ROLE OF UBIQUITIN LIGASE RNF149 IN GONOCYTE DEVELOPMENT

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Abstract

Male reproductive function depends on the formation of spermatogonial stem cells from their neonatal precursors, the gonocytes. In a previous study of the mechanisms regulating gonocyte development, we unveiled the participation of the ubiquitin proteasome system (UPS) in this process and identified several UPS enzymes dynamically altered during gonocyte differentiation. The present work focuses on understanding the role of the RING finger protein 149 (RNF149), an E3 ligase previously found strongly expressed in gonocytes and down-regulated in spermatogonia. The quantification of RNF149 mRNA by qPCR from postnatal-day (PND) 2 to 35 (puberty) in rat testis, brain, liver, kidney, and heart indicated that its highest levels are found in testis. Silencing experiments were performed in PND3 rat gonocytes by electroporation with RNF149 siRNA, a scrambled nucleotide or medium (Mock). While a proliferative cocktail of PDGF-BB and 17β-estradiol (P+E) increased both the expression levels of the cell proliferation marker PCNA and RNF149 in mock cells, the effects of P+E on both genes were reduced in cells treated with RNF149 siRNA, suggesting that RNF149 expression is regulated during gonocyte proliferation and that there might be a functional link between RNF149 and PCNA. Lastly, in order to examine RNF149 subcellular localization, EGFP-tagged RNF149 vectors were constructed, after determining rat testis RNF149 mRNA sequence. Surprisingly, two variant transcripts were expressed in rat tissues, predicting truncated proteins, one containing the PA and the other the RING functional domains. Transfection in the mouse F9 embryonal carcinoma cells and C18-4 spermatogonial cell line showed differential subcellular profiles of the two truncated proteins. The results of this study support a role for RNF149 in gonocyte proliferation and suggest its transcription to variant mRNAs resulting in two proteins with different functional

domains. Future studies will examine the respective roles of these variants proteins in the cell lines and isolated gonocytes.

Résumé

La fonction reproductrice mâle dépend de la formation de cellules souches spermatogoniales à partir de précurseurs néonataux, les gonocytes. Au cours de nos recherches sur les mécanismes régulant la differentiation des gonocytes néonataux en spermatogonies, nous avons mis en évidence la participation de plusieures enzymes du système protéasomal de l'ubiquitine (UPS), et identifié plusieures enzymes UPS dont l'expression était modifiée de façon dynamique pendant la différentiation des gonocytes. La présente étude est centrée sur la compréhension du rôle de la protéine "RING finger"149 (RNF149), une ligase de type E3 que nous avons précédemment trouvée fortement exprimée et régulée à la baisse dans les spermatogonies. La mesure des transcripts de RNF149 par PCR quantitative en temps réel des jours 2 à 35 (puberté) après la naissance dans les testicules, cerveau, foie, rein et cœur, indiquait les niveaux les plus élevés dans les testicules. Nous avons ensuite performé des expériences de blocage d'expression de RNF149 utilisant un RNA court d'interférence (siRNA), un nucléotide non-specifique brouillé, ou du milieu (mock). Alors que la combinaison proliferative de PDGF-BB et 17β-estradiol (P+E) entrainait une augmentation d'expression des transcripts du marqueur de prolifération PCNA et de RNF149 dans les cellules mock, les effets de P+E sur l'expression de ces gènes étaient réduits dans les cellules traitées avec le siRNA de RNF149. Ces résultats suggèrent que l'expression de RNF149 est regulée au cours de la prolifération des gonocytes, ainsi que l'existence d'un lien fonctionnel entre RNF149 et PCNA. Afin de déterminer la localisation subcellulaire de RNF149, nous avons généré des vecteurs d'expression fusionnant EGFP et RNF149, après avoir déterminé la séquence du transcript de RNF149 dans le testicule de rat. Curieusement, deux transcripts variants sont exprimés dans les tissus de rats, prédisant deux protéines tronquées, l'une contenant le domaine fonctionnel PA et l'autre le domaine RING de RNF149. Nous avons performé des experiences sur les lignées cellulaires de carcinoma embryonal de souris F9 et de spermatogonies de souris C18-4 qui ont révélé des différences de profiles d'expression entre les deux protéines tronquées. Les résultats de cette étude supporte l'idée d'un rôle de RNF149 dans la prolifération des gonocytes et suggère que sa transcription mène à la formation de mRNA variants codant pour deux protéines avec des domaines fonctionnels differents. Des études futures examineront les rôles respectifs de ces protéines variantes dans les lignées cellulaires et les gonocytes.

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Abbreviations

- **BMP4:** Bone morphogenic protein 4
- **CXCR-4:** C-X-C chemokine receptor type 4
- **DEHP:** Di-2-ethylhexyl phthalate
- **DNA:** Deoxyribonucleic acid
- **DNAPTP2:** DNA polymerase-transactivated protein 2
- **DRP1:** Dynamin-related protein 1
- **E_2:** 17β-estradiol
- **EEA1:** Early endosome antigen 1
- **ER:** Endoplasmic reticulum
- **ERAD:** ER-associated degradation
- **ERE:** Estrogen response elements
- **ERK:** Extracellular signal regulated protein kinase
- **ERs:** Estrogen receptors
- **FBS:** Foetal bovine serum
- **FOXO1:** Forkhead transcription factor 1
- **GAP:** GTPase activating protein
- **GRAIL:** Gene related to anergy in lymphocytes
- **hCG:** Human chorionic gonadotropin
- **HECT domain:** Homologous to E6-AP Carboxy Terminus domain
- **ICC:** Immunocytochemistry
- **IHC:** Immunohistochemistry
- **LEL:** Larger extracellular loop

- **MAPK:** Mitogen activated protein kinase
- **MAPKK:** MAPK kinase
- **MAPKKK:** MAPK kinase kinase
- **MARCH:** Membrane-associated RING-CH
- **MEK:** MAPK/ERK kinase
- **MFN2:** Mitofusin 2
- **MIS:** Mullerian inhibiting substance
- **MOM:** Mitochondrial outer membrane
- **NS:** N-terminal signal peptides
- **PA domain:** protease-associated domain
- **PDGF:** Platelet-derived growth factor
- **PDGFR:** PDGF receptor
- **PGCs:** Primordial germ cells
- **PI3K:** phosphatidylinositol 3-kinase
- **PKA:** Protein Kinase A
- **PKC:** Protein Kinase C
- **PLC-γ:** Phospholipase C-γ
- **PND:** Postnatal day
- **PRDM1:** PR domain zinc finger protein 1
- **RA:** Retinoic Acid
- **RBR:** RING between RING fingers
- **RING:** Really Interesting New Gene
- **SNARE:** Soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein

- **SSCs:** Spermatogonial stem cells
- **STATs:** Signal transducers and activators of transcription
- **TGFβ:** Transforming growth factor β
- **TGN:** Endosome/trans-Golgi network
- **TM domain:** Transmembrane domain
- **TRIM:** Tripartite motif
- **Ub:** Ubiquitin
- **UPS:** Ubiquitin proteasome system
- **VAMP3:** Vesicle-associated membrane protein 3
- **VDAC1:** Voltage-dependent anion channel
- **ZFPL1:** Zinc finger protein-like 1

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Introduction

Gonocytes are the precursors of spermatogonial stem cells (SSCs). We previously showed that the ubiquitin proteasome system (UPS) is involved in the Retinoic Acid (RA) - induced differentiation of gonocytes. Postnatal day (PND) 3 gonocytes proliferate in response to platelet-derived growth factor (PDGF)-BB and 17β-estradiol (E_2). They relocate to the basement membrane of the seminiferous cord where they differentiate to spermatogonia. Perturbations of gonocyte development can prevent the formation of SSCs, potentially resulting in infertility or testicular cancer.

Ubiquitination is a three-step process initiated with the activation of ubiquitin (Ub) by an ubiquitin-activating enzyme (E1). Ub is then transferred onto an E2 conjugating enzyme and finally linked to a target protein presented by a specific ubiquitin ligase (E3). This step can be repeated to form polyubiquitin chains. We found that the E3 ligase RNF149 in expressed at higher levels in PND3 gonocytes than PND8 spermatogonia. RNF149 is a RING (Really Interesting New Gene) - type ligase containing a PA domain, a transmembrane domain (TM), and a RING domain. PA domains are protease-associated domains containing E3 ligase activity that can interact with tetraspanin proteins. The RING domain is a specialized type of Zinc-finger of 40 to 60 residues. Two studies have shown opposite effects of RNF149 on cell proliferation. In a colon cancer cell model, RNF149 reduced proliferation by inducing BRAF proteasomal degradation. However, in Hela cells, it increased proliferation by controlling CD9 degradation, and it localized in the endoplasmic reticulum and lysosomal membranes.

Nothing is known of RNF149 role in gonocytes and spermatogenesis, in general. Thus, my goal is to determine its function in spermatogenesis, focusing on gonocytes and spermatogonia.

1. Testis development and functions

1.1 Testis structure and major cell types

Male testes, which are composed of interstitial space surrounding seminiferous tubules, are important organs that synthesize androgens and produce spermatozoa (Manku & Culty, 2015; Robaire & Chan, 2010). The interstitium consists of Leydig cells, lymphatic vessels, resident hematopoietic cells such as macrophages and mast cells, blood vessels and nerves. Germ cells, surrounded by Sertoli cells reside inside seminiferous tubules, which are enveloped by a basement membrane (tunica propria) and a layer of peritubular myoid cells. Myoid cells are smooth muscle type cells that exert peristaltic movements, moving immature spermatozoa toward the rete testis (Robaire & Chan, 2010). Leydig cells are responsible for the production of the sex steroid testosterone, as well as many other hormones in the adult testis (Robaire & Chan, 2010), whereas Sertoli cells are bound to germ cells by intercellular gap junctions (Hermo et al., 2010b; Manku & Culty, 2015) and provide nutrition and structural support to germ cells (Gnessi et al., 1995; Hermo et al., 2010b; Robaire & Chan, 2010). Although there are differences in the number of days it takes for a spermatogenic cycle to be completed between species, the process itself is similar between humans and rodents, and thus, rodents are commonly used animal models to study this process (Adler, 1996).

1.2 Spermatogenesis

Unlike the uninterrupted development process of rodent, human spermatogenesis includes two phases separated by a few years of relative testicular quiescence. The first phase starting from embryonic ages to infancy consists of successive steps of primordial germ cells (PGCs) and gonocytes (also known as pre- or pro-spermatogonia) that can be distinguished by unique features, leading to the formation of the first spermatogonia, including SSCs (Culty, 2009). Human have only three types of spermatogonia, A_{dark}, A_{pale}, and B. Once an individual has enter prepuberty, the spermatogenic cycles will start by the commitment of spermatogonia to differentiate, progressing through meiosis and spermiogenesis to end with the formation of immature spermatozoa, and this process will repeat throughout male adult life (Hermo et al., 2010a).

The rodent spermatogenic cycle includes three main phases: a mitotic phase that takes place in SSCs, undifferentiated (A_{single}, A_{pair}, and $A_{aligned}$), differentiating (A_1 to A_4, intermediate), and differentiated (type B) spermatogonia at various phases of maturation, followed by a lengthy meiotic phase including successive types of primary spermatocytes, secondary spermatocytes and the formation of haploid spermatids (round, elongated), which undergo spermiogenesis to become spermatozoa that will further achieve maturation in the epididymis (Hermo et al., 2010a).

1.3 Germ Cell Origin

PGCs originate from pluripotent stem cells in the proximal epiblast next to the embryonal ectoderm, where germ cell lineage emerges in both males and females, before gender specification and differentiation initiate (Culty, 2009; Rouiller-Fabre et al., 2003). Bone morphogenic protein 4 (BMP$_4$) and BMP$_{8b}$ are key regulators of this process, starting with the formation of PGC precursors expressing PR domain zinc finger protein 1 (PRDM$_{1,}$ also known as BLIMP$_1$) and PRDM$_{14}$, detected around embryonic day (E) $_{6.25}$ in the proximal epiblast. These cells then evolve at day E$_{7.25}$ to become alkaline phosphatase- and Stella-expressing PGCs (Kurimoto et al., 2008; Saitou, 2009), when these sexually undifferentiated PGCs start migrating toward the genital ridge, involving the interactions of the membrane receptors KIT (c-Kit/CD$_{117}$) and C-X-C chemokine receptor type 4 (CXCR-4) with their respective ligands SCF and SDF$_1$, where they will become resident and enclosed by Sertoli cells, forming testicular cords at $E_{12.5}$dpc in rodents (Jan et al., 2012). PGCs will also experience genome-wide DNA demethylation during this journey, resulting to the removal of parental imprints (Reik et al., 2001; Seki et al., 2005). Sex determination of germ cell and supporting somatic cells is established at $E_{12.5}$, partially regulated by the expression of the *Sry* gene in male somatic cells (Jameson et al., 2012). Once the germ cells become resident in the gonadal primordium, they are referred to as gonocytes where they will remain in the center of the newly formed seminiferous cord until PND3-5, when they migrate to the basement membrane to differentiate into spermatogonia (Culty, 2013; Rouiller-Fabre et al., 2003; Zhao & Garbers, 2002).

2. Gonocyte Development

The term gonocytes comprises different phases of development, encompassing a succession of cell types with distinct behaviors that are associated with different fetal and neonatal periods. In rats, gonocytes will experience a fetal phase of mitosis, followed by a phase of quiescence starting around E17.5 and ending around PND2 (also called transitional T1 pre/pro-spermatogonia), and a last phase when neonatal transitional gonocytes (also called transitional T2 pre/pro-spermatogonia) resume mitosis and migrate to the basement membrane of tubules where differentiation will take place (Culty, 2013). It is important to note that several of these phases can occur simultaneously in testis. In human, the transitional gonocyte phase takes place from around gestational weeks 20 to postnatal weeks 8-12 (Culty, 2013).

Among the complex succession of events leading to spermatozoa formation, the early phases of development, more specifically the proliferation and differentiation of transitional gonocytes are some of the least studied, although they are vital since they are at the origin of the SSC pool that will support the production of spermatozoa throughout the lifetime of a male. This is due to the difficulty of obtaining sufficient research materials, as a result of limited cell numbers and heterogeneity, and the challenge of morphologically distinguish each stage of development (Clermont & Perey, 1957). It will take a long time for scientists to find proper approaches to solve this puzzle. Today, a number of signaling pathways regulating transitional gonocyte development have been uncovered, such as those involving transforming growth factor β (TGFβ) ligands superfamily, RA, variant forms of PDGF receptors (PDGFRs), WNTs, and PTEN, which have emerged as potential regulators of transitional gonocyte differentiation (Manku & Culty, 2015). Nevertheless, the target molecules of these pathways remain unclear. Thus, it would be very interesting to understand how these pathways work with each other to fine-tune gonocyte differentiation.

2.1 Neonatal Gonocyte Development

Maintaining a functional reservoir of SSCs is very important as it assures the life-long production of sperm (Culty, 2009, 2013). This depends on the development of transitional gonocytes, founders of SSCs and type A spermatogonia of the first spermatogenic wave between PND3 and 5-6 (Culty, 2009). On the other hand, mice gonocytes will enter this journey 1–2 days earlier. In both cases, the co-existence of quiescent and proliferative gonocytes will occur in neonatal testes as they are not precisely synchronized (Culty, 2013).

During this process, gonocytes were found to be associated to Sertoli cells via gap and desmosome-like junctions, believed to regulate communication between these cells (Orth & Boehm, 1990), based on the finding of a variety of cell adhesion molecules in gonocytes and Sertoli cells, including NCAM (Orth & Jester, 1995), PB-cadherin (Wu et al., 2003), and connexin 43 (Bravo-Moreno et al., 2001; Pointis et al., 2011). In order to become spermatogonia, gonocytes must free themselves from junctions with Sertoli cells and migrate to the basement membrane of the seminiferous cord, and failure to do so leads to apoptosis (Rodriguez et al., 1997; Tres & Kierszenbaum, 2005). Therefore, the migratory ability is a unique feature of neonatal gonocytes that distinguish them from fetal gonocytes, as a result of the expression of genes related to cell migration that remain to be identified.

