Charles University

Faculty of Science

Ph.D. study program: Cell and Developmental Biology

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The study of *Xenopus tropicalis* testis-derived stem cells

Doctoral Dissertation

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Prague, 2019

Univerzite Karlova

Přírodovědecká fakulta

Doktorský studijní program: Buněčné a vývojové biologie

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Studium kmenových buněk odvozených z varlat *Xenopus tropicalis*

Disertační práce

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Praha, 2019

Declaration of Authorship

I, Nguyen Thi Minh Xuan, declare that this dissertation titled, “The study of *Xenopus tropicalis* testis-derived stem cells” and the work presented in it are my own. All the literature is properly cited, and I have not been yet awarded any other academic degree or diploma for this thesis or its substantial part.

Signed:

Date:

“Enjoy climbing the mountain”

“*Enjoy climbing the mountain*” because of no one at the destination after all,
they all went to sleep. Six years for Ph.D. is hard but leaving Prague is harder.
And to whom it may concern,
I have not mentioned your particular names on the paper
because you stay deeply in my heart.

Acknowledgments

My Ph.D. life for nearly 6 years in Czech Republic has been filled with full of fun, depression and hangovers. In some days I was a walking dead person and in others, microscope images induced me reprograming back to an enthusiastic girl-like person. I would not say doing Ph.D. here is my best thing but I can not imagine how I would live with this such colorful life elsewhere. My Ph.D. journey wouldn't have been accomplished without lovely companions. I would like to express my grateful acknowledgment to all of you.

Foremost, I would like to say "Thank you very much from bottom of my heart" to my supervisor Ass. Prof. Vladimir Krylov for giving me a chance to join our group. I have been impressed by his patience for students, immense knowledge, and his attitude for working. I have never expected he spent a whole weekend to revise this thesis and sometimes received his emails at midnight. Once again, thank him for his kind support, helpful advice, encouragement, and understanding not only with my study but also with my difficult personal life.

Besides my advisor, I would like to thank the rest of my thesis committee members for their insightful comments and the hard questions which encouraged me to widen my research from various perspectives.

My sincere thanks also go to Dr. Tereza Tlapakova, Marketa Vegrichtova, Dr. Magdalena Krulova, Dr. Lenka Doubravska, Jiri Vavra and other staff of Department of Cell Biology, Charles University for kindly helping and supporting me. Especially I would like to express my respectfulness to my beloved friend in Czech Republic, Andrea Mancikova. Life can go up and down, but that wonderful person always keeps me in a line of wonderful friendship.

I would like to acknowledge the STARS program and Charles University Grant Agency (GAUK) No. 2598461 for financial support of my Ph.D. study.

I would like to thank my parents and my younger sister who are physically and mentally taking care of my little treasure, my daughter. Their unconditional love gives me the courage to never surrender in front of challenges. In addition, many thanks to my friends for supporting me spiritually throughout writing this thesis and my life in general with cooking, traveling, shopping, drinking, dancing, laughing and fighting altogether. Last but not least, I would like to thank my husband, who immediately moved to Milano after his Ph.D. completion in Chemical Department. He has given me motivation towards obtaining a Dr. title, so I will not be only a M.S cleaner in my family. Finally, all loves to my gifts from God, my little daughter and two adorable twins, this thesis is my gift for them. Hopefully someday all of my efforts here can motivate my girls to achieve their accomplishment.

Prague, March 6th, 2019

Abstract

The substances secreted by Sertoli cells (SCs) are crucial to determine male sex characteristics in embryos and regulate spermatogenesis in adulthood. The failure in SC maturation can cause sterility in men. Before puberty, SCs keep the ability to proliferate and have been considered as immature cells. They differ remarkably from mature cells in connection with their morphology and biochemical activity and thus they probably play a part in maintaining spermatogonia stem cells in an undifferentiated stage. The transient presence of cytokeratin in immature SCs has been reported in many species, but not in *Xenopus* yet. We investigated which molecules are expressing only in immature Sertoli cells of *X. tropicalis* testes. The regulation of cytokeratin and β-catenin was revealed by fluorescent immunostaining. Cytokeratin and membrane β-catenin co-expressed in *X.tropicalis* juvenile testes and in cultured SC progenitors, called XtiSCs, but they were absent in adulthood. There was no signal of cytokeratin in migrating SCs (pre-SCs) located outside the seminiferous tubules. The suppression of cytokeratin along with the breakdown of β-catenin-based cell contacts have been observed in XtiSCs after the treatment with a small molecule drug, CHIR99021 and led to their dedifferentiation back to stem cell-like state. These findings confirm the expression of cytokeratin and a novel molecule, β-catenin along with Sox9 in SCs as markers indicating their immature state.

However, the function of CK in SC development is poorly understood. We examined interconnection between CK and β-catenin-based cell junctions in immature SCs. Reversible dissociation of CK by acrylamide in XtiSCs induced breakdown of membrane-bound β-catenin but had no effect on F-actin and β-tubulin or cell adhesion proteins (focal adhesion kinase and integrin β1). On the contrary, disruption of membrane β-catenin via uncoupling of cadherins with Ca2+ by chelator EGTA didn’t show any influence on the cytokeratin stability. The results suggest a new role of CK in the retention of β-catenin-based junctions in immature Sertoli cells, and thus serving as structural support for arrested germ cells and for the formation of proper seminiferous tubules.

Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are fundamental processes in embryonic development. In general, EMT is characterized by the conversion of sessile epithelial cells into mesenchymal cells with the migratory potential, whereas MET activates a reverse process. A mesenchymal phenotype of pre-Sertoli cells has been observed suggesting that pre-SCs must undergo a MET to differentiate into mature cells. Our laboratory has been successful in the establishment of *Xenopus tropicalis* somatic cell line from testes of juvenile frogs, called XtTSCs. The isolated cells possessed characteristics of Sertoli cells with expression of immature markers including Sox9, vimentin, cytokeratin and β-catenin, so latter called XtiSCs. The main aim of my Ph.D. project was the determination of factors responsible for the induction of EMT, a reverse differentiation process of SCs, and identification of a stemness window where cells possess the greatest differentiation potential in XtiSCs. GSK-3 inhibitor (CHIR99021), FGF2 and/or TGF-β1 ligands were employed in XtiSCs culture to induce EMT. Our results showed that XtiSCs underwent full EMT after 3-day treatment with GSK-3 inhibitor and partial EMT with FGF2, but not with TGF-beta 1. The morphology change of CHIR-treated XtiSCs to the typical spindle-like cell shape was associated with the upregulation of mesenchymal proteins (fibronectin, integrin α5β1, Snai1 and Zeb1) and low expression of the epithelial marker, cytokeratin. Moreover, this inhibitor also promoted stem cells markers (Sox2, *cd44*) and the efficient derivation of stem cells from *Xenopus* testes. CHIR-treated, but not FGF2-treated or vehicle XtiSCs can differentiate into chondrocytes *in vitro* and cardiomyocytes *in vivo* after their microinjection into the peritoneal cavity of *X. tropicalis* tadpoles. Interestingly, the EMT-shifted cells could migrate toward cervical cancer cells *in vitro* (HeLa cells) and to the injury site *in vivo*. In general, our results provide a better understanding of signaling pathways underlying the generation of testis-derived stem cells. Moreover, XtiSCs could represent a novel model for the study of the EMT process and SC maturation.

Abstrakt

Sertoliho buňky (SCs) produkují celou řadu molekul klíčových pro determinaci samčího pohlaví a pro regulaci spermatogeneze v dospělosti. Poruchy diferenciace Sertoliho buněk vedou u člověka ke sterilitě. Před nástupem puberty hrají důležitou roli v udržení spermatogoniálních buněk v kmenovém stavu a zajišťují tak jejich dostatečný počet. Zajímavým znakem odlišujícím nezralé a zralé Sertoliho buňky je dočasná přítomnost cytokeratinu, který byl u těchto buněk popsán u celé řady modelových organismů. V rámci této disertační práce jsme se zabývali rozdílnou genovou expresí proteinů spojených s regulací cytokeratinu u nezralých a zralých Sertoliho buněk odvozených z varlat Drápatky tropické (*Xenopus tropicalis*). Imunohistochemické řezy varlat z juvenilních jedinců a imunocytochemická analýza buněčné kultury odvozené ze stejného zdroje, obsahující nezralé progenitory Sertoliho a peritubulárních myoidních buněk (XtiSC) ukázaly na společnou expresi cytokeratinu a membránového β-kateninu. V případě vzorku z dospělých jedinců nebyla detekována přítomnost ani jednoho z nich. Dále pak podobný fenotyp vykazovaly i migrující Sertoliho buňky (pre-SCs), které se nacházely mimo semenotvorné kanálky. I zde byla exprese obou proteinů potlačena. Přidání inhibitoru glykogen syntázy kinázy 3, CHIR99021, k buněčné kultuře XtiSC vedlo k dediferenciaci přítomných buněk do kmenového stavu a k rozšíření jejich diferenciačního potenciálu.

Role cytokeratinu a dalších proteinů zahrnujících β-katenin a Sox9, marker Sertoliho buněk, na diferenciaci Sertoliho buněk nebyla dosud prozkoumána. V rámci předložené disertační práce jsme se zabývali vztahem mezi cytokeratinem a membránovým β-kateninem včetně příslušných mezibuněčných spojů (cell junctions) u nezralých Sertoliho buněk. Reverzibilní destrukce cytokeratinové sítě pomocí akrylamidu vedla u XtiSC buněk ke ztrátě membránového β-kateninu. F-aktin, β-tubulin nebo proteiny buněčné adheze (kináza fokálních adhezí - FAK a integrin β1) nebyly akrylamidem ovlivněny. Na druhou stranu narušení membránového β-kateninu pomocí Ca^{2+} chelatačního činidla EGTA nezpůsobilo vážnější dezintegraci cytokeratinové sítě. Výsledky ukazují na novou roli cytokeratinu pro stabilizaci

mezibuněčných spojů závislých na β-kateninu v nezralých Sertoliho buňkách. Tato stabilizace je klíčová pro udržení spermatogonií ve stavu dělících se buněk bez meiotického zrání v rámci pre-pubertálních varlat a dále pak pro správné formování semenotvorných kanálků.

