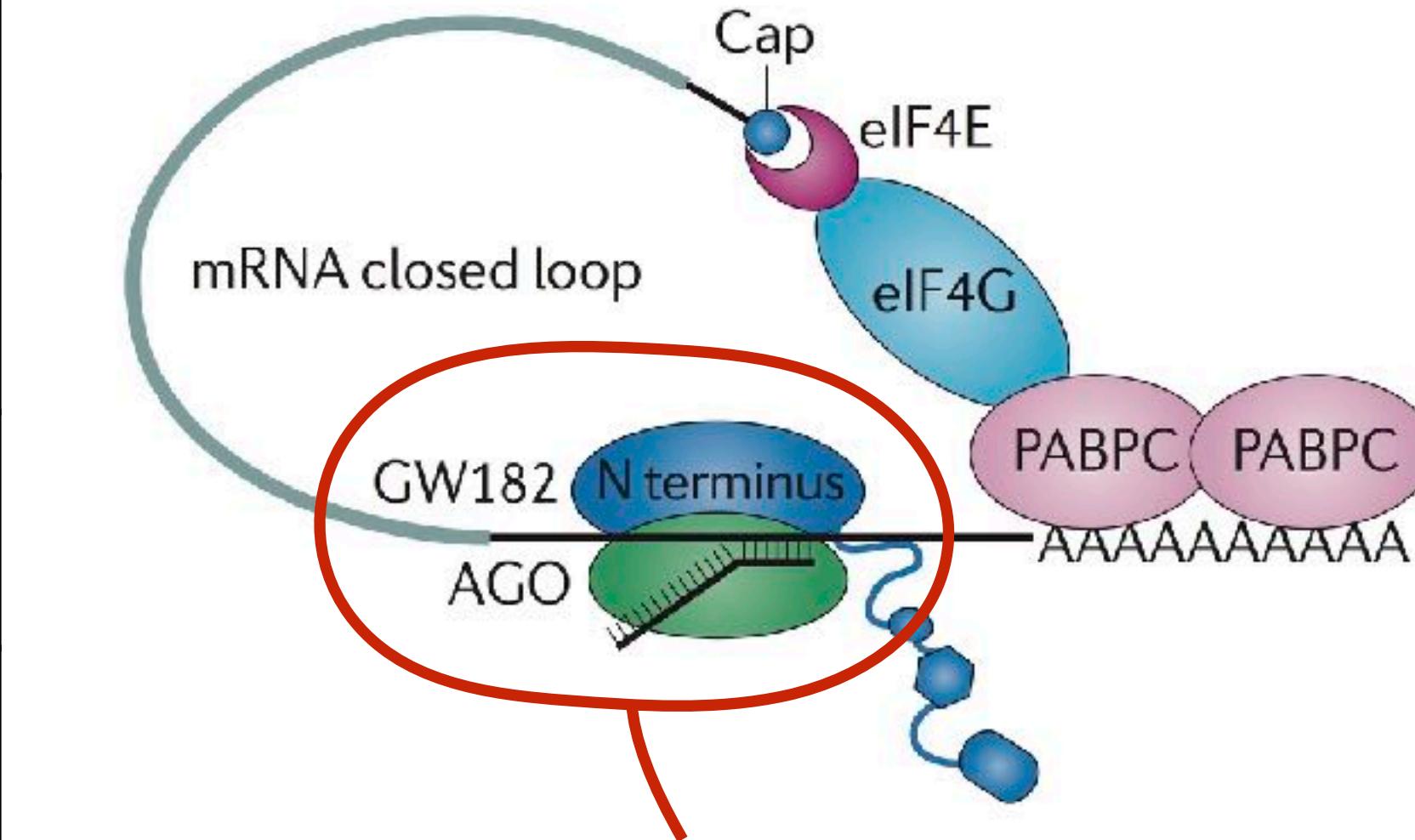
5 Criteria to Screen Transgenerational Hypoxia-Responsive miRNA-mRNA Pairs

- In order to understand the regulatory role of miRNAs on reproductive functions of marine medaka testis under hypoxia, **5 miRNA-mRNA pairs** that fulfill the following 5 criteria are selected using bioinformatic analysis:

1	Hypoxia-Responsive:	The miRNAs and their target mRNAs are hypoxia-responsive.
2	miRNA Regulation:	The mRNAs could be targeted by miRNA and regulated through RNAi.
3	Expression Patterns:	The expression of miRNA and mRNA has an inverse correlation.
4	Transgenerational:	The mRNAs are co-regulated transgenerationally.
5	Reproduction:	The mRNAs are associated with reproductive function.

Rationale for the Selected miRNA-mRNA Pairs: miRNA Regulation

Pair	miRNA	Gene symbol	Hypoxia-responsive miRNAs targeted sequences of selected genes associated with reproduction
1	miR-125-5p	PNMA1	miR-125b-5p 3' AGUGUUCAAUCCCAGAGUCCU 5' : PNMA1 5' AACAAATGGAAAATCTCAGGGT 3'
2	miR-103b	COR1C	miR-103b 3' UCGUCGUAAACAUGUCCCCGAUACU 5' : COR1C 5' ATCTCCCTTAAGAACGGCTATGT 3'
3	miR-103b	K2C8	miR-103b 3' UCGUCGUAAACAUGUCCCCGAUACU 5' : K2C8 5' ATCCACTTCCTCTCTGGCTATGA 3'
4	miR-204-5p	PPM1D	miR-204-5p 3' UCCGUAUCCUACUGUUUCCU 5' : PPM1D 5' ATAAAAGAACGTATTAAAGGGAT 3'
5	miR-451a	PIM1	miR-451a 3' UUGAGUCAUUACC AUUGCCAA 5' : : PIM1 5' GTCAAAGGAGTC TAACGGTT 3'



Partially complementary binding sites:
 The 'seed' sequence generally located in the mRNA 3' UTR and miRNA 5' end, containing nucleotides 2-7, is a major determinant in target recognition and is sufficient to trigger silencing

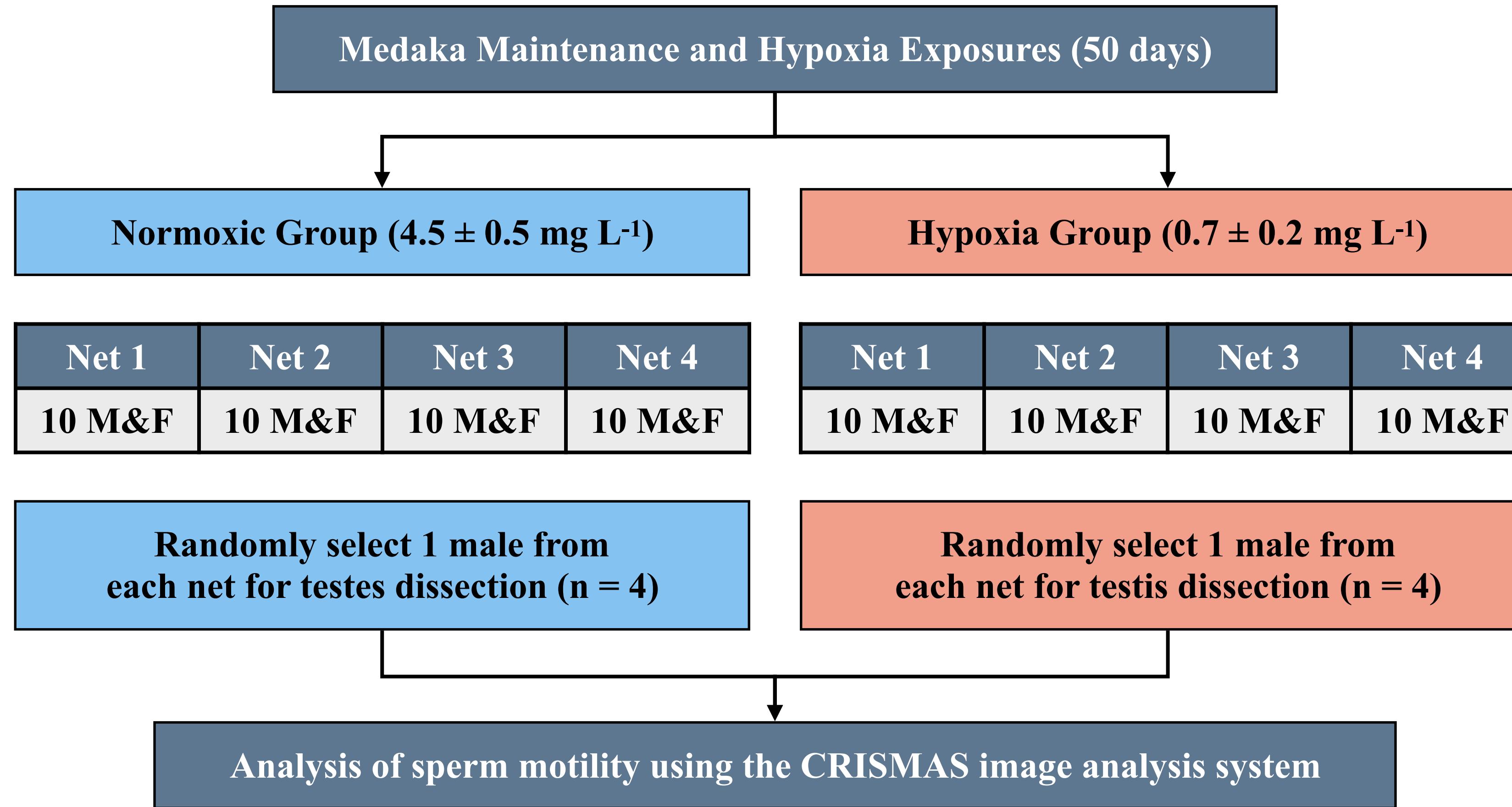
Rationale for the Selected miRNA-mRNA Pairs: Reproductive Function