2.2 Potential Regulators in Gonocytes Development

As mentioned above, scientists have spent years in searching potential regulators and pathways involved in gonocyte differentiation. Based on the hypothesis that this process is regulated by hypothalamic–pituitary–gonadal axis, human chorionic gonadotropin (hCG) was taken into consideration, but turned out to have no effect on gonocyte differentiation in neonatal mouse testis organ cultures. In contrast, the same study demonstrated that the number of gonocytes undergoing differentiation into type A spermatogonia were significantly increased by adding Mullerian inhibiting substance (MIS; also known as anti-Mullerian hormone (AMH)) produced from fetal to juvenile Sertoli cells, suggesting a role of this protein in the regulation of gonocyte fate (Zhou & Hutson, 1995). In addition, our lab previously found that PND3 rat gonocyte differentiation is induced in vitro by RA (Wang & Culty, 2007), an active metabolite of vitamin A (Collins & Mao, 1999). STRA8, a target gene of RA initially found in P19 mouse embryonal carcinoma cells, was then identified in F9 mouse embryonal teratocarcinoma cells, undifferentiated spermatogonia, and premeiotic male germ cells (Bouillet et al., 1995; Giuili et al., 2002; Oulad-Abdelghani et al., 1996; Zhou et al., 2008b), as an essential initiator of meiosis in male and female germ cells (Anderson et al., 2008; Baltus et al., 2006; Mark et al., 2008).

In different types of germ cells, STRA8 protein has been found to shuttle between nucleus and cytoplasm and to display transcriptional activity in cell lines when it is expressed in the nucleus (Tedesco et al., 2009). In both mice and rat gonocytes, RA can induce a strong and consistent increase in *Stra8* mRNA and protein, which are at low levels in freshly isolated PND3 rat gonocytes (Wang & Culty, 2007). In rats, we have also found that RA induces the expression of truncated PDGFRs and that PDGFR signaling pathway probably plays a role in gonocyte differentiation (Manku et al., 2015; Wang & Culty, 2007). This will be discussed in the next section.

This intricate process requires large amount of remodeling. We found that the ubiquitin–proteasome system (UPS) is a crucial regulator of this process, as the RA-driven induction of Stra8 expression in rat gonocytes was significantly decreased when proteasome activation was inhibited by specific inhibitors, lactacystin and bortezomib (Manku et al., 2012c; Nikov et al., 2001). Several UPS proteins preferentially expressed in gonocytes were also identified, such as the RING finger 149 (RNF149) E3 ligase, that might play a role in gonocyte development. Several UPS proteins have been implicated in the regulation of spermatogenesis, including the E3 ligase Huwe1 (Liu et al., 2007). Since these findings are relatively new, it will require further studies to identify the functional enzyme–substrate partners and their respective roles in gonocyte development.

While PDGFR signaling pathway/variant PDGFRs take part in gonocyte differentiation, PDGF-BB and E_2 were shown to induce neonatal transitional gonocyte proliferation via MAPK activation (Basciani et al., 2008; Li et al., 1997; Thuillier et al., 2009), whereas the factors inducing fetal gonocyte proliferation have not been fully identified. Rat mitotic fetal and neonatal gonocytes react differently toward various stimulations. Moreover, they are heterogeneous populations encompassing different subsets of germ cell types. In organ culture studies, E14.5 gonocytes treated with RA was found to undergo apoptosis faster than cell proliferation, resulting in overall loss of cells. A different situation is observed in PND3 gonocytes (Livera et al., 2000). Moreover, activin A and androgen were reported to be negative regulators of fetal gonocyte proliferation, while they had no effect on PND3 gonocyte proliferation in vitro (Mendis et al., 2011; Merlet et al., 2007; Thuillier et al., 2010).

2.3 Variation in Protein Subcellular Localization and Functional Differences

In germ cell development, fetal and neonatal gonocytes share characteristics such as morphological similarities, central location and active DNA methylation process, forming a distinctive period from earlier and later phases in spermatogenesis (Culty, 2009, 2013). This period, similarly to subsequent phases of spermatogonia formation, encompass subsets of germ cell types which are hard to distinguish from each other, as they do not have unique protein profiles, but gradual changes in several markers over time (Culty, 2009; Griswold & Oatley, 2013; Luo et al., 2009; Manku & Culty, 2015; Suzuki et al., 2009). Forkhead transcription factor FOXO1, a transcription factor that regulates gluconeogenesis and glycogenolysis by insulin signaling stimulation, was shown to be expressed in all mouse PND1 (mainly quiescent) and PND3 gonocytes (mainly mitotic and migratory), representing a potential marker among other few genes identified in neonatal gonocyte. As an example of dynamic protein subcellular localization, FOXO1 protein was found strictly located in the cytoplasm in PND1 mouse gonocytes, but predominantly expressed in the nucleus of PND3 gonocytes (Goertz et al., 2011). Furthermore, the nuclei of SSCs and undifferentiated spermatogonia demonstrated high levels of FOXO1 expression, but not in more mature germ cells. This makes FOXO1 a potential marker for the observation of the gonocyte to SSC transition.

In addition to the protein profile differences between cells in quiescence or mitosis phases, functional differences were also observed on these cells, as shown by PDGF-BB and E_2 co-treatment stimulating proliferation in PND3 rat gonocytes, but not in PND2 gonocytes (Culty, 2009; Mendis et al., 2011; Thuillier et al., 2010). Another example is RA, which induces both proliferation and apoptosis in fetal gonocytes (Trautmann et al., 2008), but induces differentiation in neonatal rodents (Wang & Culty, 2007; Zhou et al., 2008a). These differences further demonstrate the plasticity of these cells.

3 Platelet Derived Growth Factor

3.1 PDGFs family

The PDGF family consists of disulphide-bonded homodimers of A, B, C and D polypeptide chains, and the heterodimer PDGF-AB (Heldin, 2013). The propeptides of PDGF-A and B are activated by proteolytic cleavage in the endoplasmic reticulum, while the C and D isoform propeptides are only activated extracellularly. The PDGF isoforms are synthesized as precursor molecules. PDGF-AA, -AB and –BB are activated by proteolytic cleavage in the endoplasmic reticulum, and packed into secretory vesicles in the producer cells. In contrast, PDGF-CC and -DD are secreted as inactive precursor molecules, and only activated extracellularly (Fredriksson et al., 2004). These isoforms play critical roles during embryonic development, where they were shown to regulate the migration, proliferation, and differentiation of different cell types by acting on specific receptors (Basciani et al., 2010; Hoch & Soriano, 2003; Ricci et al., 2004). PDGFs can also act as promoter for tissue remodelling and embryonic development of the kidneys, brain, lungs, heart and testis (Basciani et al., 2010).

3.2 PDGF Receptors and Potential targets

PDGF isoforms exert their cellular effects by binding to α- and β-tyrosine kinase receptors (PDGFRα and PDGFRβ, respectively). The two PDGF receptors are structurally similar and consist of extracellular domains containing five extracellular immunoglobulin repeats, a single transmembrane and juxtamembrane domain, and intracellular parts with kinase domains which contain characteristic inserts of about 100 amino acid residues without homology to kinases. PDGFs usually bound to Ig-like domains 2 and 3, initiate dimerization of the receptors, which is further stabilized by direct receptor-receptor interactions involving Ig-like domain 4 (Omura et al., 1997; Yang et al., 2008). PDGF-induced receptor dimerization is critical for receptor activation by bringing the intracellular parts of the receptors close to each other, promoting autophosphorylation on tyrosine residues opening docking sites for Src homology 2 (Sh2) and Sh3 domains (Heldin et al., 1998). Each receptor dimer has a unique affinity for specific ligand isoforms, as PDGF-AA, AB, BB and CC induce αα receptor homodimers, with preferential affinity for AA and AB. The ββ receptor homodimer binds to PDGF-BB and PDGF-DD with highest affinity for BB, and the αβ receptor heterodimer binds to PDGF-AB, -BB, -CC and -DD (Andrae et al., 2008; Mariani et al., 2002)

Activated PDGFRs can bind to around 10 different families of SH2-domain-containing molecules, which include signaling molecules with intrinsic enzymatic activities, such as tyrosine kinases of the Src family, the SHP-2 tyrosine phosphatase, phospholipase C-γ (PLC-γ) and the GTPase activating protein (GAP) for Ras. Generally, different receptors and ligands are found to affect different tissues, and often different receptor/ligand patterns can generate contradictory actions (Basciani et al., 2010). These receptors can also bind and activate signal transducers and activators of transcription (STATs), which translocate to the nucleus where they act as transcription factors after activation. Another type of targets are adaptor molecules like regulatory subunit p85 of the phosphatidylinositol 3-kinase (PI3K), which forms complex with the p110 catalytic subunit, and Grb2 which binds the nucleotide exchange molecule SOS1, activating Ras and the Erk/MAP-kinase pathway. In addition, the PDGF receptors bind other adaptors, e.g. Shc, Nck, Crk and GAB, which mediate interactions with a plethora of different downstream signaling molecules. The activation of these signaling pathways leads to cell proliferation and survival, as well as to actin reorganization and cell migration (Heldin, 2013). Moreover, activated PDGFRs are also often internalized into the cell and degraded in lysosomes (Dai, 2010).

3.4 PDGFs and PDGFRs roles in Gonocytes

PDGFR signaling is an important pathway in the regulation of gonocyte development, but it requires cooperation with other regulators at different stages of gonocyte development (Li et al., 1997; Manku et al., 2015; Thuillier et al., 2010; Wang & Culty, 2007). Our lab has previously shown that PND3 gonocytes are stimulated to proliferate by a combination of PDGF-BB and E_2, conducted with highly enriched gonocytes cultures in order to prevent the influence of other factors from other testicular cell types (Li et al., 1997; Thuillier et al., 2010). The crosstalk between the PDGF and estrogen pathways provides a way for perinatal Sertoli cells, which produce both PDGF and E_2, to tightly regulate the timing and amplitude of gonocyte reentry into mitosis in vivo (Thuillier et al., 2010). At PND3, gonocytes express the full length PDGFRβ at the cell surface, as well as variant forms of PDGFRβ and PDGFRα (Manku et al., 2015; Thuillier et al., 2003; Wang & Culty, 2007). Fetal exposure to various exogenous estrogenic compounds caused up-regulation of the gonocyte expression of PDGF receptor β (Thuillier et al., 2003), suggesting that the estrogen pathway may be involved in the regulation of PDGFRβs expression in gonocytes. While the full length PDGFRβ mediates gonocyte proliferation by binding PDGF-BB, the two variant PDGFRs α and β appear to be involved in gonocyte differentiation. Indeed, we have found that the expression of both V1-PDGFRβ and variant PDGFRα are increased by retinoic acid (RA) treatment (Manku et al., 2015; Wang & Culty, 2007). V1-PDGFRβ does not contain ligand binding domain but maintains tyrosine kinase autophosphorylation domain, and is localized in the cytosol (Wang & Culty, 2007). Similarly, PND3 gonocytes express a RA-induced variant PDGFRα that comprises part of the transmembrane domain and the kinase domain. Moreover, PDGFR activation was required for RA-induced gonocyte differentiation, since addition of a PDGFR inhibitor prevented RA effects (Manku et al., 2015). Thus, PDGFRβ crosstalk with estrogen regulates gonocyte proliferation, whereas a crosstalk between PDGFRs (likely variant forms of PDGFRα and β) and RA is involved in differentiation.

4. Estrogens

4.1 Endogenous and exogenous Estrogens

Although estrogens are mainly female sex hormones, they are also necessary for many processes in the male body. Estrogens are crucial for the development, maintenance and function of both male and female secondary sex characteristics. In female, this includes developing breasts and regulating the menstrual cycle during reproductive age. In males, the main biologically active estrogen is E_2, produced from the conversion (aromatization) of testosterone, which binds on estrogen receptors (ERs) (Ge et al., 2014). In addition to their sexual functions, estrogens regulate growth, bone mineralization, brain masculinisation and cardiovascular functions in males as well as females (Luconi et al., 2002). They also play a role in the immune system and the central nervous system (Heldring et al., 2007). E_2, estrone and estriol are the three major types of endogenous estrogens found in the endocrine system, with E_2 being the strongest and most potent of the three. Estrogens are secreted as endocrine messengers and therefore circulate through the bloodstream until reaching their target tissue where they will bind the ERs, triggering the expression of specific genes regulated by estrogen (Heldring et al., 2007). In addition to the endogenous estrogens, xenoestrogens stands for the artificial or other natural sources originating molecules that have the ability to bind and affect the activity of ERs (Nikov et al., 2001). While the chemical structure of these compounds is not always similar to estrogens, they are binding ERs as well as other types of receptors with different affinities, while some may act as potential carcinogens (Nadal et al., 2000). Although many of these molecules may disrupt endocrine signallings, whether they bring positive or negative effects depends on the dosages and the length of exposure. In addition, some exogenous estrogen compounds might be able to prevent cancer in the right doses and period of exposure, such as found with genistein, a common phytoestrogen in soy which has a high affinity for ERβ. Xenoestrogens can also interact in vivo with other environmental chemicals, leading to disruption of the male reproductive system, as found in male rats following in utero exposure to the combination of genistein and , the phthalate plasticizer di-(2-ethylhexyl) phthalate (DEHP) (Jones et al., 2014).

4.2 Functions of the Estrogen Receptors

As members of the steroid nuclear receptor superfamily and key factors in estrogen signaling pathway, ERs mediate estrogens action in target cells by regulating gene expression in the role of transcription factors. The two major types of ERs are ERα (ESR1/NR3A1) and ERβ (ESR2/NR3A2) (Segars & Driggers, 2002). Their expression varies according to the organs, species and developmental stage, as in testis, where ERβ is involved in the control of neonatal gametogenesis, whereas ERα regulates fetal and neonatal steroidogenesis, based on studies conducted on knockout (KO) mice (Fisher et al., 1998; Korach, 1994; Robertson et al., 1999).

Once ERs are bound to estrogen, ERs change their conformation and become stable homologous dimers, then translocate from the cytosol to nucleus and bind to estrogen response elements (ERE) found near or in the promoter region of the targeted genes. With other coregulatory proteins, ERs can regulate the expression of their target genes (Heldring et al., 2007; Luconi et al., 2002; Nikov et al., 2001; Segars & Driggers, 2002). In addition to the genomic signaling pathway of ERs, many studies have shown that ERs can also cooperate with second messengers such as calcium, activated kinases and tyrosine kinases, Protein Kinase A (PKA) and Protein Kinase C (PKC) as well as the ERK pathway to induce a very rapid cascade of responses to estrogen signals. These effects occur in the cytoplasm or on the cellular membrane, and possibly trigger different functions from the known ER genomic effects, some via types of ER unrelated to the two classical receptors, such as the estrogen-activating membrane bound G protein-coupled receptor GPR30 (Delbès et al., 2006; Filardo et al., 2000; Luconi et al., 2002; Nadal et al., 2000; Segars & Driggers, 2002).

4.2 Estrogens and ERs in Male Reproduction

There are no doubts that estrogens are essential for spermatogenesis and the regulation of adult male reproductive system through specific ERs. In males, estrogens can be synthesized from testosterone by aromatase cytochrome P450 in testes (Carreau et al., 2003), present in different cells of the male reproductive system, including somatic and germ cells in the testis and epididymal cells (Berensztein et al., 2006; Carreau et al., 2003; Nitta et al., 1993; Robertson et al., 1999). The sites of aromatase expression, estrogen production and estrogen sensitivity vary according to the age, with aromatase being mostly expressed in Sertoli cells and germ cells in human and mouse fetal testes, while in the adult, the primary source of estrogen are Leydig cells (Berensztein et al., 2006; Delbès et al., 2006; Delbes et al., 2007; Nitta et al., 1993). Environmental estrogens, absorbed from the environment as natural phytoestrogens like genistein, derivatives of plastics, food additives, pesticides and other industrial chemicals represent another source of compounds capable of inducing ER activation and the expression of estrogen-responsive genes, although in a non-physiological context that can be potentially harmful (Delbes et al., 2007; Jones et al., 2014; Sharpe & Irvine, 2004; Toppari et al., 1996).