Epitelo-mezenchymální tranzice (EMT) a mezenchymo-epiteliální tranzice (MET) patří mezi základní buněčné procesy v zárodečném vývoji. EMT je charakterizovaná jako přeměna adhezivních buněk epiteliálního typu na mezenchymální typ schopný migrace. MET pak představuje obrácený proces. Nezralé Sertoliho buňky (XtiSC) vykazují mezenchymální fenotyp. Za fyziologických *in vivo* podmínek musí tyto buňky podstoupit MET a diferencovat se ve zralé Sertoliho buňky. V laboratoři školitele se podařilo založit dlouhodobou buněčnou kulturu odvozenou z varlat juvenilních jedinců *X. tropicalis*. Expresní profil buněk obsahoval markery nezralých Sertoliho buněk jako Sox9, vimentin, cytokeratin a β-katenin. V první publikaci, která je součástí disertační práce byly tyto buňky zkracovány jako XtTSC (Xenopus tropicalis Testicular Somatic Cells). V dalších dvou rukopisech jsou popisovány jako nezralé Sertoliho buňky, XtiSC (Xenopus tropicalis immature Sertoli Cells). Cílem předložené disertační práce byla identifikace faktorů odpovědných za indukci epitelo-mezenchymální tranzice u XtiSC a nalezení tzv. “kmenového okna” (stemness window), ve kterém buňky vykazují nejširší diferenciační potenciál. K tomuto účelu jsme zvolili GSK-3 inhibitor (CHIR99021) a dále pak FGF2 a/nebo TGF-β1 ligandy. Třídenní ošetření XtiSC buněčné kultury GSK-3 inhibitorem vedlo ke kompletní EMT. FGF2 vedl pouze k částečné tranzici do mezenchymálního fenotypu. Na druhou stranu TGF-β1 měl vliv na senescenci nikoliv na EMT. Působení CHIR99021 bylo patrné jak na úrovni změny buněčné morfologie (vřetenovitý tvar buněk), ale také na zvýšené expresi mezenchymálních proteinů jako je fibronektin, integrin α5β1, Snai1 a Zeb1 a na snížené expresi epiteliálního markeru, cytokeratinu. Navíc ošetření buněk GSK-3 inhibitorem vedlo ke zvýšení markerů typických pro kmenové buňky, jako např. Sox2 a *cd44*. To mělo za následek schopnost XtiSC diferencovat do chondrocytů *in vitro* a kardiomyocytů *in vivo* po jejich mikroinjekci do peritoneálního prostoru pulců *X. tropicalis.* XtiSC buňky po EMT tranzici migrovaly k nádorovým buňkám cervikálního karcinomu a do místa po

indukovaném poranění. Výsledky této disertační práce umožní lepší pochopení mezibuněčné signalizace, která stojí za přípravou kmenových buněk odvozených z varlat. Navíc XtiSC buňky představují nový model pro studium epitelo-mezenchymální tranzice v rámci zrání Sertoliho buněk a spermatogeneze.

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Abbreviations

01.	AMH	anti-Mullerian hormone
02.	BTB	Blood-testis barrier
03.	CK	Cytokeratin
04.	ECM	Extracellular matrix
05.	EMT	Epithelial-mesenchymal transition
06.	FBS	Fetal bovine serum
07.	FSH	Follicle-stimulating hormone
08.	GnRH	Gonadotropin-releasing hormone
09.	H&E staining	Haematoxylin-Eosin staining
10.	hCG	Human chorionic gonadotropin
11.	HGF	Hepatocyte growth factor
12.	IF	Intermediate filament
13.	LH	Luteinizing hormone
14.	MET	Mesenchymal-epithelial transition
15.	MDIF	Mullerian duct inhibitory factor
16.	MMP	Matrix metalloproteinases
17.	MSCs	Mesenchymal stem cells
18.	PBS	Phosphate buffer saline
19.	PBSTr	PBS+0.1% Triton-X 100
20.	PGCs	Primordial germ cells
21.	PMCs	Peritubular myoid cells
22.	qRT-PCR	quantitative Reverse transcription-polymerase chain reaction
23.	RFP	Red fluorescent protein
24.	RT-PCR	Reverse transcription-polymerase chain reaction
25.	SCs	Sertoli cells
26.	Sma/Acta2	Smooth muscle actin
27.	Snai1/SNAI1	Snail family zinc finger 1
28.	Sox9	(Sex-determining region Y)-box 9
29.	SRY	Sex-determining region Y
30.	SSCs	Spermatogonial stem cells
31.	TNF	Tumor necrosis factor
32.	TSCs	Testis-derived stem cells
33.	Twist	Twist-family bHLH transcription factor 1
34.	XtiSCs	*Xenopus tropicalis* immature Sertoli cells
35.	XtTSCs	*Xenopus tropicalis* testicular somatic cells
36.	Zeb1	Zinc finger E-box-binding homeobox 1