Pair	miRNA	Gene symbol	Gene target	Gene functions
1	miR-125-5p	PNMA1	Neuron- and testis-specific protein 1	Positive regulation of apoptosis (RefSeq, 2016; Chen & D'Mello, 2010); Spermatogenesis (Dalmau et al., 1999)
2	miR-103b	COR1C	Coronin-3	Modulator of actin cytoskeleton dynamics (Behrens et al., 2016); Spermatogenesis (Larance et al., 2011); Apoptosis (RefSeq, 2016)
3	miR-103b	K2C8	Type-II keratin Kb8	Ovarian tumorigenicity; Apoptosis (Collodoro et al., 2012)
4	miR-204-5p	PPM1D	p53-induced protein phosphatase 1	Progesterone receptor activity (Proia, Nannenga, Donehower, & Weigel, 2006), Negative regulation of cell proliferation
5	miR-451a	PIM1	Serine/threonine-protein kinase pim-1	Cell cycle progression, apoptosis and tumorigenesis (Wang et al., 2001; Bachmann & Möröy, 2005); Oogenesis (Guzmán et al., 2014)

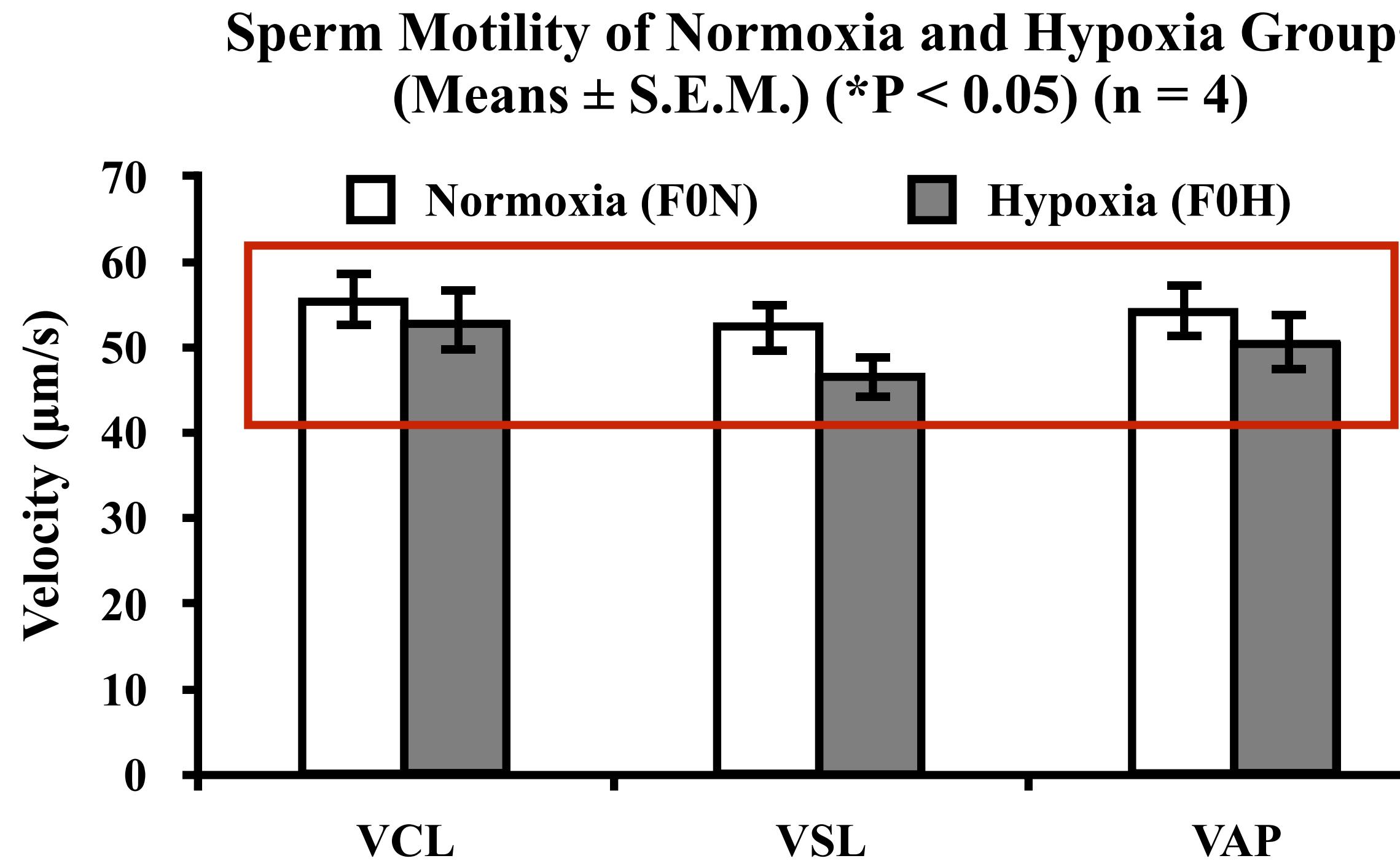
- Base on literature search, these 5 genes are associated with **reproductive functions** such as steroidogenesis, spermatogenesis, oogenesis, sexual development or apoptosis.

Certain roles in spermatogenesis?

Effect of Hypoxia on Sperm Motility



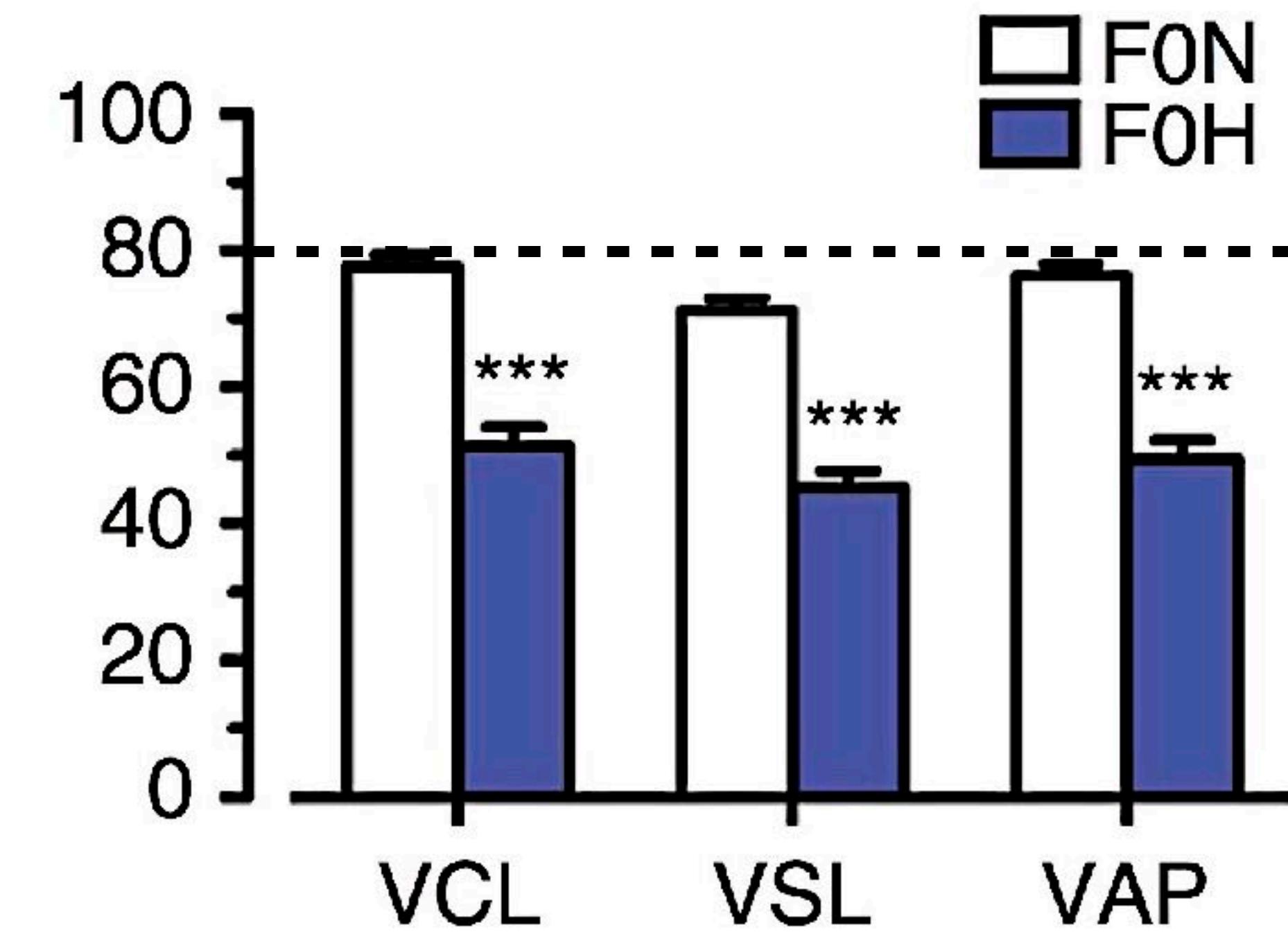
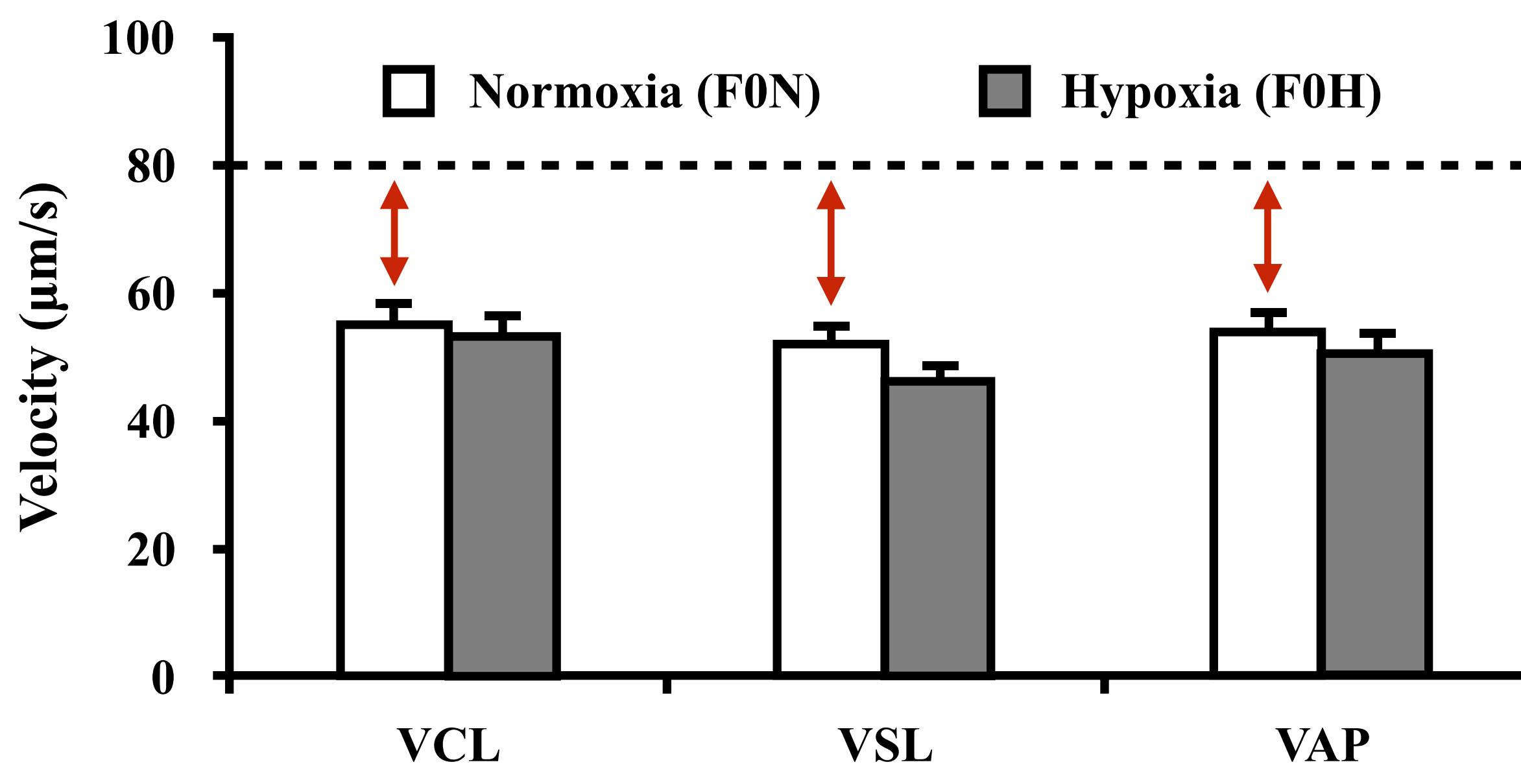
Effect of Hypoxia on Sperm Motility