Our lab have previously shown that the proliferative effect of PDGF/estrogens co-treatment was most likely conducted through ERβ, which is strongly expressed in isolated gonocytes (Li et al., 1997; Thuillier et al., 2010). In contrast, no effect of exogenous estrogens were observed on gonocytes in organ cultures or co-cultures with Sertoli cells naturally producing E2 (Delbes et al., 2007). Using KO mice for ERα (ERαKO), ERβ (ERβKO), both ERs (ERαβKO), or the CYP19/aromatase (ArKO), scientists demonstrated that estrogens are important regulators of testicular germ cells at different periods of spermatogenesis (Filardo et al., 2000; Korach, 1994; Robertson et al., 1999).

The balance between androgens and estrogens is critical for proper testis development, during gametogenesis and steroidogenesis in fetal period. The disruption of estrogen and androgen homeostasis during periods of reproductive system development can lead to reproductive disorders like cryptorchidism, hypospadias, testicular cancer, and low sperm count (Sharpe & Irvine, 2004; Toppari et al., 1996). In adult males, estrogen deficiency give rise to reduced sperm production and sperm motility in humans (Hess, 2003), as excess of estrogens deteriorate sperm production and maturation (Atanassova et al., 2000). In addition, the finding that the polymorphisms *ESR1 Pvu*II and *ESR2 Rsa*I of ER genes were significantly associated with male infertility risk (Ge et al., 2014), suggest that genetic abnormalities of ERs might be another major contributing factors of male infertility (Kovac et al., 2013).

5. Raf-MEK-ERK mitogen-activated protein kinase cascade

The MAPK cascades are key signaling pathways involved in proliferation, gene transcription, migration, differentiation, development, learning, survival as well as apoptosis (Robinson & Cobb, 1997). They function downstream of cell surface receptors to activate transcription factors that regulate gene expression (McCubrey et al., 2007). Deregulation of these cascades will leads to cancer and other human pathologic disorders (Roberts & Der, 2007).

In mammalian cells, these cascades are comprised of MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK (Johnson & Lapadat, 2002), acting as a phosphorylation regulated signaling relay. The terminal serine/threonine kinases (MAPKs) are the extracellular signal regulated protein kinase (ERK1/2), the c-Jun amino-terminal kinases (JNK12/3; also called stress activated protein kinase), p38 kinases (p38alpha/beta/italic gamma/delta) and ERK5. The ERK pathway is often involved in proliferation and is activated by growth factor-stimulated cell surface receptors (Rumora & Grubisic, 2009). The MAPKKK component of the ERK cascade is comprised of the Raf serine/threonine kinases (A-Raf, B-Raf and c-Raf-1) (Schreck & Rapp, 2006; Wellbrock et al., 2004).

6. Ubiquitin proteasome system

The UPS is the main post-translational modification in eukaryotes that conjugates ubiquitin to lysine residues of target proteins and determines their intracellular fate (d'Azzo et al., 2005; Daviet & Colland, 2008; Reinstein & Ciechanover, 2006). They perform both proteolytic and non-proteolytic functions, such as regulating cell cycle progression, protein interactions, signal transduction, kinase activation, DNA repair, endocytosis, protein trafficking, (Bedford et al., 2011; d'Azzo et al., 2005; Daviet & Colland, 2008; Reinstein & Ciechanover, 2006), intercellular communication (Kjenseth et al., 2010), cell proliferation (Lu et al., 2009) and apoptosis (Ramakrishna et al., 2011). In addition, a number of oncogene and tumor suppressor gene products were found to be targets of ubiquitination, indicating that malfunction of proteasomal degradation could lead to neurological and immune disorders, cancer (Mani & Gelmann, 2005), diabetes and obesity (Wing, 2008), and infertility (Bedard et al., 2011; Crimmins et al., 2009; Ryu et al., 2008; Zimmerman & Sutovsky, 2009).

6.1 Ubiquitination

The conjugation of ubiquitin is catalyzed by the sequential action of three enzymes: E1-activating enzyme, E2-conjugating enzyme and E3-ubiquitin ligase. The initial step is when the E1 enzyme activates the ubiquitin molecule, leading to the formation of a high energy thioester bond between the C-terminal glycine (Gly76) of Ub and the cysteine residue in the active site of the E1 enzyme. Activated ubiquitin is then transferred to the catalytic cysteine residues of the E2 conjugating enzyme. Finally, the E3 ligase will attach the ubiquitin tag onto its specific substrate, by an isopeptide bond between the C-terminus of Ub and an ε-amino group of a lysine residue of the target protein. This process can be repeat multiple times to lengthen the Ub chain (polyubiquitination) (Glickman & Ciechanover, 2002).

Ub contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) that can be used to form poly-Ub chains (Ikeda & Dikic, 2008). Generally, the function of ubiquitination is to cause protein degradation (ubiquitins linked at the Lys48) in the 26S proteasome (Chau et al., 1989; Thrower et al., 2000), but in some cases (ubiquitins linked via Lys63), it leads to the regulation of protein synthesis, protein stabilization, chromatin remodeling (O'Neill, 2009), signal transduction and endocytosis (Bhoj & Chen, 2009; Mukhopadhyay &

Riezman, 2007; Spence et al., 1995). Ubiquitination is also reversible by the deubiquitinating enzymes (DUB) that cleave isopepetide bonds between Ub and target protein or within a poly-Ub chain (Reyes-Turcu et al., 2009). E3 ligases can also catalyze conjugations of Ub-like modifiers, including small Ub-like modifier (SUMO), neural precursor cell-expressed developmentally downregulated protein 8 (Nedd8) and interferon-stimulated gene 15 kDa (ISG-15) in similar manner, and result in distinct functions in a number of different cellular processes (Nakamura, 2011).

6.2 E3 ligases

The E3 ligases play an important role in substrates recognition, which explain the presence of such a large number of them compared to the E1 and E2 enzymes. Based on the presence of the E3 catalytic core domain, E3 ligases can be categorized into three classes, which are HECT-domain ligases (Homologous to E6-AP Carboxy Terminus), the RNF-type ligases and the U-box protein families (Ardley & Robinson, 2005).

RNF domain usually comprises 40–80 amino acid residues with eight highly conserved cysteine and histidine residues that provide two zinc ions to form a unique three-dimensional structure known as a “cross-brace” (Deshaies & Joazeiro, 2009). They can be classified into at least three subgroups based on the presence of cysteine and histidine residues in the fourth and fifth positions: C3HC4 (RING-HC), C3H2C3 (RING-H2) and C4HC3 (RING-CH) fingers. They provide a stable platform for efficient binding to E2 enzymes in close proximity to substrate proteins. To date, at least 49 RNF proteins have been identified, containing hydrophobic regions predicted to be transmembrane (TM) domains, implying that they are embedded in the cellular membrane and directly participate in the biological processes of both the cellular membrane and membranous organelles (Cao et al., 2008; Li et al., 2008; Neutzner et al., 2011).

More recently, E3 ligases have been shown to participate in spermiogenesis and the condensation of the sperm nucleus; the regulation of junctional complexes between cells of the seminiferous epithelium; the process of meiosis in spermatocytes; and the regulation of germ cell apoptosis (Richburg et al., 2014), positioning them as key regulators of spermatogenesis.

6.3 RNF Proteins

According to the location of RING motif, RNF proteins can be classified into four groups, which include members of the tripartite motif (TRIM) containing, PA (protease-associated domain)-TM (transmembrane domain)-RING E3s, RING between RNFs (RBR) and membrane-associated RING-CH (MARCH) families. However, not all of the RNF proteins has E3 ligase function (Nakamura, 2011).

TRIM proteins contain three characteristic structures, the RING-HC finger domain followed by one to two B-box domains (zinc-finger-like motifs) and a coiled-coil region. Although the clear function of B-box domain has not been identified, recent studies have shown that B-box domain may modify E3 activity (Du et al., 2014). The coiled-coil regions are important for the subcellular localization and oligomerization of TRIM proteins (Reymond et al., 2001).

Nine proteins have been identified so far in this group, including the RNF149 E3 ligase that was mentioned earlier. The PA-TM-RING class RNF Proteins shares three conserved domains, the protease-associated domain, the transmembrane domain and the RING-H2 finger domain. Most of them have signal peptide sequences at their N-termini. The PA domain is also considered as extracellular or luminal domain, which is proposed to serve as substrate recognition and binding module, as in GRAIL (gene related to anergy in lymphocytes, also known as RNF128) (Anandasabapathy et al., 2003; Mahon & Bateman, 2000). In addition, a recent study indicates that PA domain is essential for the endosomal localization of these proteins, as observed on human RNF13 and RNF167 (van Dijk et al., 2014).

The majority of MARCH RNF Proteins are found located on cellular membrane. The specific motif in this family is the PDZ domain-binding motifs located on either N-terminal or C-terminal. This motif recognize the PDZ domain in scaffold proteins, facilitating the trafficking, localization and assembly of membrane proteins such as receptors, ion channels and transporters (Cao et al., 2008).

Having two RNF motif that are linked by an in-between-ring (IBR) domain, the RNF motif on C-termini of RBR proteins only binds one metal ion, whereas the N-terminal RNF motif has the original cross-brace structure for E3 ligase activity. Many RBR proteins are found across eukaryotes with a diverse range of biological functions, including protein quality control, signaling, cell cycle and apoptosis (Eisenhaber et al., 2007).

6.4 Known Cellular Functions of the RNF Proteins and RNF149

In humans, most of the RNF proteins possess transmembrane domains, which explains their specific localization on membrane compartments in both secretory and endocytic pathways, as well as in mitochondria and peroxisomes. In addition, an increasing number of studies have shown that transmembrane RNF proteins are key factors in many cellular and physiological processes, as they control the stability, trafficking and activity of proteins involved in these events.

The endoplasmic reticulum (ER) is a network of tubules, vesicles and flattened sacs that serve as the protein factory of the cell, and covers the synthesis, assembly and processing of both integral membrane proteins and secreted proteins. Protein misfolding, genetic mutations and cellular stresses happen during protein production, which can lead to the malfunction of protein products. The accumulation of these impaired proteins can induce cellular stress which eventually leads to cell death. To maintain the stability of the secretory pathway of ER, a highly conservative process called ER-associated degradation (ERAD) is in charge of the degradation of these proteins in both prokaryotic and eukaryotic cells. In addition to the misfolding proteins, native ER proteins are also the substrates of ERAD, indicating that ERAD participates in both metabolism and intracellular signaling (Meusser et al., 2005). Several E3 ligases have been found to be resident on ER and to share common features like multiple transmembrane segments, such as Hrd1p and Doa10p in yeast, and mammalian ortholog of Hrd1p, HRD1. These ER-resident E3 ligases can form a complex with the adaptor protein, which recruits the ERAD machinery (Hirsch et al., 2009).

Downstream of the ER is the Golgi apparatus, which is the processing and sorting site for newly synthesized proteins received from the ER. Zinc finger protein-like 1 (ZFPL1) is a single TM protein that resides on the cis-Golgi membrane. The N-terminal region contains a variant RNF domain and a zinc finger motif that mediates the interaction of ZFPL1 with the C-terminal coiled-coil segment of GM130, providing a scaffold function for the cis-Golgi matrix proteins to assist membrane tethering and fusion (Chiu et al., 2008).

Endocytosed proteins, endosomal receptors, enzymes and the furin peptidase, are known to cycle between the endosome/trans-Golgi network (TGN) and plasma membrane through the retrograde transport pathway (Johannes & Wunder, 2011). Studies show that the RNF172 (MARCH2) endosomal E3 enzyme is likely to be involved in at least the regulation of early/recycling endosome-to-TGN (EE/RE–TGN) transport, and is essential in sustaining cell polarity and epithelial integrity (Nakamura et al., 2005).

Mitochondrial fission is known to be controlled by the cytosolic GTPase dynamin-related protein 1 (DRP1) (Smirnova et al., 2001). The mammalian mitochondrial E3 enzyme MARCH5 (RNF153) is located inside the mitochondrial outer membrane (MOM). It acts as a regulator of mitochondrial fission, as it ubiquitinates DRP1 and Fis1, a MOM-anchored protein involved in mitochondrial fission, and loss of its activity results in mitochondrial fragmentation (Yonashiro et al., 2006). Others MOM related RNF have recently been found, such as RNF185 and cytosolic E3 Parkin that ubiquitinates dynamin-related GTPases mitofusin 2 (MFN2) and the voltage-dependent anion channel (VDAC1) (Tang et al., 2011).

During male germ cell development, spermatogenesis requires unique mechanisms regulating gene expression, chromatin condensation, protein transport, signal transduction and protein degradation. In the mouse genome, near 4% of the genes are uniquely expressed in male germ cell (Schultz et al., 2003), and many testis-specific RNF proteins have been found to be essential in spermatogenesis and fertilization processes. RNF133, a PA-TM-RING type RNF E3 ligase is highly and specifically expressed in spermatids. In the mouse spermatocyte cell line GC-2, RNF133 was found located to the ER, and further studies indicated that RNF133 is able to perform self-ubiquitination in human embryonic kidney 293T (HEK-293T, which stably express the SV40 large T antigen) cells. These findings implicate that RNF133 may be involved in ER protein quality control during spermatogenesis (Nian et al., 2008). In rat, MARCH11, a member of MARCH family, is specifically expressed in the early developmental stages of spermatids (Morokuma et al., 2007). Another RNF protein is RNF19a, which is found in the Golgi apparatus, acrosomal membrane and tail. It can interact with the 26S proteasome subunit Psmc3, potentially associated with the UPS in shaping the spermatid head and tail (Rivkin et al., 2009).

Our lab previously demonstrated the differential expression profiles of UPS in neonatal rat gonocytes and spermatogonia. Among those, RNF149 is strongly expressed in gonocytes and down-regulated in spermatogonia (Manku et al., 2012c), but the role it plays in this process remains unclear. RNF149 is a transmembrane PA-TM-RING E3 ligase mostly found on ER (Endoplasmic reticulum) and lysosomal membrane (Li et al., 2013). It is homologous to GRAIL family (Lineberry et al., 2008), which is a type I transmembrane protein that localizes to the endocytic pathway (Borden, 2000) and is able to recognize LEL (larger extracellular loop) domain of tetraspanin proteins through their PA domain (Bocock et al., 2009).

Interestingly, RNF149 has been reported in human colon cancer cells to ubiquitinate B-Raf by direct conjugation of ubiquitin to the C-terminal of B-Raf, and to reduce cell proliferation through degradation of B-Raf (Hong et al., 2012). Among three known Rafs, B-Raf is the most effective Raf kinase in terms of induction of MEK/ERK activity, but the mechanisms involved in B-Raf regulation remain unclear. In contrast, a recent study published in Chinese indicates RNF149 can ubiquitinate tetraspanin CD9, leading to its degradation, and increase HEK 293 cell proliferation (Li et al., 2013). CD9 has also been shown to be expressed in SSCs and in spermatogonia presenting long term repopulation potential in transplantation studies (Hofmann et al., 2005). In addition, our lab observed that the expression level of RNF149 were significantly decreased when compared to other UPS genes in both PND8 rat spermatogonia and isolated rat gonocytes treated with retinoic acid, which indicates that Rnf149 is actively down-regulated during both in vitro and in vivo differentiation (Manku et al., 2012c). Furthermore, the other name of RNF149 is DNA polymerase-transactivated protein 2 (DNAPTP2), in relation to RNF149 functions in RNA processing, cell-cycle control and peroxisomal biogenesis, where the RING domain is considered as a potential transcriptional activation domain that functions in organizing large protein complexes contributing to diverse cellular processes (Borden, 2000).

7. Conclusion

While our studies showed that RNF149 is down-regulated during the transition from gonocyte to spermatogonia, the two studies discussed above indicate that RNF149 may either positively or negatively regulates cell proliferation in different cell types. In view of this knowledge and our findings, we hypothesized that RNF149 may be involved positively in proliferation and needs to be down-regulated for the cells to progress toward differentiation. In this context, the goal of our research was to clarify whether RNF149 plays a role in gonocyte proliferation and its mechanism of action.

Materials and Methods

1. Animals

Newborn male Sprague-Dawley rats were purchased from Charles Rivers Laboratories (Saint-Constant, Quebec). PND2 to PND35 pups were euthanized and handled according to protocols approved by the McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care.

2. Gonocyte Isolation and RNF149 silencing

Gonocytes were isolated from PND3 rat testes using 30–40 pups per preparation as previously described (Manku et al., 2012a). In short, decapsulated testes were submitted to sequential enzymatic tissue digestion consisting of a first step of collagenase and hyaluronidase to separate the interstitium from the tubules, then trypsin digestion to dissociate seminiferous tubule cells into mixtures of isolated germ cells, Sertoli and myoid cells, with DNAse I added in all dissociation steps to prevent denatured DNA to trap cells, inhibition of trypsin with fetal bovine serum (FBS) and filtration. Cells were transferred into serum-free medium for electroporation. For RNF149 silencing, transfections were performed on Sertoli cells, myoid cells and gonocytes mixtures. Cell numbers were fixed at $25x10^6$ cells/ml RPMI 1640 (Life Technologies, Canada), then electroporated in a Lonza 4D-Nucleofector™ System at 17ml per well with either RPMI 1640 medium, RNF149 siRNA or Scramble as negative control (siRNA: Screening DsiRNA TriFECTa Kit; Scramble: DS NC1, Both purchased from IDT Inc., Canada). Electroporated cell mixtures are then plated overnight with 5% FBS (Invitrogen, Burlington, ON, CA) to separate gonocytes from somatic cells, as gonocytes maintain floating while somatic cells attach to the culture dish.

After overnight differential plating, media were collected and applied onto a 2-4% BSA gradient for further cell separation. Fractions that contained the most gonocytes (as judged by their morphology and large size) were chosen and pooled to obtain final cell suspensions presenting 80-90% purity; the remaining fractions that still contained many gonocytes were also collected as “tail fraction” and treated as described below before processing for immunocytochemical analysis. High purity gonocyte fractions are centrifuged after collection and small aliquots are frozen for later RNA extraction as day 0 controls. The remaining cells are aliquoted and cultured as described below. Tail section were either collected directly on microscopic slides by cytospin centrifugation (day zero), or kept for 24 and 48 hrs with medium alone (control) or a proliferation cocktail of 10 ng/ml PDGF-BB and 1 µM E_2 (Both purchased from Sigma-Aldrich, USA) before collection on cytospin slides.

3. Gonocyte Culture

Gonocytes transfected with either medium, siRNA or scramble nucleotide were cultured for one or two days to examine the effects of of PDGF-BB and E_2 as previously described (Thuillier et al., 2010). Gonocytes from the final BSA pool (with a purity of ~80-90%) were resuspended in RPMI 1640 medium supplemented with 2.5% FBS (Invitrogen), 2% penicillin/streptomycin, and 1% amphotericin B (All antibiotics are purchased from CellGro; Mediatech, USA). Gonocytes were then plated in 24-well plates at a density of 10,000 cells in 500µL final volume containing medium alone. Because of the limited numbers of gonocytes per isolation and the previous determination of the lack of effect of the solvent (0.1% ethanol) as compared to medium alone, control samples consisting of medium alone were used in subsequent experiments. These cells were cultured in a 3.5% CO_2 incubator at 37°C. At the end of the each incubation condition, gonocytes were collected for sequential RNA extraction, cDNA synthesis, and qPCR analysis. Each condition was tested in duplicate wells, and at least three independent experiments were performed for each condition examined.

4. RNA extraction and PCR analysis

Total RNA of gonocytes was extracted from cell pellets using the Arcturus® PicoPure® RNA Isolation Kit (Life Technologies, Canada) as previously described (Manku et al., 2012c). Tissue total RNA of testis and other organs in PND2 to PND35 rat pups were extracted using QIAGEN RNeasy Mini kit (Qiagen, Santa Clarita, CA) as previously described (Wang & Culty, 2007). All samples were digested with DNase I before extraction (Qiagen, Santa Clarita, CA). cDNA was synthesized from isolated RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics).

RNF149 gene expression in various tissue samples was examined by PCR and electrophoresis in 1.5% agar gel. Two primer sets were used: for RNF149 cloning primers (Reverse, 5'- CGA GCG GTC TCA CTC TTC C; Forward, 3'- TGA GGC TGT CAA TGA AGA CG), and variant (VA) form testing primers (Reverse, 5'- AAG GAA TTC CAG TAA AAA TGA GG; Forward, 3'- TTA AAG TTT TCA ATA CAC ACT GC). PCR reactions were carried out using GoTaq® DNA polymerase (Promega) and amplified using the iCycler thermal cycler from Bio-Rad (Hercules, CA). PCR cycle conditions were 95°C for 3 min; 45 cycles of 95°C for 60 sec, 55°C for 60 sec, and 72°C for 2 min; followed by a 10 minute extension at 72°C, then cool down to 4°C.

5. Real-time Quantitative PCR (qPCR)

qPCR was performed as previously described on the Roche LightCycler 480 using SYBR Green PCR Master Mix kit (Roche Diagnostics, Laval QC) (Manku et al., 2012c) Primers specific for the genes of interest were designed with the Roche primer design software, include RNF149, markers of proliferation/self-renewal (PCNA, Plzf, Nanog, Oct4), undifferentiated spermatogonia (Foxo1) and differentiated spermatogonia (Stra8, c-Kit, Dazl). (Table 1).

Ct values of the various genes were normalized to the endogenous reference (18S rRNA) by calculating the value of 2ΔCt, with ΔCt being the difference between the cycle threshold (Ct) of the gene of interest and that of 18S rRNA. We initially determined the Ct values of three potential housekeeping genes, GAPDH, α-Tubulin and 18S rRNA in cDNA samples from isolated gonocytes cultured for 1 day after siRNA interference, and 18S rRNA showed that it presented minimal changes in Ct values between samples. The final data were expressed in arbitrary units representing the mRNA levels of the genes of interest present in the samples tested. Assays were

Table 1. List of primers

Name	**Ref Seq**	**Primers** (5' to 3')	**F/R**
RNF149	XM_343561.7	TGCACCTTCAAGGACAAGGT	F
		ACGACTATGCTGCCTGTTCC	R
PCNA	NM_022381.3	GAACTTTTTCACAAAAGCCACTC	F
		GTGTCCCATGTCAGCAATTTT	R
Stra8	XM_575429.2	TGCTTTTGATGTGGCGAGCT	F
		GCGCTGATGTTAGACAGACGCT	R
PLZF	NM_001013181	CGCCCAGTTCTCAAAGGA	F
		AGACAGAAGACAGCCATGTCC	R
FOXO1	NM_001191846.2	TCAGGCTAGGAGTTAGTGAGCA	F
		GGGGTGAAGGGCATCTTT	R
C-KIT	NM_022264.1	ACGTGGTAAAAGGAAATGCACG	F
		AAAATGCTCTCCGGTGCCA	R
DAZL	NM_001109414	CGGTTGATGTTAGGATGGATGAAACCG	F
		AGCCCTTCGACACACCAGTTC	R
Nanog	AB275459.1	CGTTGGGAGAAGTTTTGCTGCAACG	F
		ACGCTGCTCCGCTCCATAAC	R
OCT 4	NM_001009178.2	CGGTTTTCGAGTGTGGTTCTGTAACCG	F
		GCGGCCTCATACTCTTCTCGT	R
GAPDH	NM_017008	CGGAGCAAAAGGGTCATCATCTCCG	F
		TGGTTCACACCCATCACAAACAT	R
α-Tubulin	BC062238	CGGGGGAGAGTTCTCTGAGGCCCG	F
		CAGAATCCACACCAACCTCCTC	R
18S	X01117.1	CGGTTGATGTTAGGATGGATGAAACCG	F
		AGCCCTTCGACACACCAGTTC	R

performed in triplicate. The results were expressed as the means ± SEM of the fold changes in relative expression levels normalized to 18S rRNA.

6. Immunohistochemistry

Protein expression of RNF149 and PCNA was determined using formaldehyde-fixed, paraffin-embedded sections of PND3 and PND8 testes. As previously described, slides were dewaxed and rehydrated using Citrosolv (Fisher Scientific, Toronto, ON, CA) and Trilogy solution (Cell Marque IVD, Rocklin, CA, USA) (Manku et al., 2012b; Manku et al., 2012c). Following treatment with boiled Dako Target Retrieval solution (DAKO, Burlington, ON, CA), the sections were incubated with PBS (Invitrogen) containing 10% goat serum (Vector Laboratories, Burlington, ON, CA), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega, Madison, WI, USA) for 1 h to block nonspecific protein interactions. Sections were then incubated with RNF149 (G-12, Santa Cruz) or PCNA antibody (PC10, Santa Cruz) diluted in PBS (200x dilution, Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) overnight at 4°C. The sections were then incubated with biotin-conjugated goat anti-rabbit or anti-mouse secondary antibody (BD Pharmingen, Mississauga, ON, CA), in PBS (Invitrogen) diluted 1% BSA (Roche Diagnostics) Master Mix for 60 min at room temperature. Immunoreactivity was detected using streptavidin-peroxidase (Invitrogen) and AEC single use solution (Invitrogen). Sections were counter-stained with filtered hematoxylin (Sigma-Aldrich) and processed for cover-slipping. Slides were examined under bright-field microscopy with a BX 40 Olympus microscope (Olympus, Center Valley, PA, USA) coupled to a DP70 Olympus digital camera (Olympus). Negative controls were done by incubating some sections with Rabbit IgG (preimmune rabbit serum) (Invitrogen)

7. Immunocytochemistry

The expression of RNF149 and PCNA were examined by immunocytochemistry, following a previously described protocol (Manku et al., 2012a; Thuillier et al., 2010). Briefly, gonocytes (or cell lines) are collected right after treatment, washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min, centrifuged, washed, and then collected onto microscopic slides by cytospin centrifugation. The slides are dried and treated with a mixture of acetone/methanol (60/40), followed by DakoCytomation target retrieval solution (Dako North America Inc., Carpinteria, CA). The slides are incubated overnight at 4°C with RNF149 and PCNA antibodies. The next day, the slides are treated for colorimetric staining using biotin-coupled secondary antibodies (BD Pharmingen, Mississauga, ON, CA) and streptavidin-coupled horseradish-peroxidase (HRP)/colorimetric HRP chromogen (AEC; Invitrogen), further counterstained with hematoxylin (Invitrogen), and cover-slipped. Negative controls are produced by incubating some samples with nonspecific rabbit IgG (Invitrogen).

Microscopic slides of isolated gonocytes and spermatogonia were prepared by cytospin centrifugation of germ cells at lower purity, using tail fractions of the BSA gradient that contained Sertoli and myoid cells, since high purity was not required for this type of end-point, and the presence of somatic cells could potentially provide internal controls by showing the immunostainings of other cell types.

8. Recombinant DNA constructs and amplification

Template RNF149 cDNA were cloned from PND3 testis total cDNA with RNF149 cloning primers by GoTaq® DNA polymerase (Promega) to create poly-A tailing, template concentrations are all set to 100ng/μL. PCR products were then separated and extracted from 1.5% agar gel.

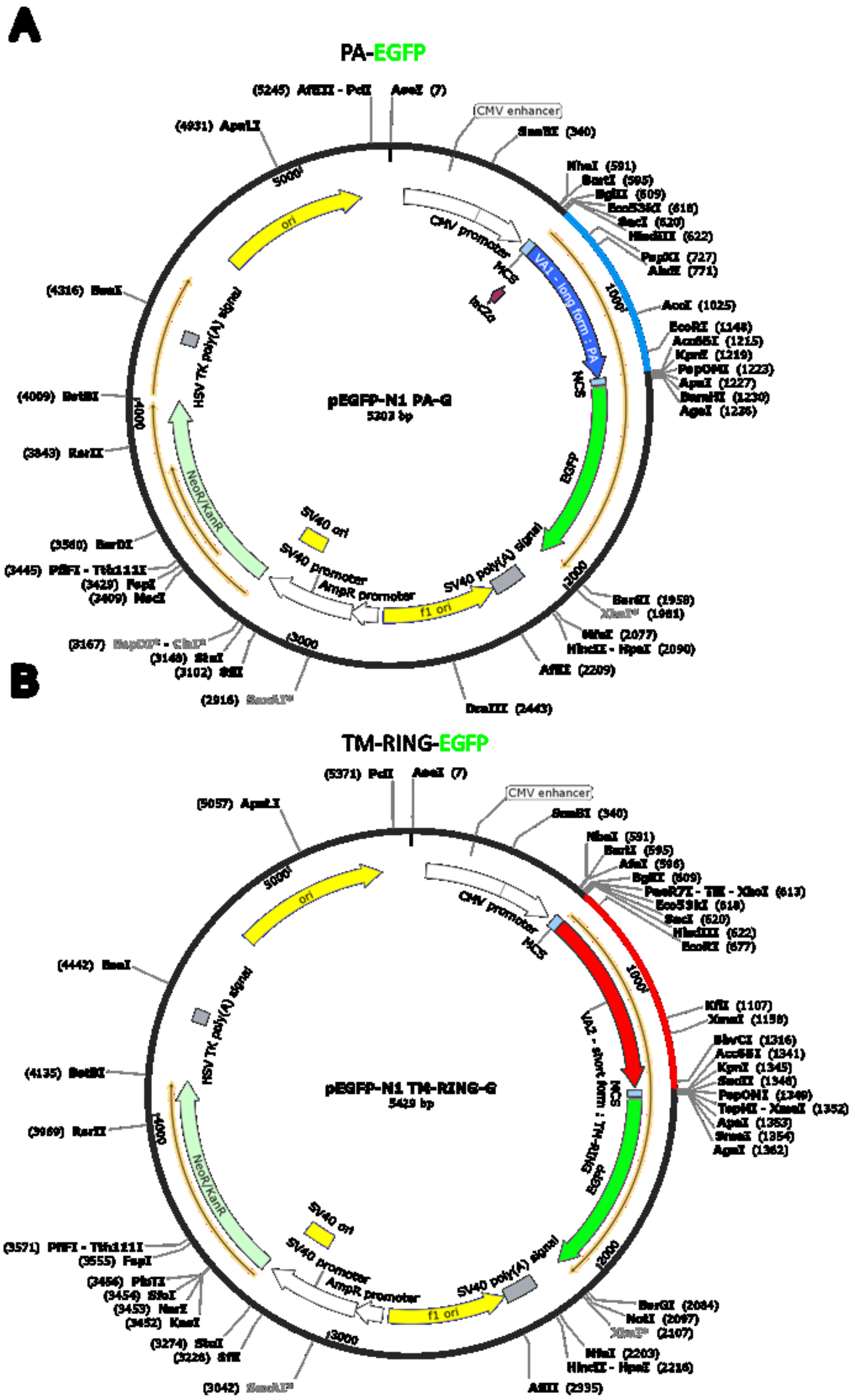


Figure 1. Gene maps of RNF149-EGFP plasmids.

(A) RNF149 VA1-EGFP gene map. (B) RNF149 VA2-EGFP gene map.