Sperm motility of marine medaka in normoxia and hypoxia groups. VCL, mean curvilinear velocity; VSL, mean straight line velocity; VAP, mean angular path velocity. Data are presented as the means \pm S.E.M. (*P < 0.05) (n = 4).

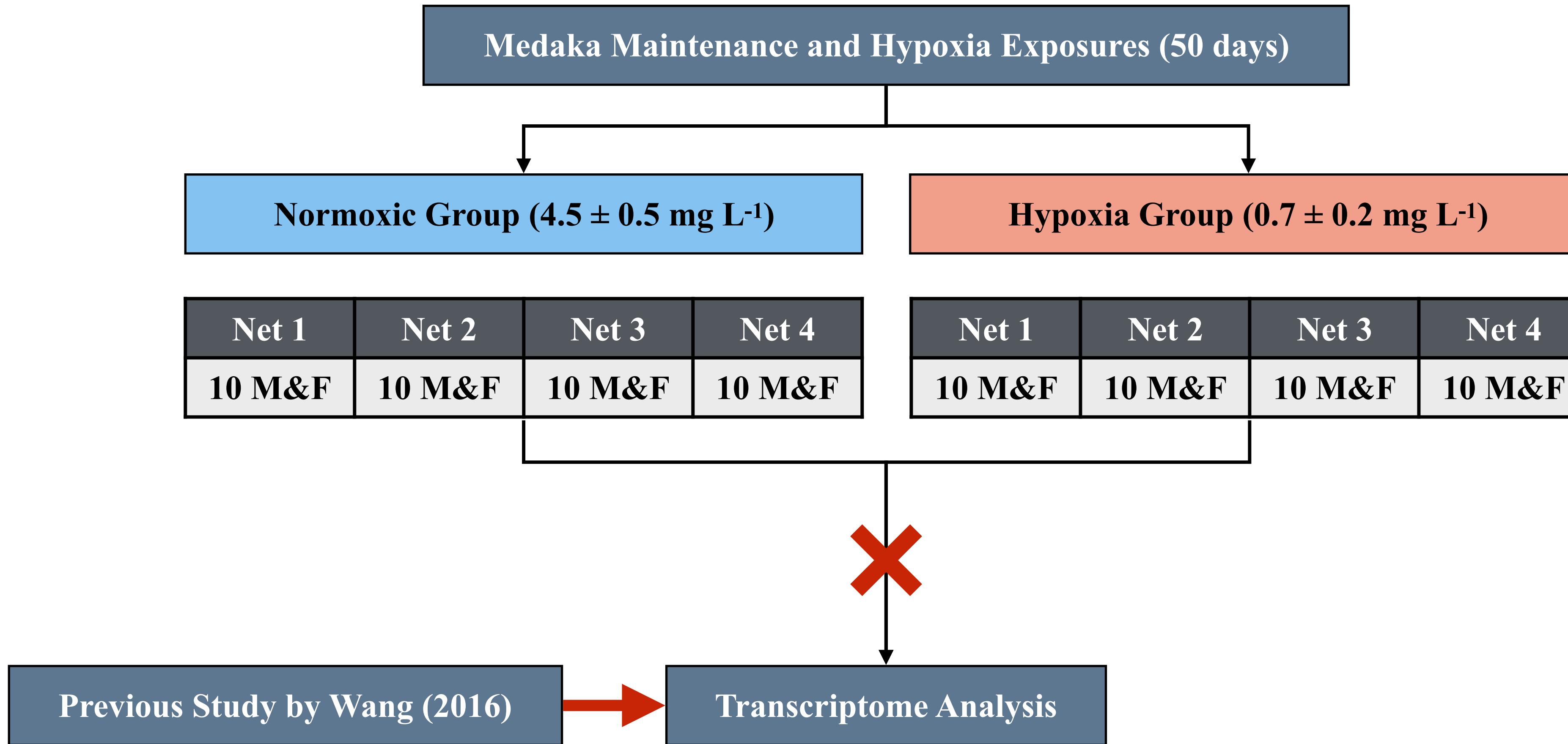
Effect of Hypoxia on Sperm Motility

Sperm Motility of Normoxia and Hypoxia Groups
(Means \pm S.E.M.) (*P < 0.05) (n = 4)

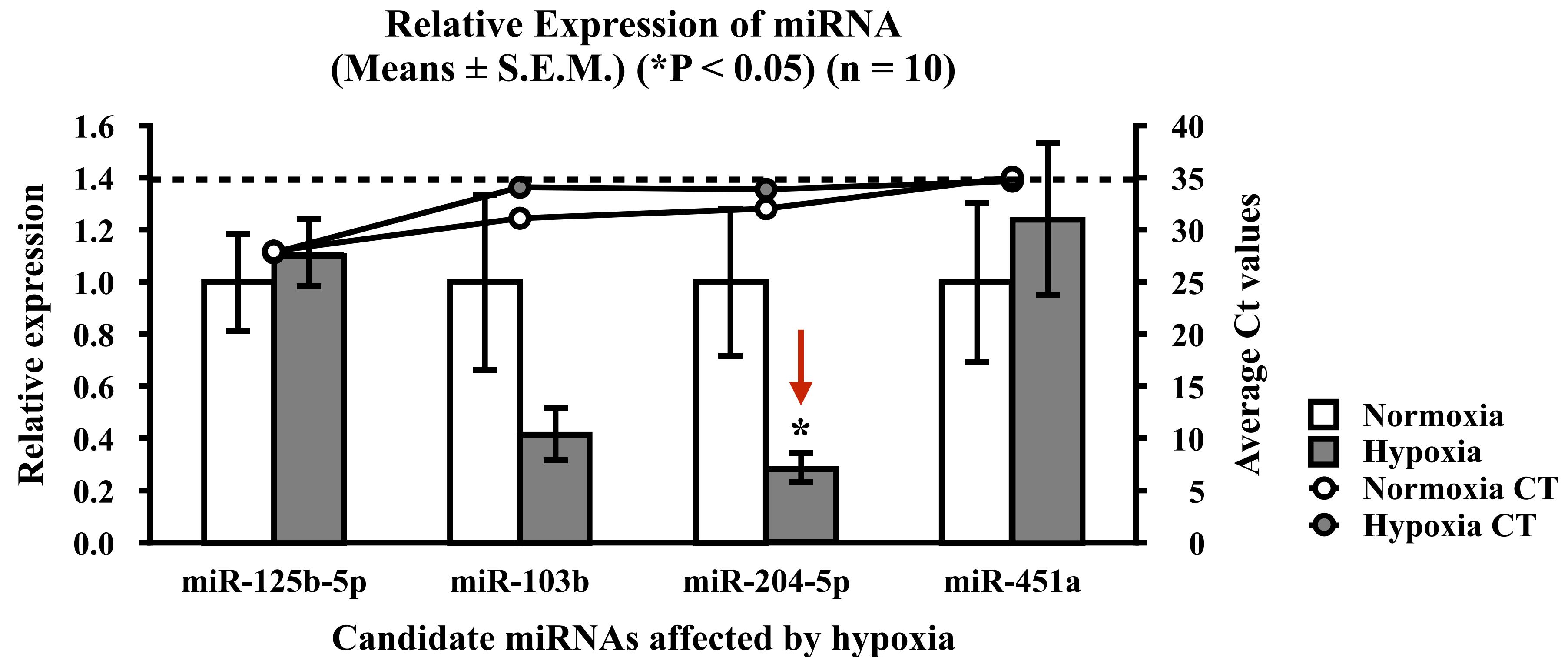


(Modified from Wang et al. 2016)

Effect of Hypoxia on Sperm Motility

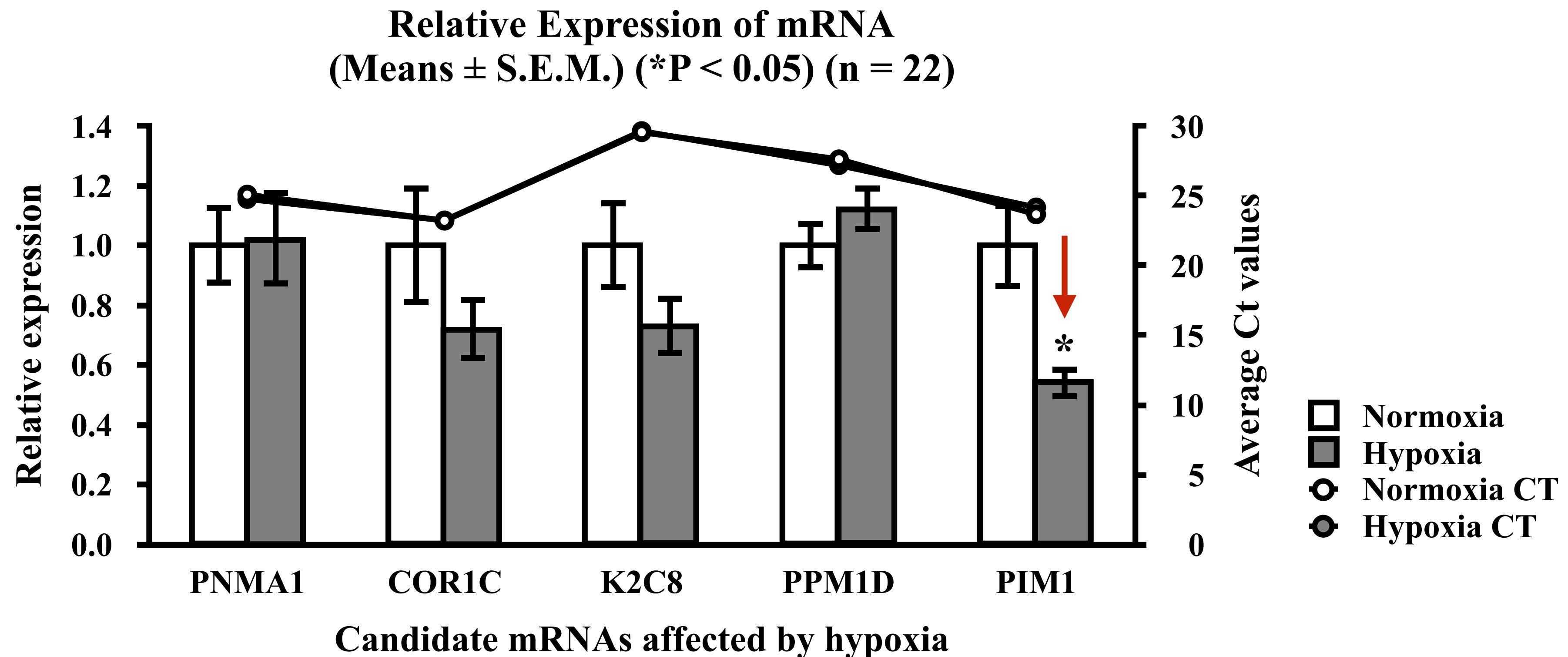


Effect of Hypoxia on Transcriptome (miRNA)



Validation of hypoxia-responsive miRNAs identified by small RNA sequencing using qRT-PCR. TaqMan® MicroRNA Assay was used to determine the deregulation of miR-125b-5p, miR-103b, miR-204-5p and miR-451a caused by hypoxia. Data are presented as the means \pm S.E.M. (*P < 0.05) (n = 10).

Effect of Hypoxia on Transcriptome (mRNA)

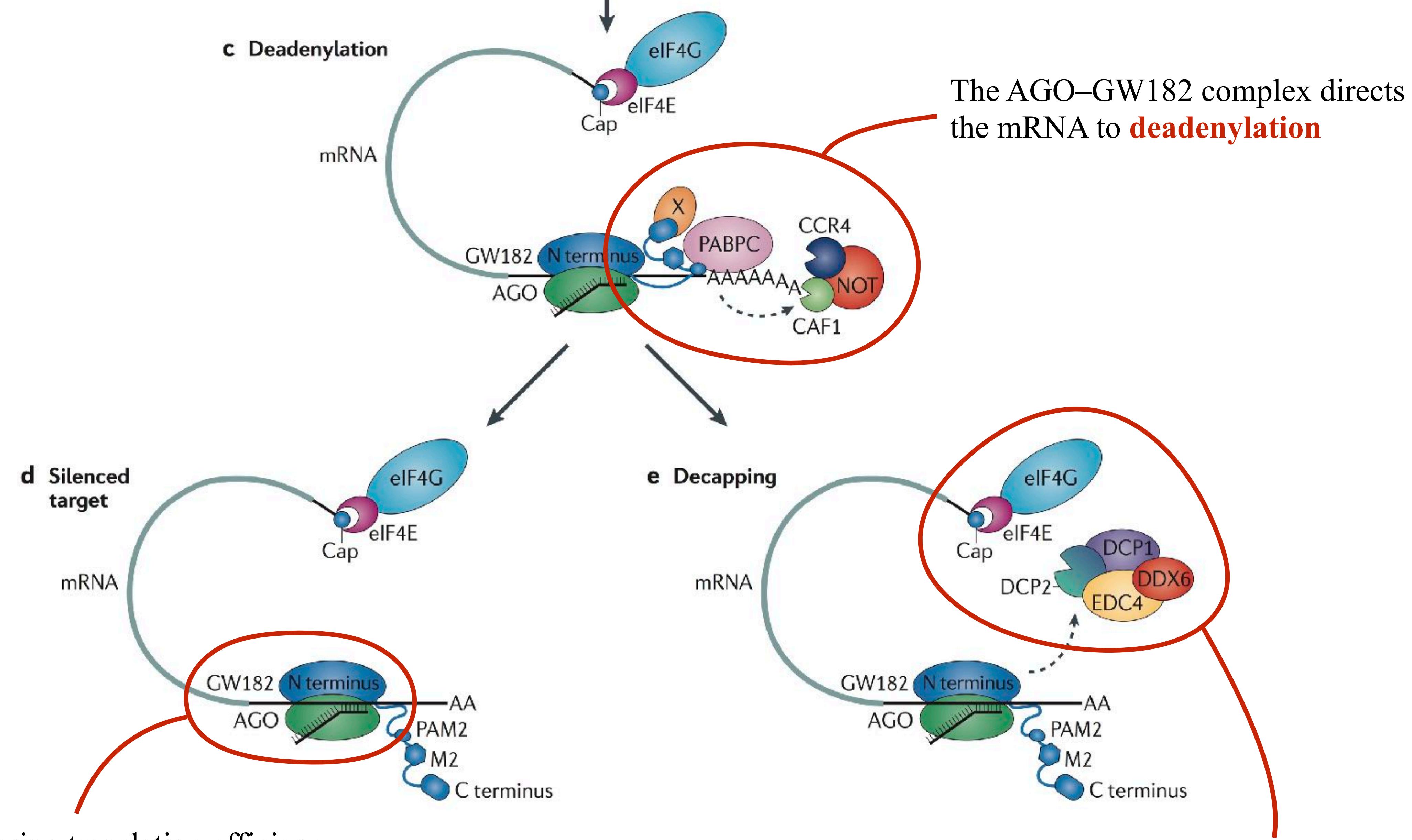


Validation of hypoxia-responsive genes identified by RNA-seq using qRT-PCR. GoTaq® qPCR Assay was used to examine the hypoxia-induced differential gene expression of PNMA1, COR1C, K2C8, PPM1D, and PIM1. Data are presented as the means \pm S.E.M. (* $P < 0.05$) ($n = 22$).