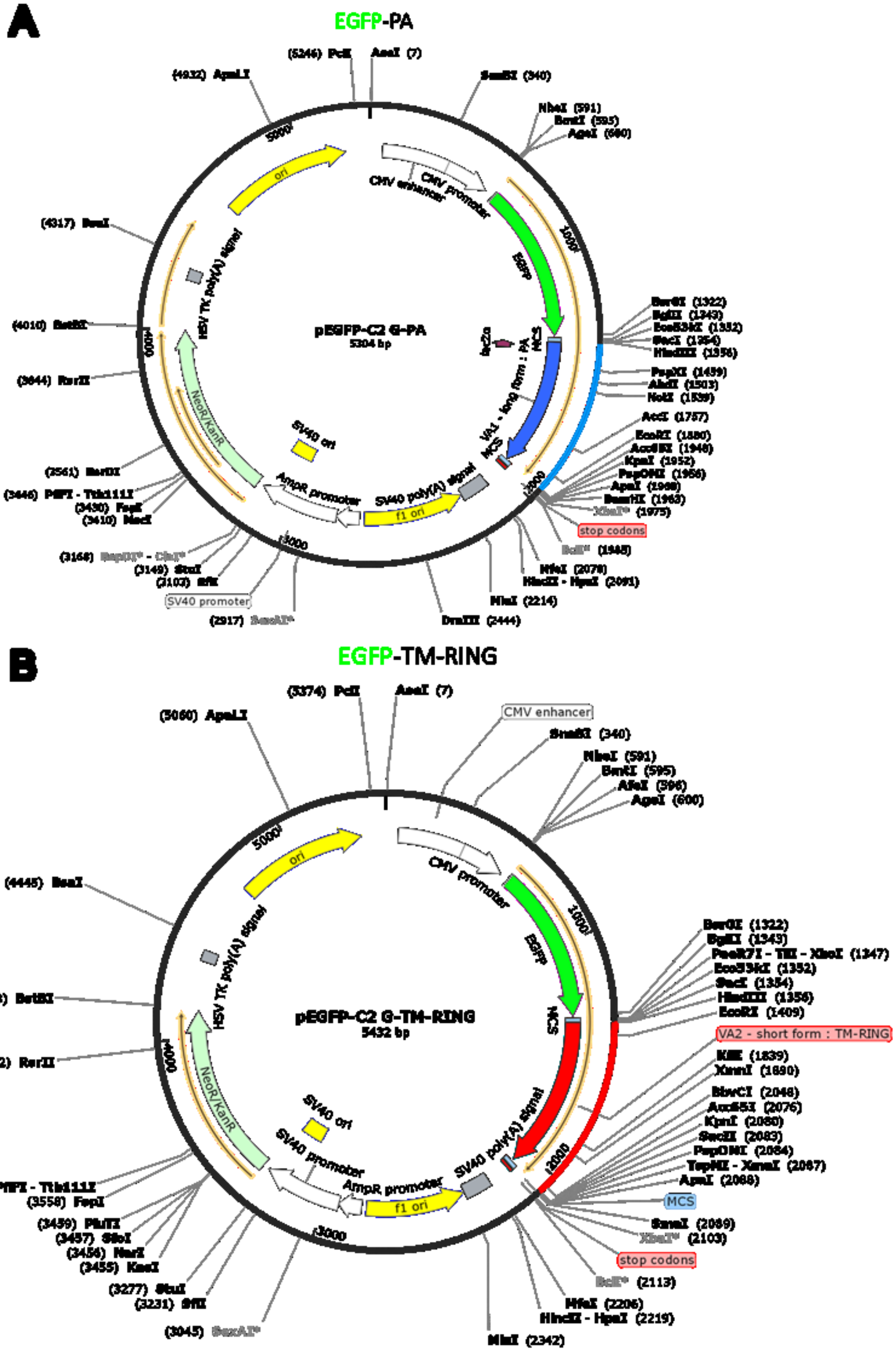


Figure 2. Gene maps of EGFP-RNF149 plasmids.

(A) RNF149 EGFP-VA1 gene map. (B) RNF149 EGFP-VA2 gene map.

Purified segments were then ligated to pGEM®-T Easy Vector System I (Promega) and transformed into DH5-α competent cells (Life Technologies) overnight in incubator at 37°C. Single colonies were collected and cultured in LB (Invitrogen) for 8 hours, then plasmid were purified by QIAprep Spin Miniprep kit (Qiagen) and sent to sequencing. After sequencing, two confirmed variant forms of RNF149 were then further amplified and purified with HiSpeed Plasmid Maxi Kit (Qiagen). These two variant forms were then ligated into pEGFP-N1 and pEGFP-C2 (Clontech). Based on the gene maps constructed on SnapGene® software (Version 2.8, GSL Biotech), restriction sites were selected at HindIII and KpnI with 2.1 buffer (New England Biolabs, USA). The following constructs were used in the present study: N-terminal EGFP-tagged VA1 (pPA-EGFP) and VA2 (pRING-EGFP) (Fig 1), C-terminal EGFP-tagged VA1 (pEGFP-PA) and VA2 (pEGFP-RING) (Fig 2).

9. Transfections and Live Cell Imaging

F9 cells (mouse embryonal teratocarcinoma cell line) and C18-4 cells (spermatogonial cell line) were cultured in DMEM (Life Technologies), containing 10% FBS, 100 units/ml penicillin and 2.5 μg/ml streptomycin. The C18-4 cell line is a gift from MC Hofmann, created by Large T antigen immortalization of PND6 mouse spermatogonia (Hofmann et al., 2005). For F9 cells, 0.1 % gelatin (Millipore, USA) is coated on culture dish before adding cells. Both cell lines were grown on 35mm fluorodish cell culture dish (World Precision Instruments, USA) at 25 million cells before transfection. F9 cells were incubated in 37°C, 5% CO_2, whereas C18-4 cells were incubated in 34°C, 5% CO_2. Cells were transfected with the plasmids mentioned above with either Set1: BFP-KDEL (Blue ER tubules mark, Addgene, USA) and DsRed-Mito (Red mitochondria mark, Clontech, USA) or Set2: pDsRed2-ER (Red ER mark, Clontech, USA), one day before confocal microscopy observation, using Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol before observation. LysoTracker Blue DND-22 (Life Technologies) is added to Set2 cells before observation, final concentration is 60nM, then incubate for 30 min at either 37°C or 34°C depending on cell types.

Before observation under confocal microscope, cells are gently washed with culture medium, then 1 ml Opti-MEM medium (Life Technologies) are added in replace of culture medium. Cell sample were analysed by Zeiss LSM880 Laser Scanning Confocal and Super-Res SIM/PALM/dSTORM system (Zeiss) in McGill University Health Centre Research Institute Molecular Imaging Core laboratory. Images were collected from each sample for 15–60 min.

10. Statistical Analysis

All statistical analysis was performed using unpaired two-tailed t-test with statistical analysis functions in GraphPad Prism version 6.0 software (GraphPad Inc., San Diego, CA).

Results

1. Expression Profile of RNF149 in Neonatal to pubertal Rat Organs

Our previous studies showed that RA-induced gonocyte differentiation requires an active ubiquitin proteasome system (UPS), and identified a number of UPS genes and proteins differentially expressed between PND3 gonocytes and PND8 spermatogonia (Manku et al., 2012c). Among those, RNF149 was found to be more abundant in gonocytes than spermatogonia, suggesting that this UPS gene is decreased during the process of differentiation and remains low thereafter. In order to confirm this hypothesis and to understand the role of RNF149 in rat development, tissues such as testis, kidney, liver, heart and brain were collected from rat pups age from PND2 to PND35 for gene expression and immunohistochemistry studies.

Through qPCR analysis, RNF149 mRNA seems mainly expressed in testis at different time point from PND2 to PND10 and PND15, PND21, PND35 when compared to other major organ in rat, such as brain, liver, kidney, and heart (Fig 3A). In neonatal testis, RNF149 is mainly expressed in gonocytes and is found in Sertoli cells at older ages. Interestingly, RNF149 was found highly expressed in the nucleus of PND2 and 3 gonocytes, with a weaker staining in gonocyte cytoplasm, , and to translocate to the cytoplasm in spermatogonia (Fig 3B), which indicates the RNF149 might works as FOXO1, bybeing dynamically expressed in either nuclear or cytoplasm at different stages of development. Although gene expression level of RNF149 in other organs was lower than in testis, we also observed weak RNF149 staining in brain, liver and heart at PND3, a more robust staining in PND10 liver and a strong signal in cells of kidney tubules at both ages (Fig 3C). This suggests that RNF149 might play an important role in regulating the development of different

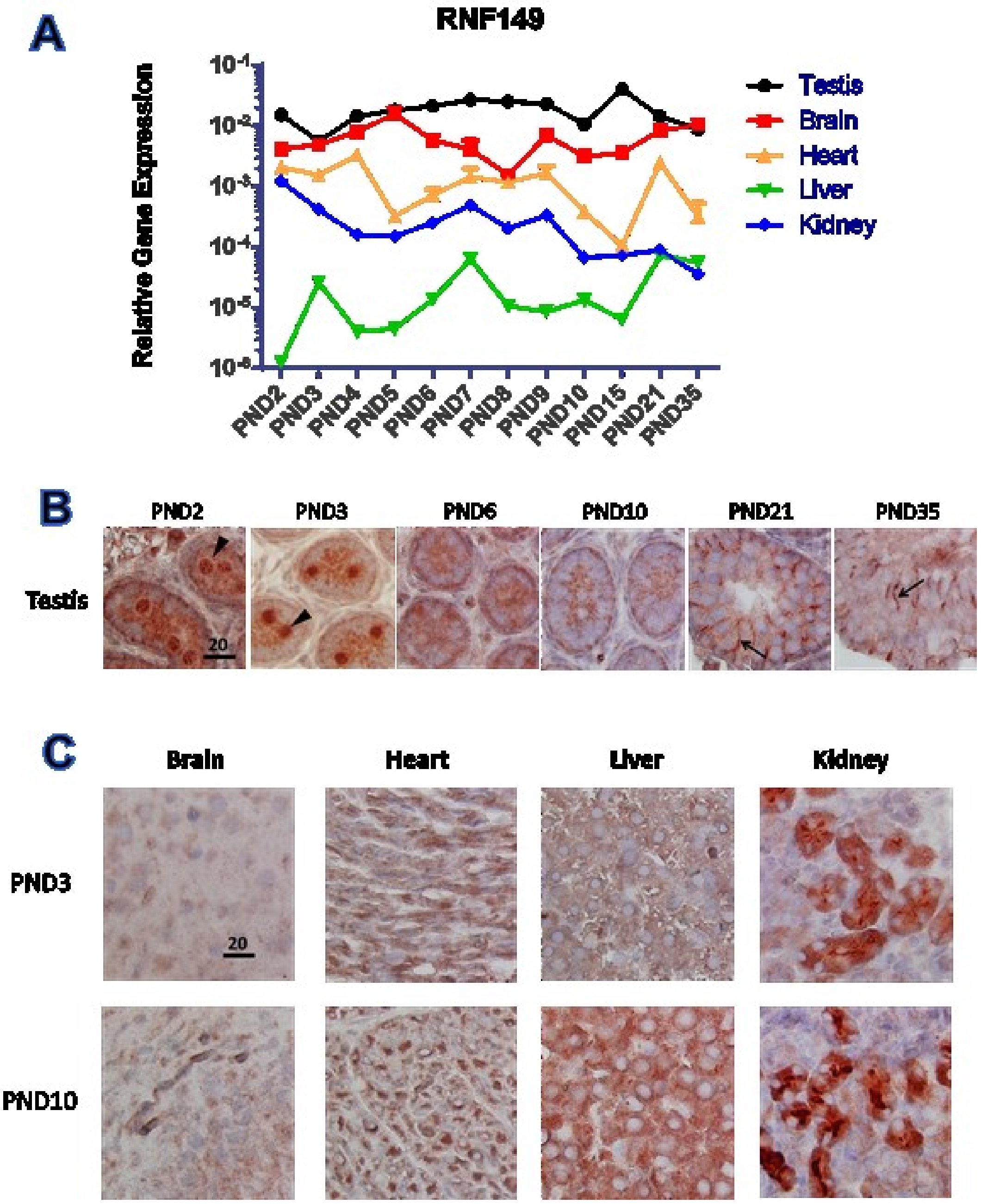


Figure 3. RNF149 tissues and developmental profiles in rat.

(A) RNF149 mRNA levels in various tissues from PND2 to PND35. (B) RNF149 protein expression in testis from PND2 to 35. Arrowhead: germ cell; arrow: Sertoli cell. (C) RNF149 expression in brain, heart, liver, kidney at PND3 and PND10. Scales in µm. Representative pictures are shown.

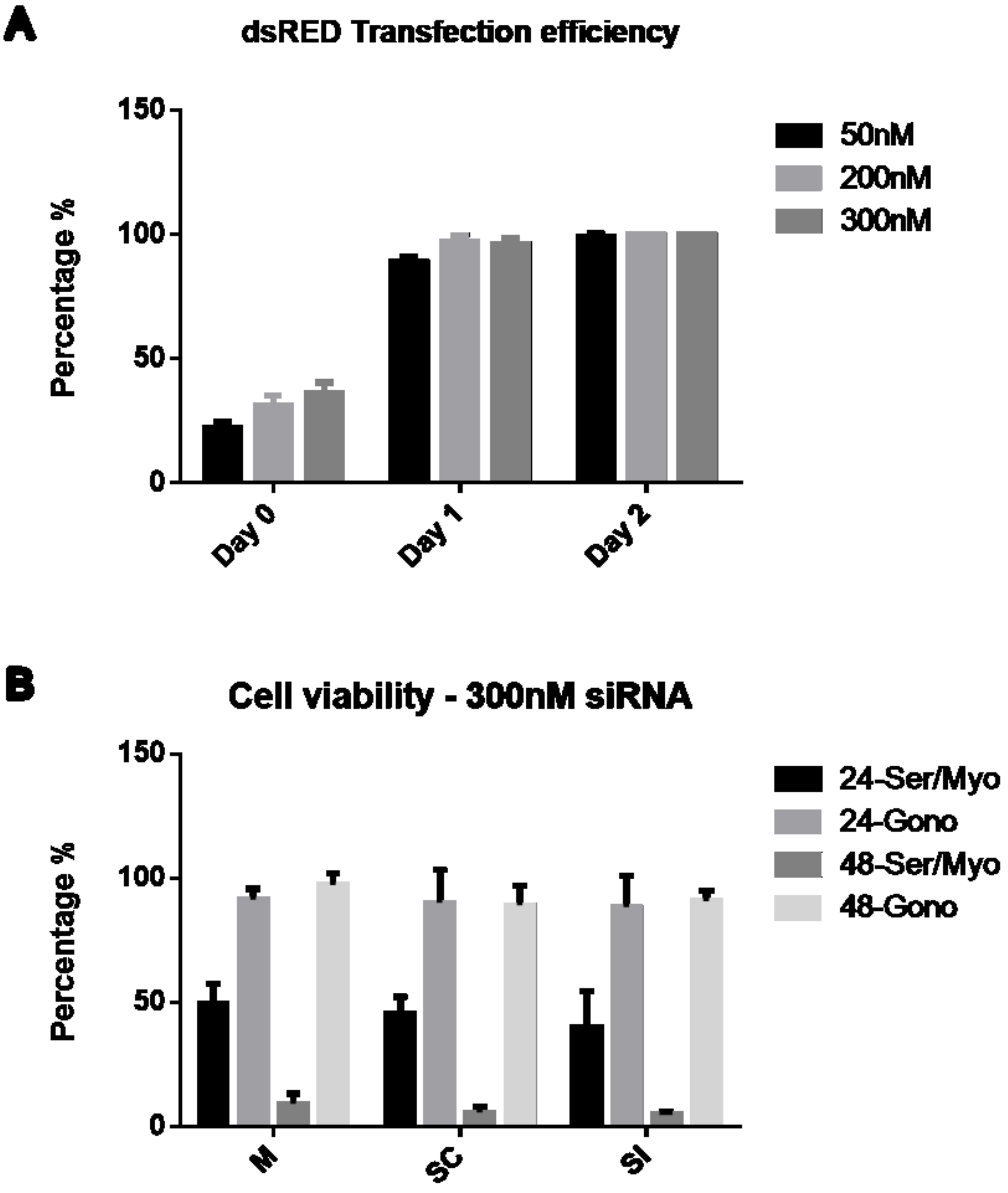


Figure 4. dsRED Transfection Efficiency and Cell Viability Test.

(A) Transfection efficiency of dsRED in GSM cell mixture at 50nM, 200nM, and 300nM after 24 and 48 hours of transfection. (B) Cell viability of gonocyte and Sertoli cell/myoid cell under 300nM (100nM of each duplex) of siRNA and other conditions 24 and 48 hours after transfection. After electroporation, all cells in different conditions were cultured in 5% FBS RPMI1640 medium overnight, and redistribute to serum-free RPMI1640 medium for the next 24 hours.

tissues, mainly testis and kidney. However, whether it regulates proliferation or differentiation in gonocytes remains unclear.