The Regulatory Role of miRNA-mediated Gene Silencing

- Based on the qRT-PCR results, the expression pattern of miRNAs and mRNAs are only **marginally consistent**, respectively, with the previous results of small RNA sequencing by Tse et al. (2016) and transcriptome sequencing by Wang et al. (2016), because most of the results are not statistically significant.

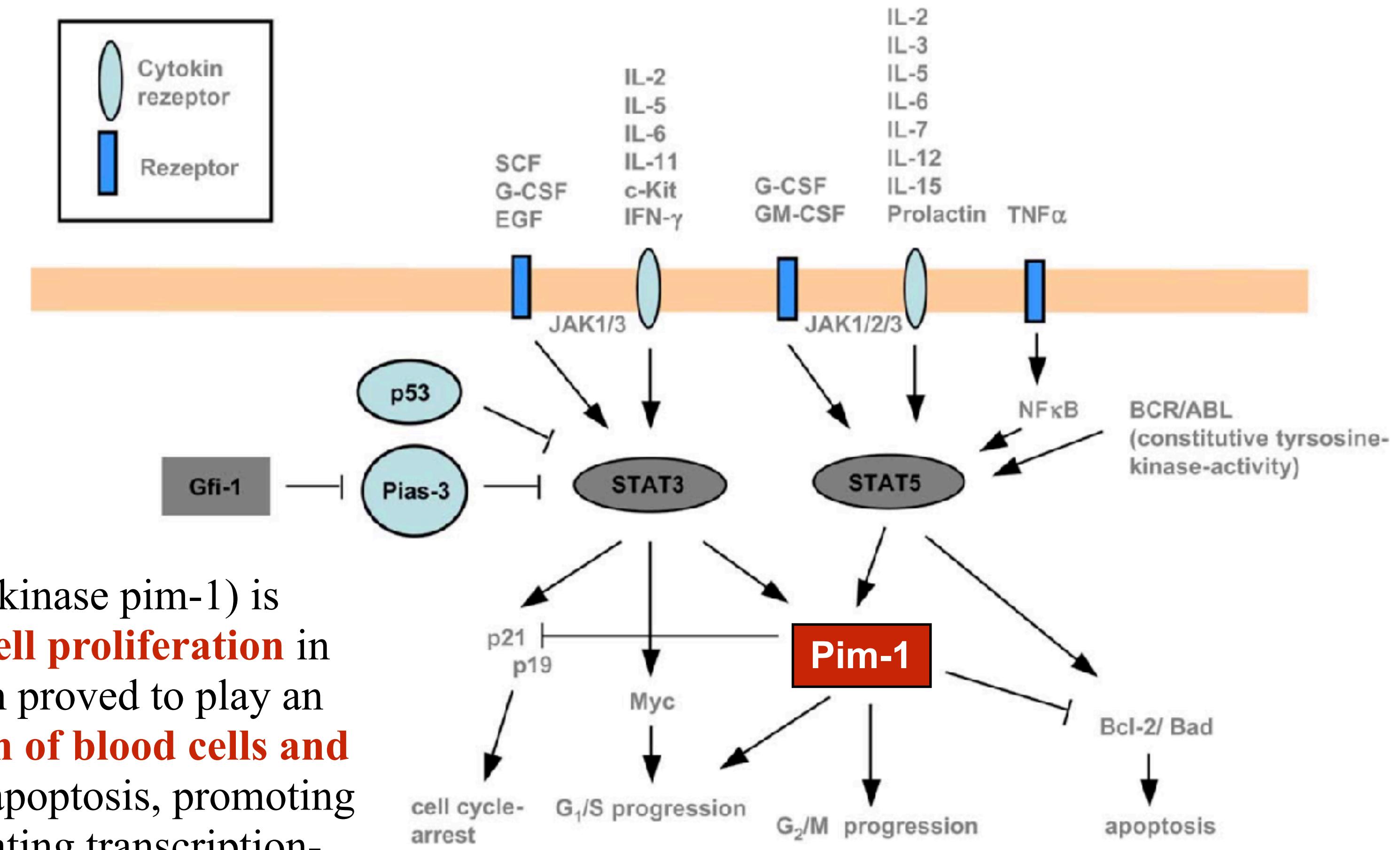
Pair	miRNAs	Results of small RNA sequencing	Result of qRT-PCR on miRNAs	mRNAs	Results of transcriptome sequencing	Result of qRT-PCR on mRNAs
1	miR-125-5p	Downregulated	Upregulated	PNMA1	Upregulated	Upregulated
2	miR-103b	Downregulated	Downregulated	COR1C	Upregulated	Downregulated
3	miR-103b	Downregulated	Downregulated	K2C8	Upregulated	Downregulated
4	miR-204-5p	Downregulated [#]	Downregulated*	PPM1D	Upregulated	Upregulated
5	miR-451a	Upregulated	Upregulated	PIM1	Downregulated [#]	Downregulated*



Repressing translation efficiency
reduces **the protein expression**

mRNA degradation reduces **the mRNA expression**

PIM1 Sequence Alignment and Its Possible Roles



- PIM1 (Serine/threonine-protein kinase pim-1) is associated with **apoptosis** and **cell proliferation** in mice and human. PIM1 has been proved to play an important role in **the production of blood cells and lymphatics cells** by repressing apoptosis, promoting cell cycle progression and mediating transcription-repressing proteins. (Bachmann & Möröy, 2005).

PIM1 Sequence Alignment and Its Possible Roles

- omPIM1 shared **high sequence identity** with the PIM1 of Japanese Medaka (63%) (highlighted in BLUE). The PIM1s of most fishes have 400-500 amino acids (highlighted in ORANGE), compared to around 300 amino acids in zebrafish, rat, human and frog (highlighted in TEAL).

Abbreviation	Common Name	Scientific Name	Identity	Length	Sources
omPIM1	Marine Medaka	<i>Oryzias melastigma</i>	-	501	Lai et al., 2015
olPIM1	Japanese Medaka	<i>Oryzias latipes</i>	63%	443	Unpublished, Genbank: XP_011492215
lcPIM1	Barramundi Perch	<i>Lates calcarifer</i>	42%	455	Unpublished, Genbank: XP_018548135
mzPIM1	Zebra Mbuna	<i>Maylandia zebra</i>	42%	495	Unpublished, Genbank: XP_014269581
onPIM1	Nile Tilapia	<i>Oreochromis niloticus</i>	44%	414	Unpublished, Genbank: XP_019217914
croakerPIM1	Large Yellow Croaker	<i>Larimichthys crocea</i>	38%	524	Genbank: KKF24757
zebraPIM1	Zebrafish	<i>Danio rerio</i>	34%	336	Genbank: NP_001116186
ratPIM1	Rat	<i>Rattus norvegicus</i>	35%	313	Genbank: NP_058730
humanPIM1	Human	<i>Homo sapiens</i>	34%	313	Genbank: AAH20224
frogPIM1	Frog	<i>Xenopus laevis</i>	32%	337	Genbank: NP_001088619

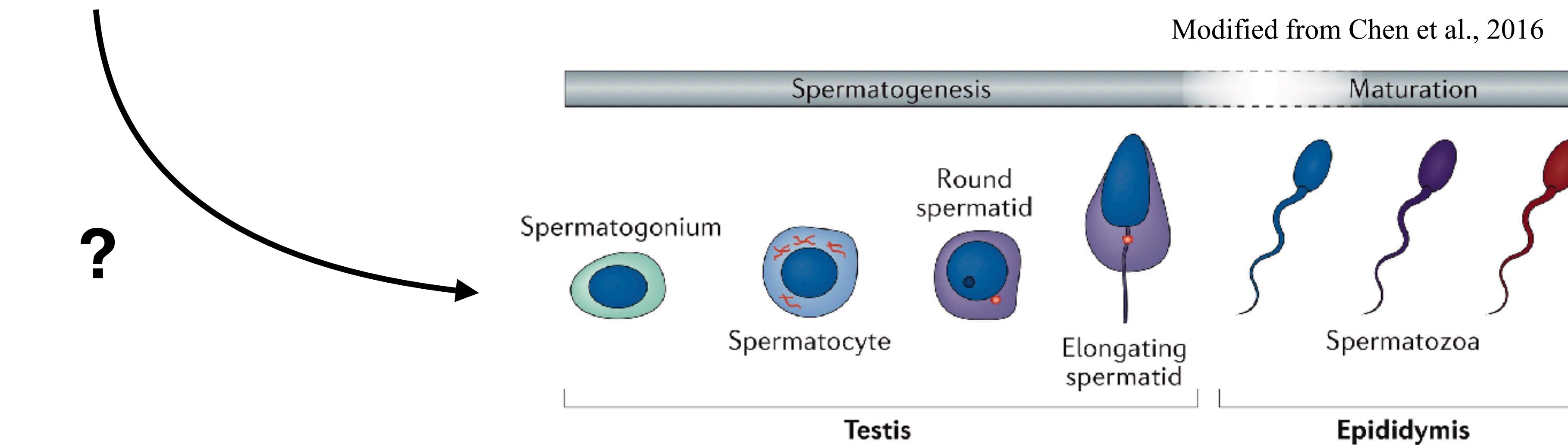
- The result shows that Marine Medaka PIM1 contains all of the **8 functional domains** that are highly conserved in other vertebrate species. Studies suggested that these regions are involved in ATP binding, phosphorylation or substrate binding. Based on the sequence identities, marine medaka PIM1 represents the **homolog of vertebrate PIM1.**

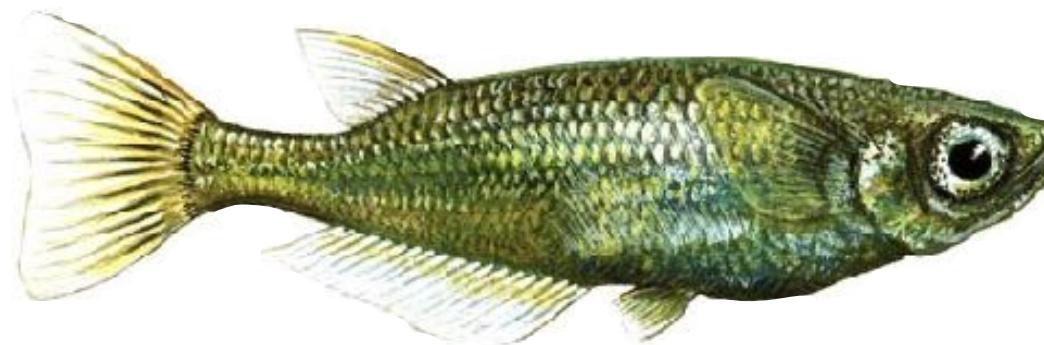
	Active Site	ATP Binding	
omPIM1	RPIPCEDMYIENKGGKLQEEEAKIIMKQLIHTAIDLEERNIFHQDIKIDNILIQTSSD		389
olPIM1	RPIPCEDLFHYIDTKGGTLEEEEAKIIMTQLIRTAIDLEDRNIFHQDIKSENILIQSHSN		331
lcPIM1	RPVPCEDLLQYIEDNGGSLQEDEAKIILKQLVYAATELQEMKIFHRDIKVENILIE TDSE		342
mzPIM1	RPVPAVDLFEYIRENGGCLPEGKAKVILKQLVDAAKDLEEKQIFHRDIKSDNILIE TGSD		383
onPIM1	RPVPAVLHDYITENGGLPEEKAKVILKQLVDAAKDLEDKHIFHRDIKSENILIE TGSD		302
croakerPIM1	RPVPAV DLLKYIQGKGGSIDENEAKIIMKQLVDAAKELKDKCIFHRDIKVENILIE TGSG		411
zebraPIM1	RPAPCQDLQSFCCEEN-GCLDEPLAKVVLVQLIAALKHCESRRVLHRDVKPENLLISTDSH		224
ratPIM1	RPEPVQDLFDFITER-GALQEELARSFFWQVLEAVRHCHNCGVLRDIDENILIDLNRG		180
humanPIM1	RPEPVQDLFDFITER-GALQEELARSFFWQVLEAVRHCHNCGVLRDIDENILIDLNRG		180
frogPIM1	RPEPVQDLFDFITER-GALGEELASNFFRQVVEAVRHCHSCDVVRDIDENILVDLRTA		197
	*** * * : : . * : * * . : * : : : . . . : * : * : * : * : * : .		
	Region 3	Region 4	
	Autophosphorylation	Catalytic Domain	
omPIM1	VPRARLIDFGLSCMAEKDTIFRVFSGTPAHPGPPEYFKGF-SSPGSTTVWQLGVVLYESLH		448
olPIM1	TPQARLIDFGVSCMAEKDSILRGFSGTPINAPPEAFKGF-CSPGSTTVWQLGVVLYETLH		390
lcPIM1	VPRVRLIDFGLSCFFKKRSLYRVFYGTPAHPPEFYSRKTYWAGPTTVWQVGVVLFEMLH		402
mzPIM1	VPRVRIIDFGLSCFATEQSQFCFFYGTPIHSPPECYWGKKYRPGPTTVWQMGVVLYEALH		443
onPIM1	VPRVRIIDFGLSCFAKARSLYRVFYGTPIHTPPECYGCKKYKAGPTTVWQMGVVLYEALH		362
croakerPIM1	TPRLRLIDFGLSCFVKEKSRYHIFYGTPDHIPPEWFRHNTYHAGPTTVWQVGVVLYESVH		471
zebraPIM1	--DIKLLDFGCGDLM-KDSAYRYFACTPAFAPPFWFRHRYHASPLTVWSIGVTLYNLC		281
ratPIM1	--ELKLIDFGSGALL-KDTVYTDFDGTRVYSPPPEWIRYHRYHGRSAAVWSLGILLYDMVC		237
humanPIM1	--ELKLIDFGSGALL-KDTVYTDFDGTRVYSPPPEWIRYHRYHGRSAAVWSLGILLYDMVC		237
frogPIM1	--ELKLIDFGSGALL-RDAVYTDFDGTRVYSPPPEWIRFHKYHGKSATVWSLGILLYDMVC		254
	: : : * * * . : : * * ***	: * * . : * : : :	
	Region 5	Region 6	Region 7

PIM1 Sequence Alignment and Its Possible Roles

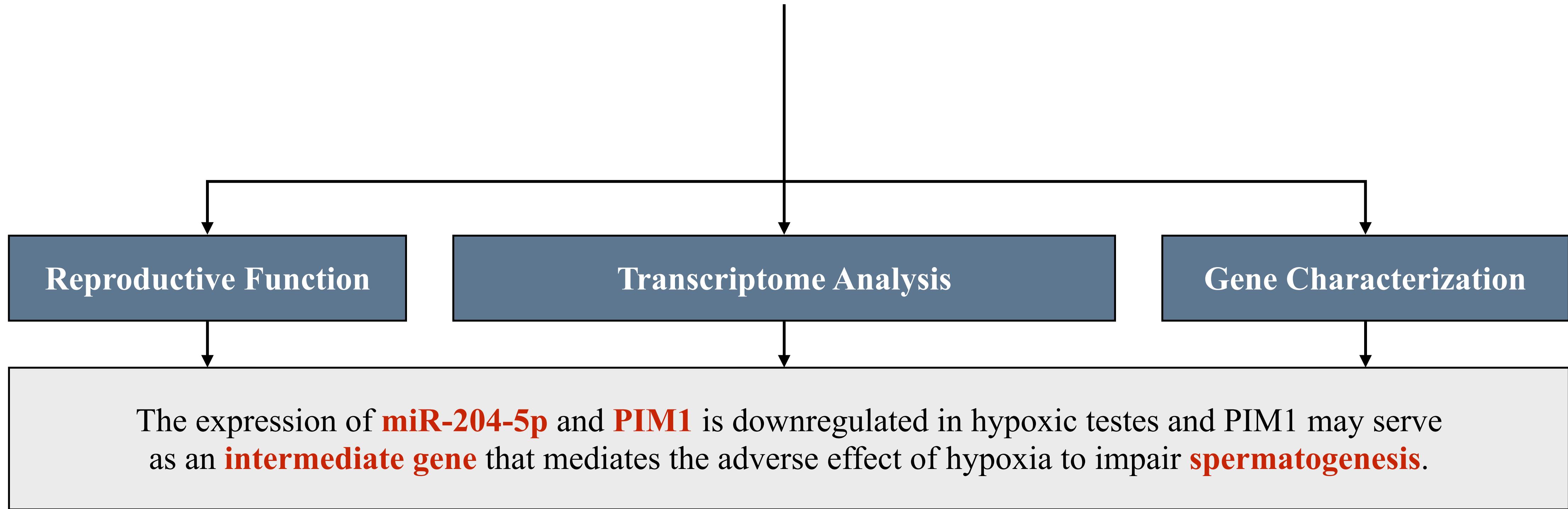
Pim-1

- Considering the role of PIM1 in **apoptosis** and **cell cycle progression** in mice and human, its down-regulation during hypoxia may impair **spermatogenesis**, ultimately leading to a decrease in sperm number and motility