2. RNF149 silencing might leads to reduced cell proliferation

To understand the mechanism of RNF149 in regulating cells development, we started by testing the function of RNF149 in proliferation, as previous studies indicated that RNF149 is an E3 ligase highly expressed in PND3 gonocyte (Manku et al., 2012c), and it had been shown to regulate cell proliferation via different pathways in other cell lines (Hong et al., 2012; Li et al., 2013). To optimize silencing condition for functional studies, the gonocyte/Sertoli cell/myoid cell (GSM) mixture was collected from dissociation of PND3 SD rat testes, and aliquoted into three parts for electroporation with either mock, scramble, and siRNA treatements. The siRNA kit used was the siRNA Trifecta kit, which includes three siRNA sequences against RNF149 (XM_343561). Transfection efficiency was confirmed by dsRED control. Cell viability after electroporation was confirmed by trypan blue assay. Based on dsRED result, over 90% of the GSM mixture cells were transfected with dsRED (Fig 4A). Under 300nM of siRNA treatment, over 90% of gonocyte can survive at least 48 hours, whereas most Sertoli cell and myoid cell died in serum-free medium (Fig 4B).

RNF149 silencing efficiency and marker for cell proliferation (PCNA) were determined by qPCR. With 300nM of siRNA (100nM for each siRNA sequence), an average of 66% and 58% of silencing efficiency for 24 and 48 hours after electroporation was observed (Fig 5A), whereas the cell proliferation marker PCNA expression level increased at 48 hours after electroporation in mock, but was reduced in cell treated with siRNA against RNF149 (Fig 5B). To increase silencing efficiency, higher concentration of siRNA was applied. With higher siRNA concentration (900nM,

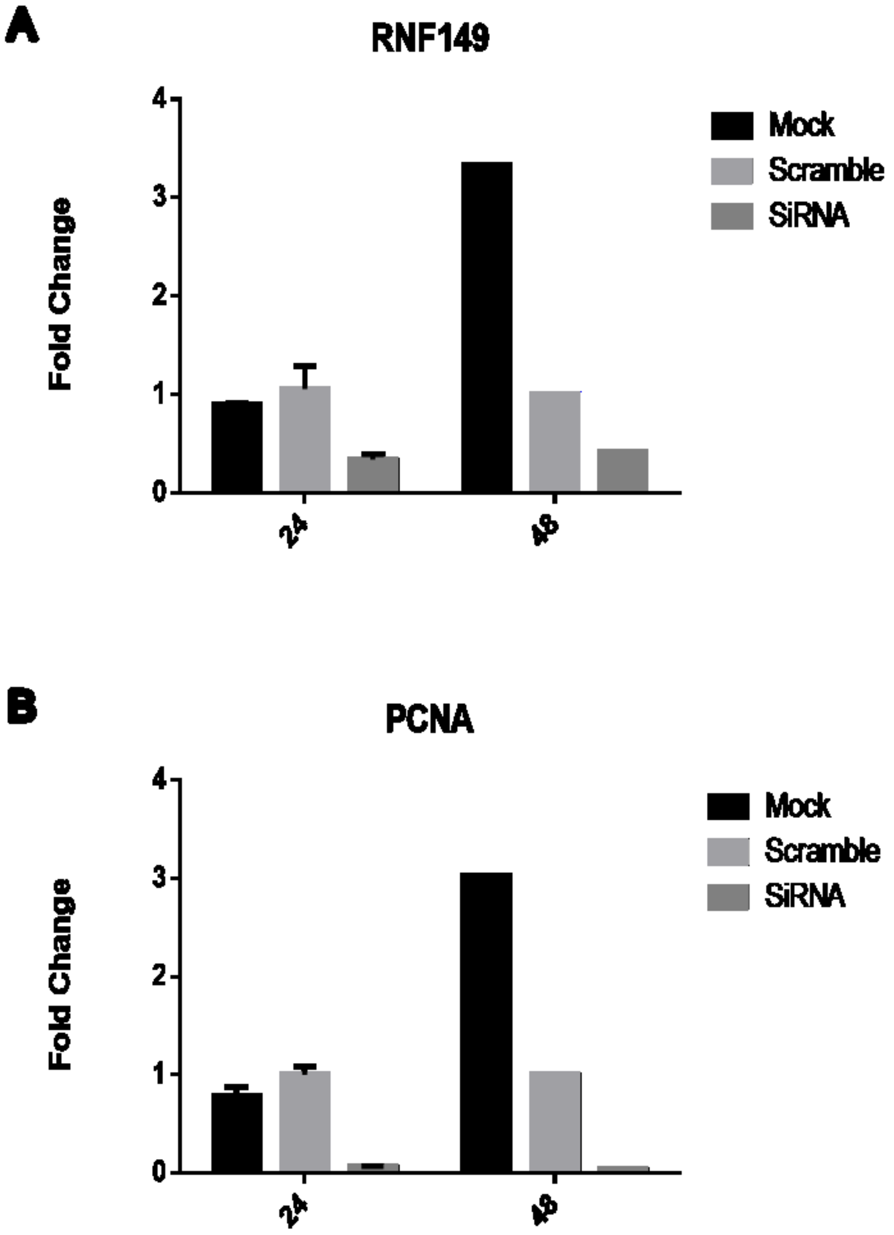
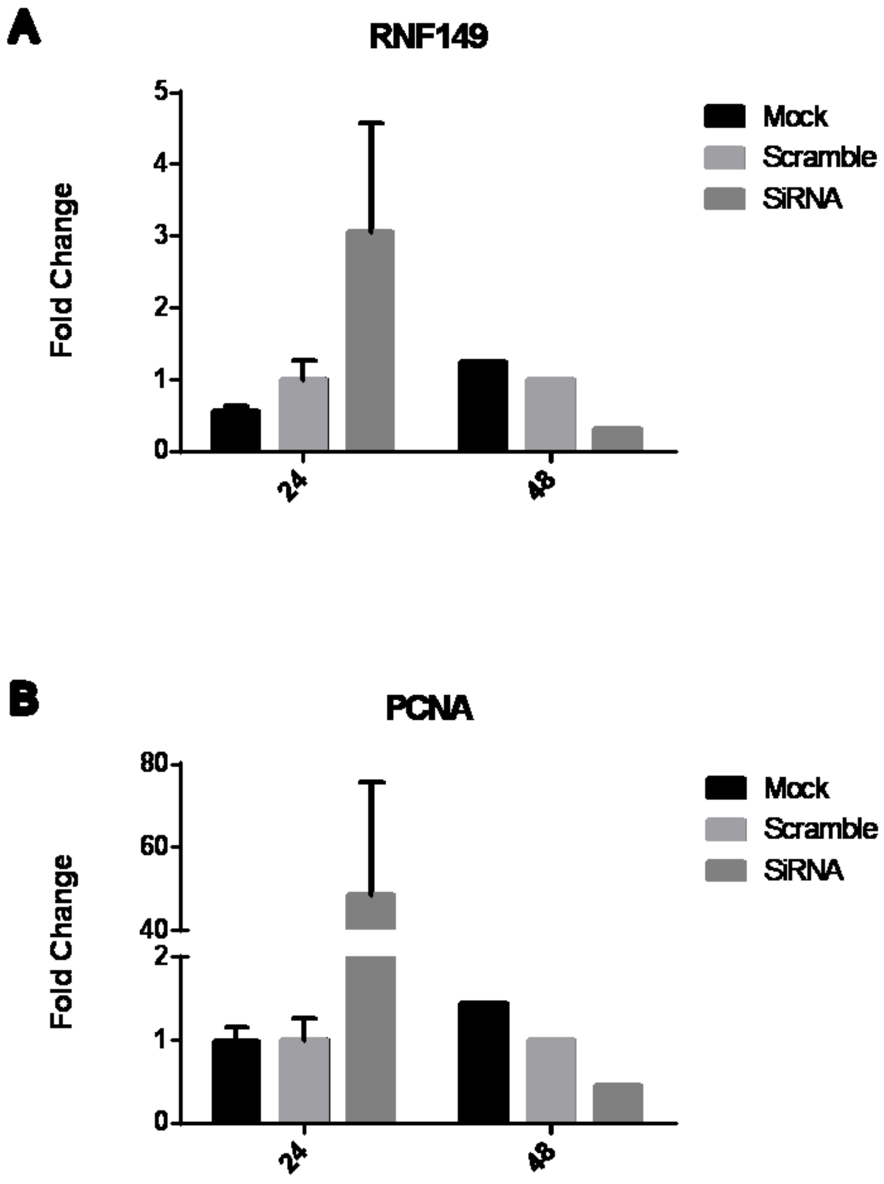


Figure 5. qPCR Analysis of RNF149 Silencing Efficiency and Marker of Gonocyte Proliferation of Low Concentration siRNA.

(A) RNF149 silencing efficiency 24 and 48 hours after electroporation with 300nM siRNA (Mock: cells treated with RPMI1640 and electroporation only; Scramble: cells treated with 100nM scramble sequence; siRNA: cell treated with three siRNA duplex, 100nM each). (B) qPCR analysis of proliferation marker PCNA. All data were normalized to average scramble relative expression level of each time point, N≥3.



<u>Figure 6. qPCR Analysis of RNF149 Silencing Efficiency and Markers of Gonocyte Proliferation with High Concentration of siRNA.</u>

(A) RNF149 silencing efficiency 24 and 48 hours after electroporation with 900nM siRNA (Mock: cells treated with RPMI1640 and electroporation only; Scramble: cells treated with 300nM scramble sequence; siRNA: cell treated with three siRNA duplex, 300nM each). (B) qPCR analysis of proliferation marker PCNA. All data normalized to average scramble relative expression level of each time point, N≥3

300nM for each siRNA sequence), RNF149 silencing efficiency for 48 hours increased to 70%, but high levels of RNF149 mRNA expression was observed 24 hours after electroporation (Fig 6A), same trend was observed in PCNA mRNA expression (Fig 6B).

Since we had observed that silencing efficiency increased in 48 hours with higher concentration of siRNA, and knowing there was less Sertoli cell and myoid cell that might affect RNF149 expression at that time point, BSA gradient was applied to obtain enriched gonocytes preparations after electroporation. Purified gonocyte were treated with or without PDGF-BB and E_2 in serum-free medium at 24-hours' time point and collected at 48 hours' time point to measure proliferation. In purified gonocytes, the RNF149 mRNA level was reduced in siRNA compared to scramble at 24 hours after electroporation, and 48 hours after electroporation with P+E treatment for 24 hours.

However, RNF149 expression level in both scramble and siRNA was higher than mock in all group, except when gonocytes were treated with P+E to induce proliferation for 24 hours, after 24 hours of electroporation (time point 48 hrs) (Fig 7A). In protein expression level, immunocytochemistry was used instead of western blot due to limited cell numbers. Result showed, when compared to control, that P+E treated mock and scramble gonocytes had higher RNF149+ cells than siRNA, similar to mRNA result (Fig 7C). In mock gonocytes, 24 hours of P+E treatment (time 48 hrs from start) increased PCNA mRNA levels, as expected from previous studies, whereas the PCNA expression in siRNA treated gonocyte was ten time less (Fig 7B). These data suggest that higher RNF149 expression is associated with higher PCNA expression, and that silencing RNF149 prevents proliferation-induced PCNA increases. Moreover, P+E treatment seems to increase RNF149 expression in the absence of siRNA, and the addition of siRNA reduces the

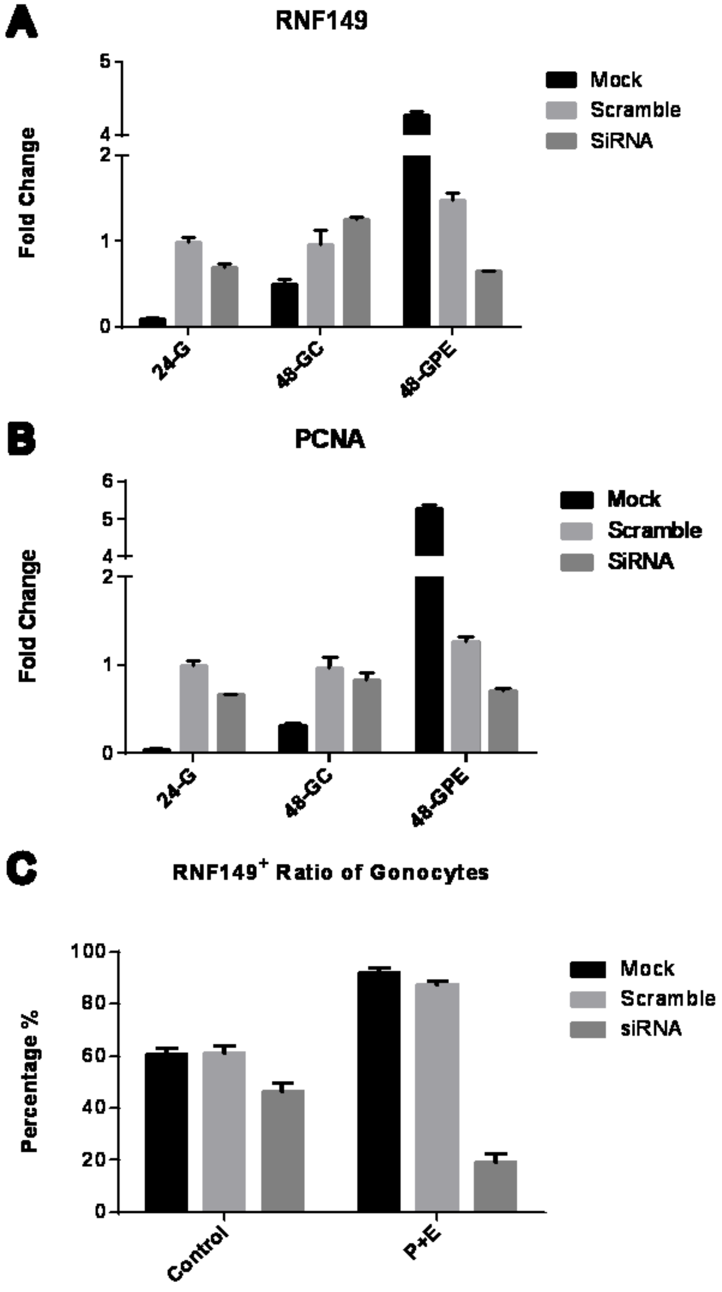


Figure 7. RNF149 and PCNA Expression Using Low Concentration siRNA.

Following mixed cell electroporation, gonocytes were isolated on BSA gradiant before treatment +/- proliferation cocktail. (A) RNF149 silencing efficiency 24 and 48 hours after electroporation with 300nM siRNA (Mock: RPMI1640 and electroporation only; Scramble: 100nM scramble sequence; siRNA: three siRNA duplex, 100nM each). (B) qPCR analysis of proliferation marker PCNA. C. Percentage of RNF149-positive gonocytes. All data were normalized to average scramble relative expression level of each time point, N≥3.

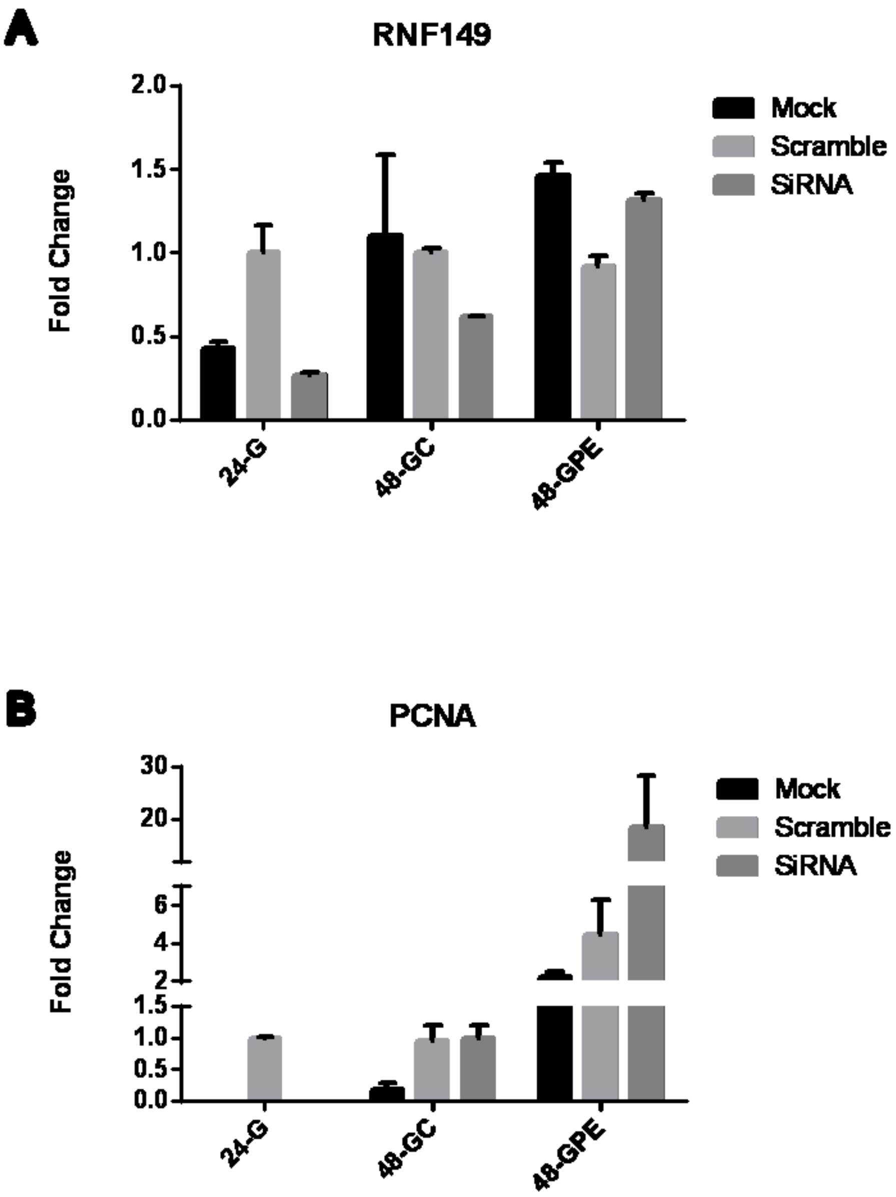


Figure 8. qPCR Analysis of RNF149 Silencing Efficiency and Markers of Gonocyte Proliferation with a Higher Concentration of siRNA.

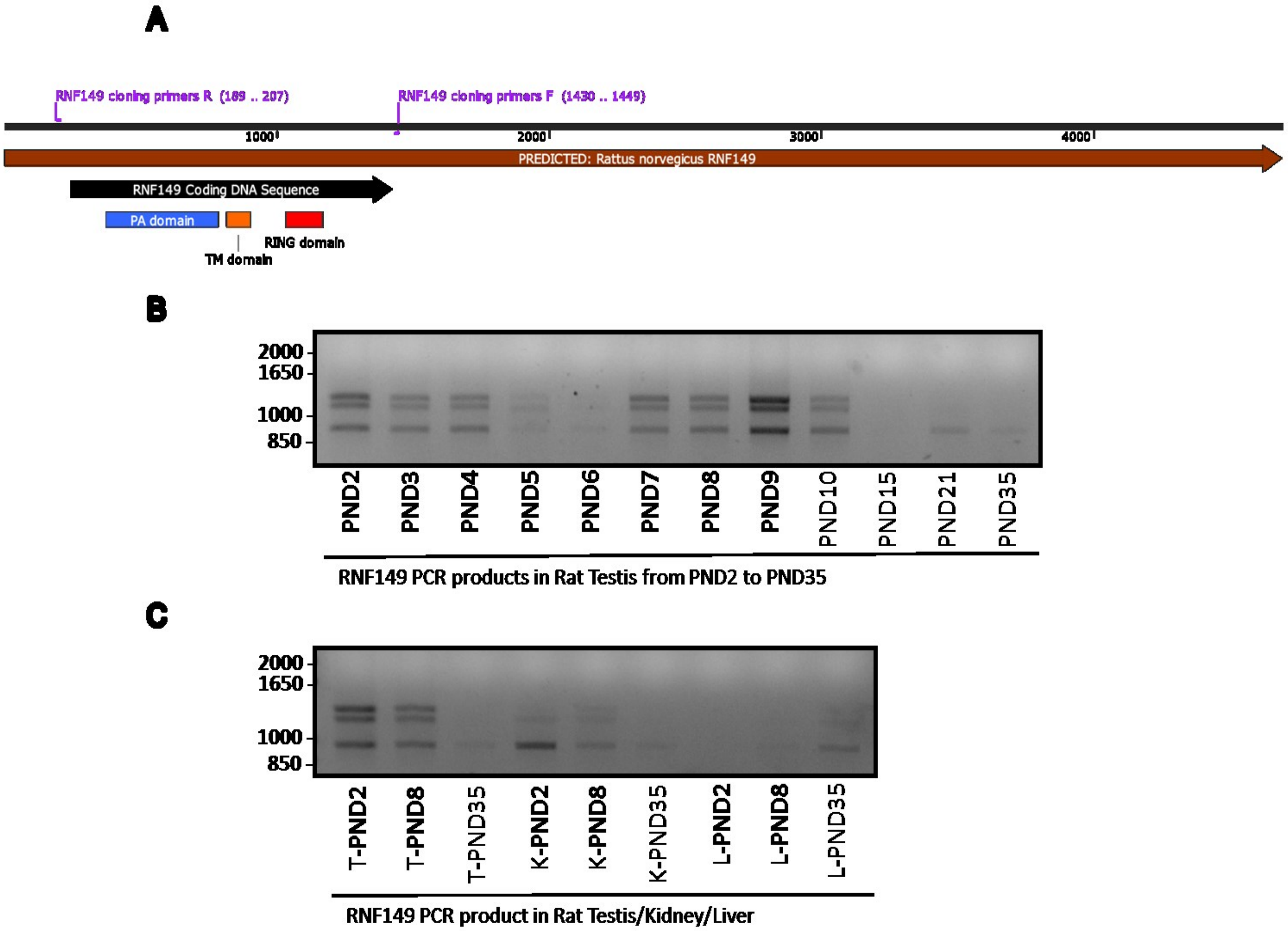
(A) RNF149 silencing efficiency 24 and 48 hours after electroporation with 1800nM siRNA (Mock: cells treated with RPMI1640 and electroporation only; Scramble: cells treated with 1800nM scramble sequence; siRNA: cell treated with three siRNA duplex, 600nM each). Here the cells were isolated at PND2, but keeping them overnight in Sertoli-gonocytes co-cultures allows them to become responsive to proliferative agents on the next day. (B) qPCR analysis of proliferation marker PCNA. All data normalized to average scramble relative expression level of each time point. N=2.

positive effect of P+E on RNF149 expression, possibly by degrading the newly formed RNF149 mRNA in response to P+E.

To introduce maximum siRNA entry into the cells, the manufacturer of the electroporation company recommends higher siRNA concentration than those we had used in previous experiments. In order to do that, we then tested the effects of 1800 nM RNF149 siRNA (using 3 duplex at 600nM each) on gonocytes. This gave a better silencing at 24 and 48 hrs in control samples. However, in the presence of P+E, the siRNA did not decrease RNF149 levels (Fig 8), and PCNA was increased by P+E treatment, which correspond to the results of lower siRNA concentration. Although these changes were less satisfactory than with lower siRNA concentrations, it indicated that when RNF149 silencing fails, then PCNA remains elevated upon PE stimulation.

3. Two forms of RNF149 transcripts exist in rat tissues

To date, the PA domain and RING domain of PA-TM-RING type RNF have been found serving various functions in regulating cell development either by working along or cooperating with other proteins. A recent report demonstrated a potential function for PA domain as a determinant for endosomal localization in HEK 293 cells (van Dijk et al., 2014). In addition, both human and mice RNF149 genes have been sequenced in the last decade, but the RNF149 sequence in rat remains unconfirmed. Because nothing was known on RNF149 role in germ cells, and only two articles had been published about its function, we propose to build EGFP tagged RNF149 vectors for better understand its cell localisation and mechanism of action. Interestingly, with primers designed based on a predicted sequence that should generate a single product with a size around 1185bp (Fig 9A), three major variant forms were found in all tissue samples, showing



<u>Figure 9. PCR Analysis of RNF149 sequence cloned from tissues from rat pups at different ages.</u>

(A) Gene map of RNF149, brown feature represent total predicted gene, black feature represent RNF149 coding sequence which includes PA, TM and RING domain. (B) PCR products of RNF149 in testes of rat pups at different ages. (C) PCR products of RNF149 in testes of rat pups in compare to Kidney and Liver at different ages.

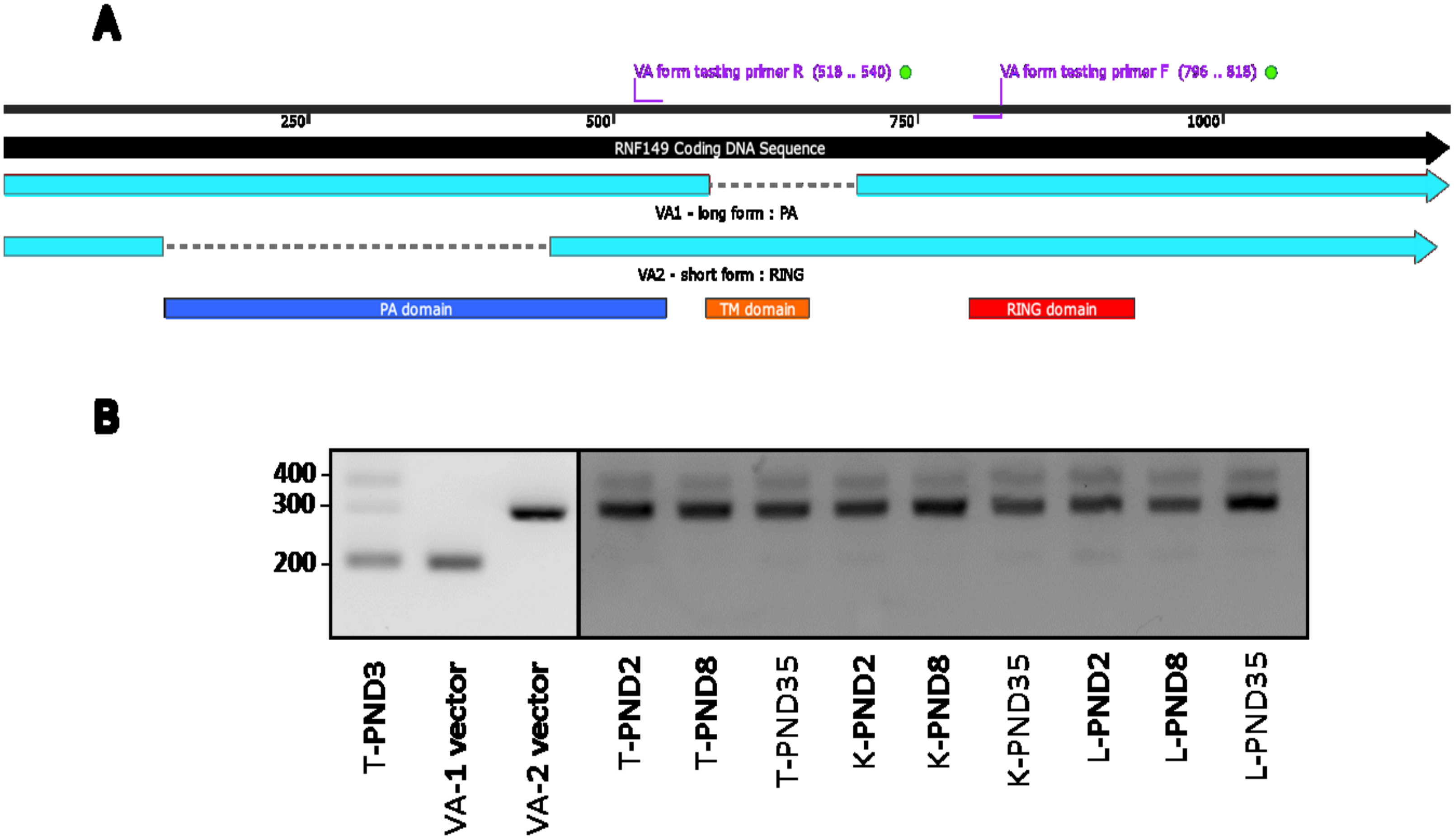


Figure 10. PCR Analysis for RNF149 variant forms confirmation in Testes, Kidneys and Livers of rat pups at different ages.

(A) Gene map of RNF149 CDS, black feature represent RNF149 coding sequence which includes PA, TM and RING domain. Two light blue feature represent two variant **form of RNF149 found in Rat. (B) PCR products of RNF149 in PND3 testis of rat pups, VA1 plasmid and VA2 plasmid in compare to Kidney and Liver at different ages.**

stronger expression in testis and kidney than liver,such as Kidney and liver, in agreement with the qPCR and tissue staining results (Fig 9B and 9C). Two PCR products that have sizes around 1000 were then cloned into pGEM®-T Easy Vector for sequencing. As the largest band (near 1400bp) was way over the expected size of RNF149 full length (1185bp), this band was not further used. However, one cannot exclude that it may correspond to a true RNF149 variant mRNA with intron retention, as found for a number of germ cell and cancer variant transcripts.

The sequencing results indicated that there are two variant forms in rat tissues: VA1 (1066bp) that expresses the PA domain and VA2 (862bp) that expresses the TM-RING domain (Fig 10A). Although a great number of mutated RNF protein expressions have been found in cancer patients, this might be the first naturally expressed variant forms of RNF proteins in rat. Based on the sequencing result, a primer set was designed to verify the expression of these two variant forms in other organs, which for VA1 should produce an 182bp long product, and for VA2 a 301bp. The results showed that almost all organs tested had three bands, two of them matching to our prediction (Fig 10B). The largest band, however, might be the same as seen in Figure 9B and 9C. VA1 and VA2 plasmid were then designed for EGFP-tagged RNF149 vector construction.

4. Expression of EGFP-tagged RNF149 isoforms in C18-4 and F9 cell lines

Our lab has shown that F9 cells, considered as surrogate for embryonic stem cells, also share similar traits with gonocytes, especially in their ability to express the spermatogonial marker STRA8 in responses to RA treatment, and in the existence of crosstalk between RA and PDGFR signaling pathways (Manku et al., 2015; Wang & Culty, 2007). Moreover, F9 cells proliferate in response to PDGF-AA, similarly to gonocytes that proliferate in response to PDGF_BB. This suggests that F9 cells can be used as a model for the study of gonocyte differentiation. On the other