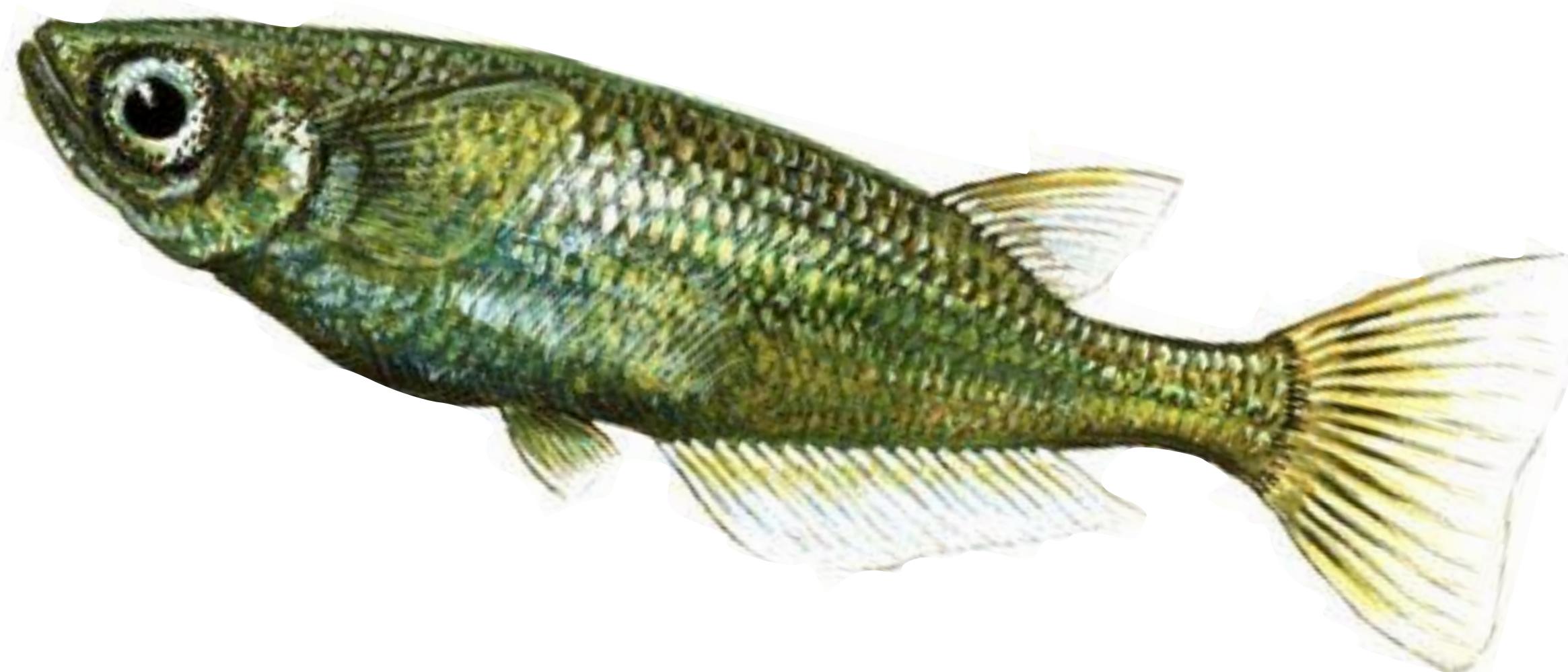


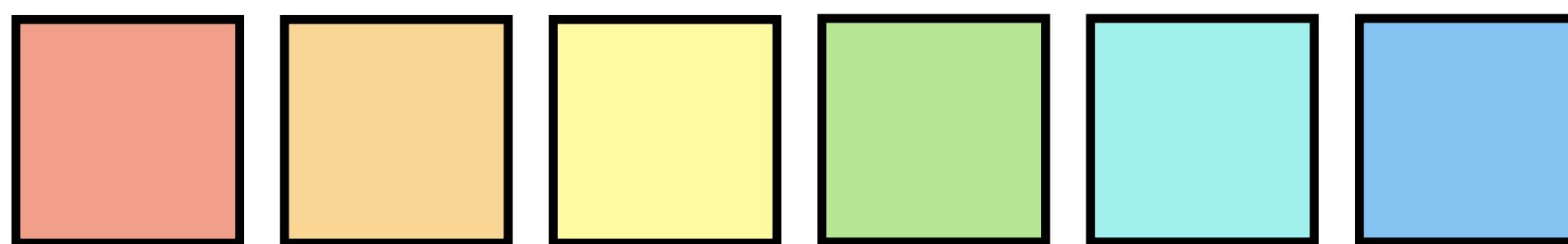
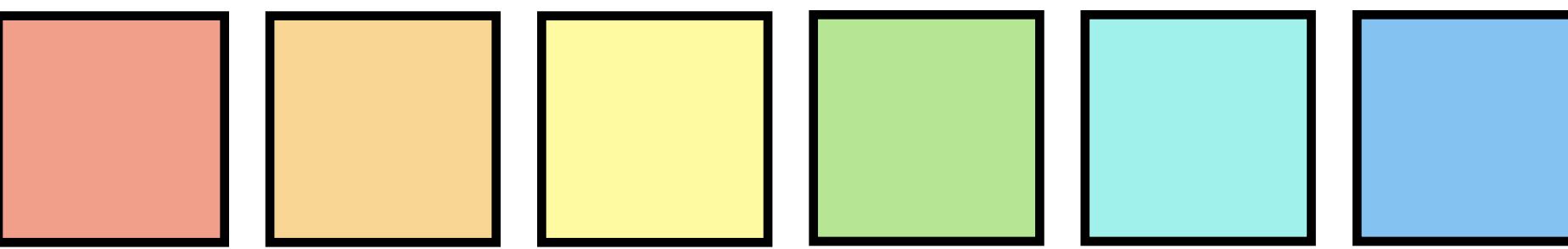


Conclusion

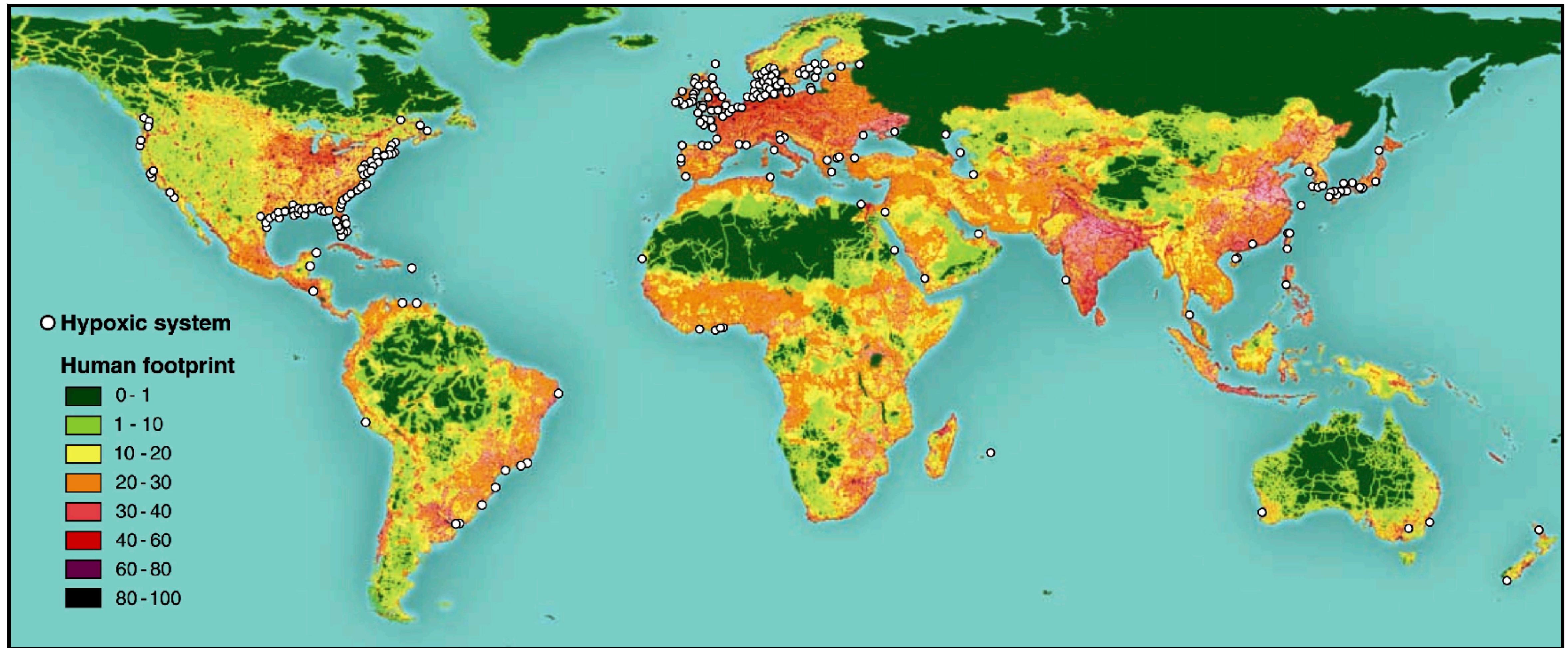


Questions ?!



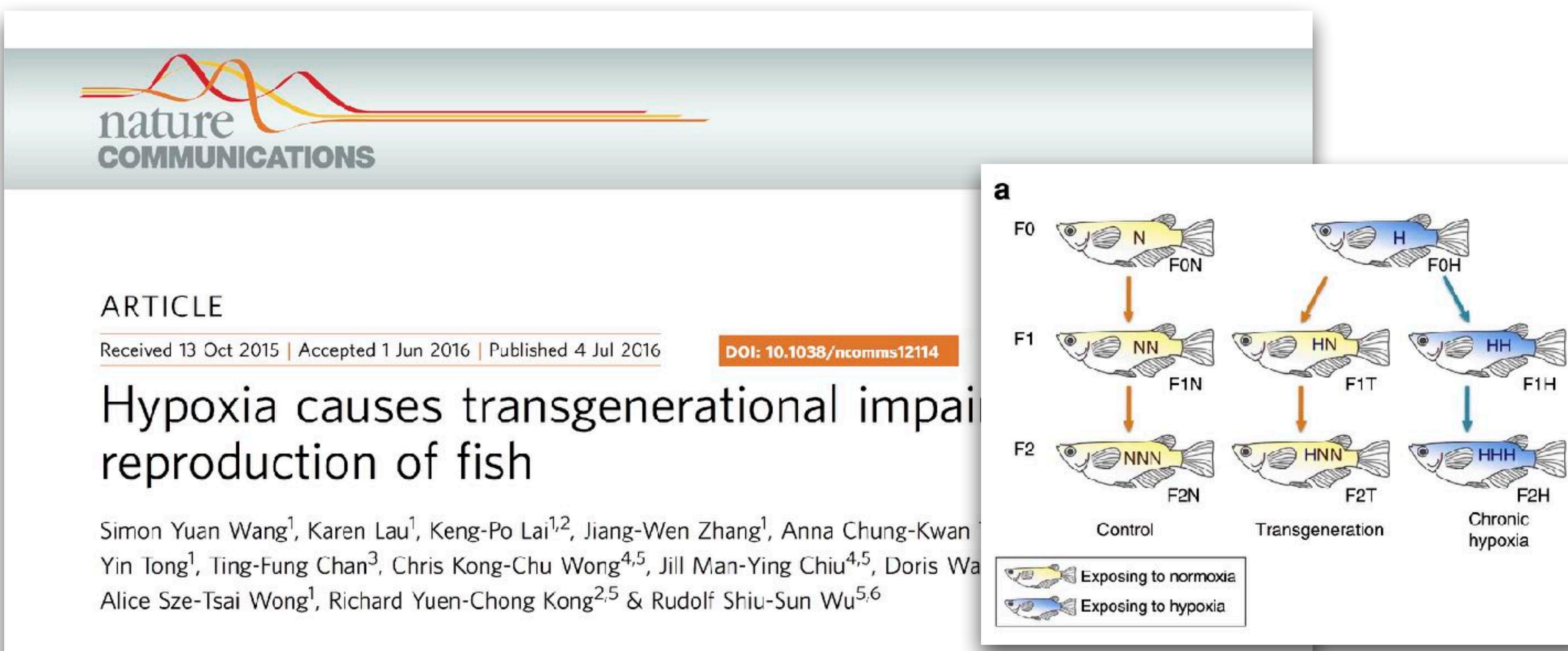


Spreading Dead Zones (Aquatic Hypoxic Area)



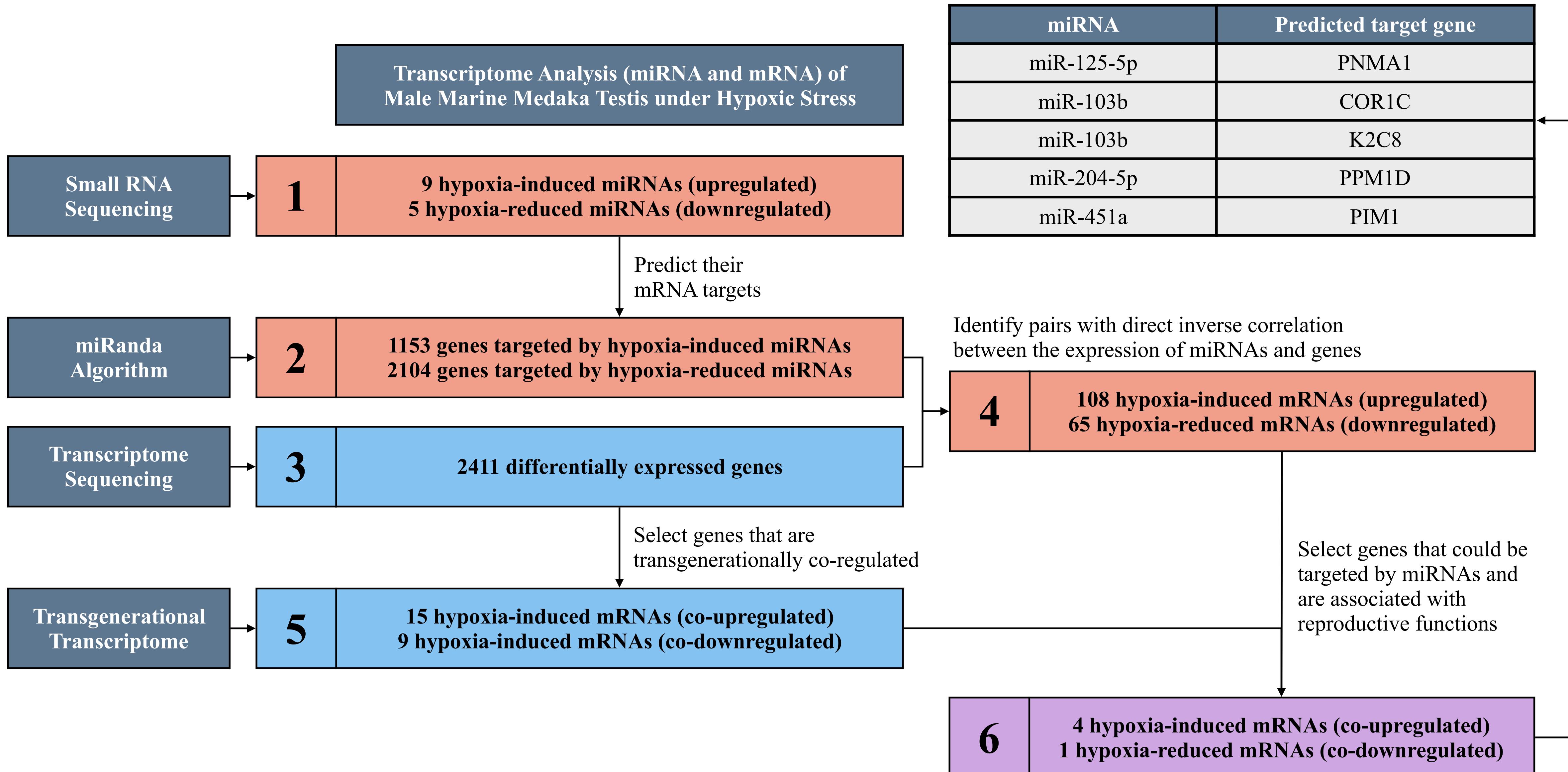
Hypoxia Induces Transgenerational Reproductive Impairments

- An recent discovery reported that hypoxia can cause **transgenerational reproductive impairments** in male marine medaka through **inheritable epigenetic changes** in testis, indicating that the adverse impact of aquatic hypoxia is potentially more serious than we previously thought (Wang et al., 2016).



The workflow of screening transgenerational hypoxia-responsive miRNA-mRNA pairs.

miRNA-mRNA pairs that are associated with reproductive functions and have transgenerational direct inverse correlation in expression level



PIM1 Sequence Alignment and Its Possible Roles

GCAACACGCTTCCAC	AACACAAAGAGTTT	AGTCGGTAGCTGACC	ATTCGCAAGTAGCTG	AGAAAAACTTGATC	1
GTTCACTCGTGTT	GGGAAAAAACAGCAA	AAGATCATGGTTAAA	AATTCTGCTAAGGAT	AATCCAAAATGTACT	76
M V K	N S A K D	N P K C T			
GATTCCAGCAGTAAA	ACACGTCAAAAACCT	TTTGACAATAAACAA	ACCAAAAAGGTTGAG	AGGAAACCCAAGTCT	151
D S S S K T R Q K P F	D N K Q T K K V E	R K P K S			
GAAACCAAGAACCGAG	AAGAAAAAGTGAAA	TTCTTGAAACCACGT	GAGTCTCCAGAACCT	GAACCAACACGGCCA	226
E T K K Q K K V K F	L K P R E S P E P	E P T R P			
TCTACCAGCACCAA	GACAACCCCCCAGTG	AGAGGACTAAAGAGA	AAACGACAGGAAGAC	CAACAGGAAGAAGAA	301
S T S T Q D N P P V R G L K R K R Q E D	Q Q E E E				
ATCATTGAGCCAAAG	AAGAGGAGACCTTGT	CTTAAAGCTTCCAGC	AGCAGTGCAGCCAA	CAAAGCAGCAAAACG	376
I I E P K K R R P C L	K A S S S S A A Q Q S S K T				
TTTTCTGATGACCAA	ACCAAAGAAGTCAAG	AGAAAAGCCAAACCT	GAACCAAAGAAAAAG	AAGAAAAAGTGAAA	451
F S D D Q T K E V K R K P K P E	P K K K K K K V K				
CACTGTGAACAGACC	GAGTCTGATATCGGT	CAGGACAACACCGA	GTGAGAGGACTAAAG	AGGAAAAGACAGGAG	526
H C E Q T E S D I G Q D N T R V R G L K R K R Q E					
GACGAAGACGAAGAC	ACAAGAGACCAACCA	AAGATAATGAAGCCA	TCTTTGAAGAGCTAC	CAAGAACTGAGGGAT	601
D E D E D T R D Q P K I M K P S L K S Y Q E L R D					
TTATACAATAAGATG	AAAGGAAACAAAACG	AGTGGCCAAGAAAAG	ACACTTTAAAGAGT	TCAGCAAATGAAAAAA	676
L Y N K M K G N K T S G Q E K T L L K S S A N E K					
GAATCAAACCAAGAA	ATCCCAATCAAAAAG	ACGGAGAGAGACGAC	GTGATCGACCAAAAAA	GCCGAGTTGAAGGCC	751
E S N Q E I P I K K T E R D D V I D Q K A E F E A					
AAATATGTGGAAGAA	GATCAGTTGGATCA	GGAGGTTTGGATCC	ATTCATGCCGGCTTT	CGCAGATCTGACAAT	826
K Y V E E D Q F G S G G F G S I H A G F R R S D N					

PIM1 Sequence Alignment and Its Possible Roles

- Except for **regions 6, 7 and 8** which are more conserved in bonyfishes than other vertebrates, there is no substantial difference between bonyfishes and other vertebrates. Further investigation can be done to find out the functional importance of such difference. Based on these sequence identities, omPIM1 represents the **marine medaka homolog of vertebrate PIM1 serine/threonine-protein kinase**.

Region	Percent sequence identity of omPIM1 and:								
	olPIM1	lcPIM1	mzPIM1	onPIM1	croakerPIM1	zebraPIM1	ratPIM1	humanPIM1	frogPIM1
1	58.33	58.33	58.33	50.00	58.33	66.67	66.67	66.67	50.00
2	100.00	100.00	100.00	75.00	100.00	100.00	100.00	100.00	100.00
3	100.00	85.71	57.14	57.14	57.14	71.43	57.14	57.14	57.14
4	80.00	70.00	80.00	70.00	70.00	50.00	70.00	70.00	70.00
5	87.50	100.00	87.50	87.50	100.00	50.00	62.50	62.50	62.50
6	75.00	75.00	62.50	75.00	50.00	62.50	37.50	37.50	37.50
7	70.59	58.82	68.75	68.75	64.71	47.06	35.29	35.29	35.29
8	72.73	63.64	36.36	45.45	54.55	45.45	36.36	36.36	36.36