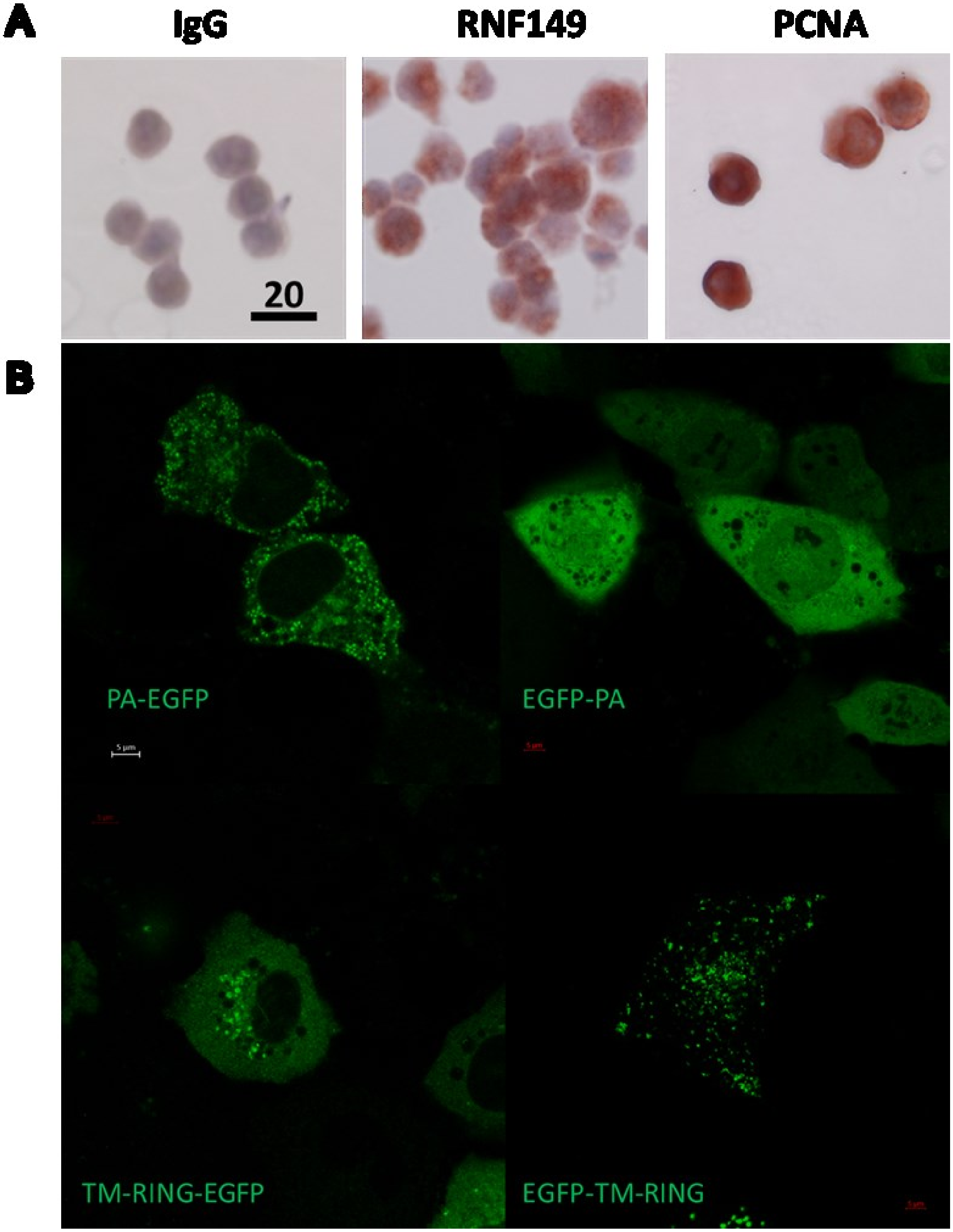


__**Figure 11. C18-4 cells as a potential model for studying RNF149 function.**__

(A) C18-4 express RNF149 and PCNA. (B) Single transfection of EGFP-tagged RNF149 isoforms in C18-4 cells.

hand, C18-4 cells are immortalized spermatogonial cells that exhibits the general properties of type-A spermatogonia, that we found to express cytosolic RNF149, in agreement with the cytosolic expression found in PND8 spermatogonia (Fig. 3), and PCNA (Fig 11A). Together, these findings suggest that F9 and C18-4 cell lines can be used as potential models for studying proliferation and differentiation mechanisms in gonocytes. Four vectors that express either C-termini or N-termini EGFP tagged VA1 and VA2 forms of RNF149 were transfected into both F9 and C18-4 cell lines independently, with or without the co-transfection of either blue ER marking plasmid or red ER marking plasmid with lysotracker.

Although other PA-TM-RING ubiquitin E3 ligase members like Godzilla or its human isoform RNF167 are able to induce the formation of giant endosomes in HEK-293 cells through perturbation of endosomal recycling processes, such giant bubble were not observed in any isoforms of EGFP tagged RNF149 transfected to C18-4. However, the location of EGFP on either C-termini or N-termini does change the distribution of RNF149 (Fig 11B), as EGFP tagged on N-termini might hinder the function of the putative signal peptide that also located on N-termini (Nakamura, 2011). Therefore, C-terminal EGFP plasmids were used for subsequent studies. To test where RNF149 isoforms are located in C18-4 cells, two C-terminal EGFP plasmids were co-transfected ER mark and mitochondria mark. The RING type RNF149 isoform was widely expressed in C18-4 cytoplasm and nuclear (Fig 12A), whereas the PA isoform co-localized with ER, but not with mitochondria or lysosome (Figs 12B and 13), which indicates that the PA domain might be an essential factor for RNF149 to reside in ER membrane.

Interestingly, the same expression patterns were observed in F9 cell transfected with PA-EGFP plasmid with blue ER mark and red mitochondria mark, where PA-EGFP RNF149 co-localized with ER rather than mitochondria (Fig 14). When co-transfected with red ER mark and

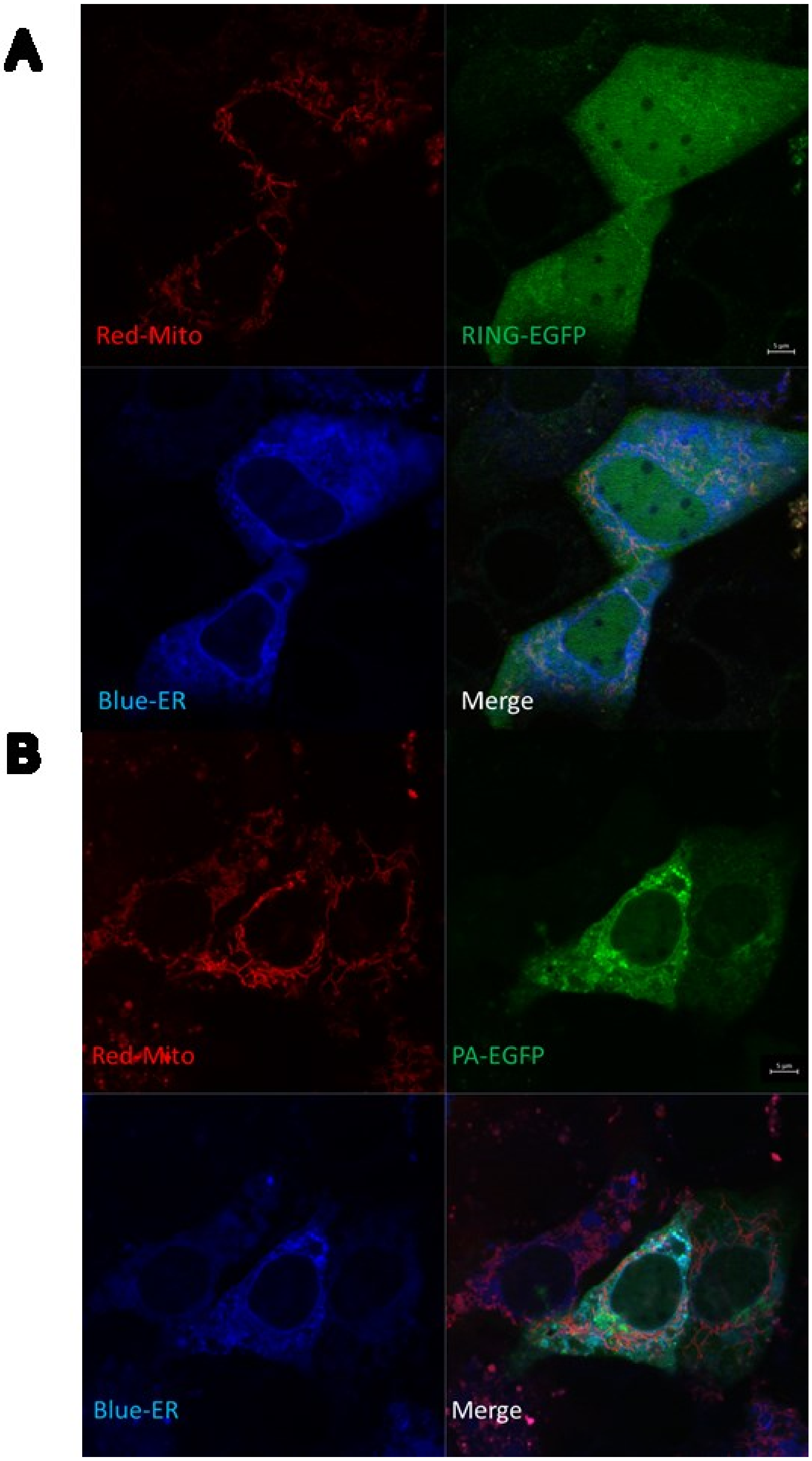
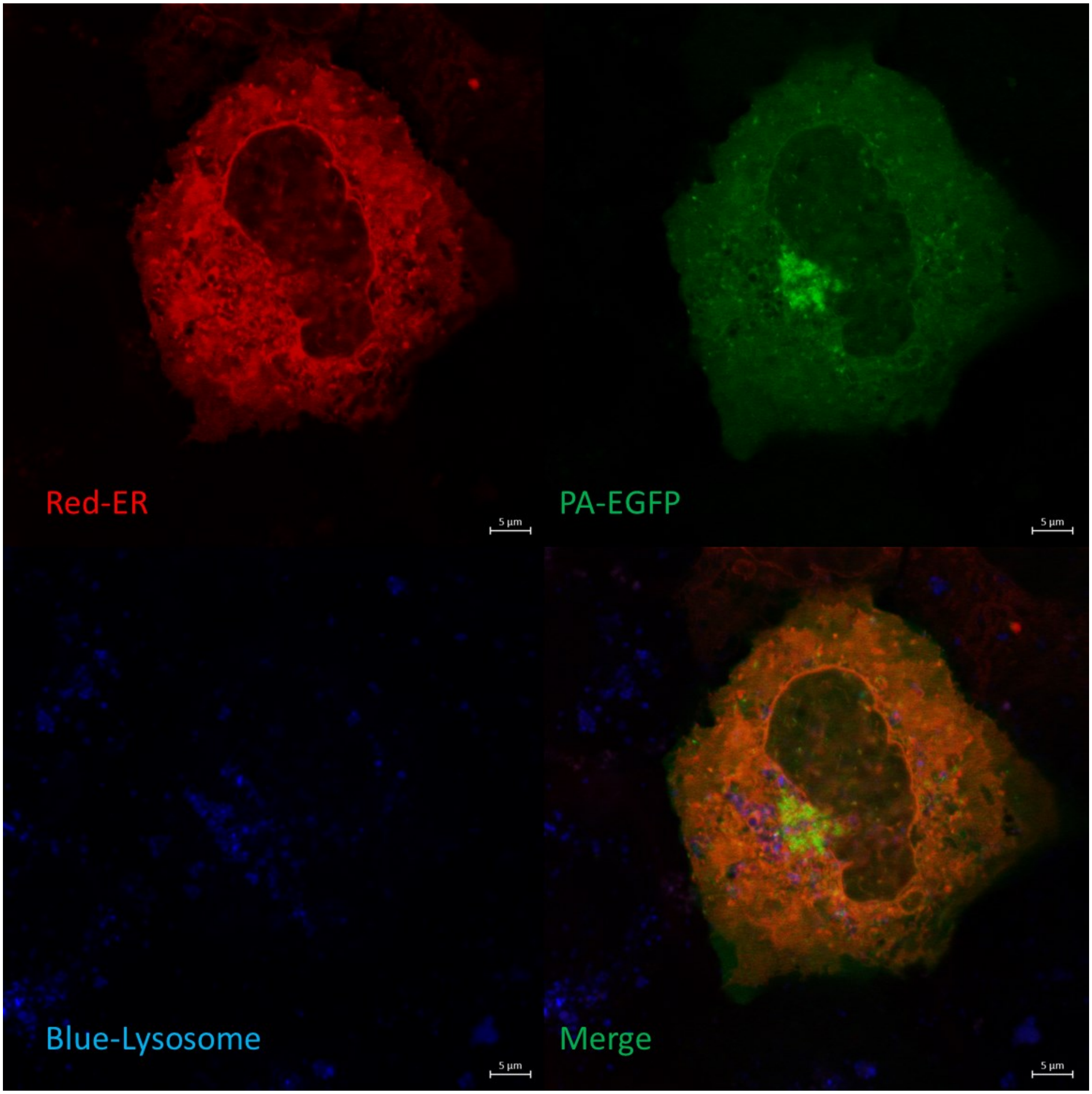


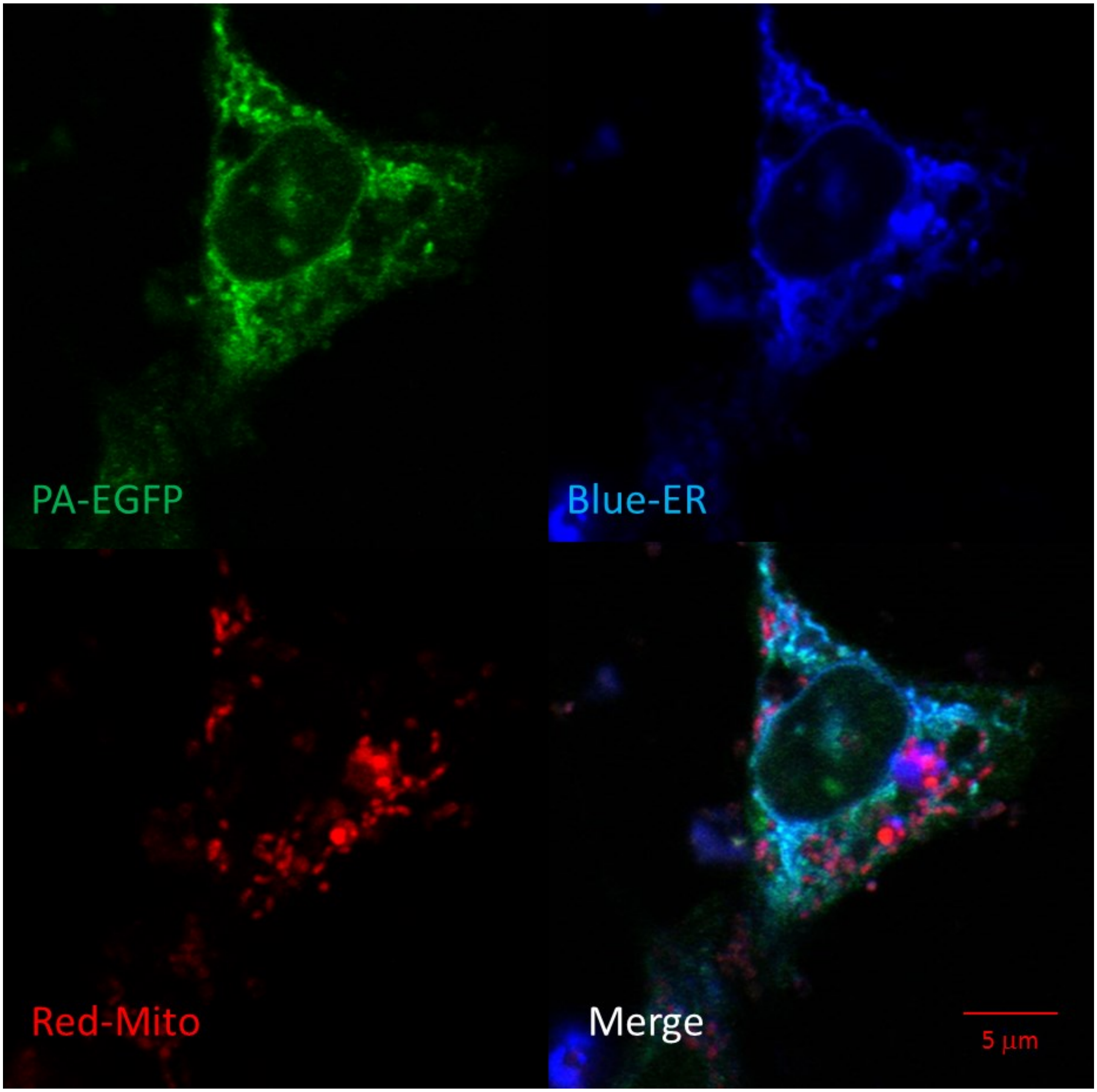
Figure 12. RNF149 are co-localized with ER by PA domain in C18-4 cells.

(A) RING-EGFP vector are co-transfected with mitochondria mark and ER mark in C18-4 cell. (B) PA-EGFP vector are co-transfected with mitochondria mark and ER mark in C18-4 cell. Both co-transfection are performed with Lipofectamine™ 3000 (Invitrogen) 24 hours before observation according to the manufacturer's protocol before observation.



<u>**Figure 13. RNF149 are co-localized with ER by PA domain but not lysosome in C18-4 cells.**</u>

PA type RNF149 isoform are co-transfected with Red ER mark through Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol 24 hours before observation. LysoTracker Blue DND-22 (Life Technologies) is added to cells before observation, final concentration is 60nM, then incubate for 30 min at 34°C.



<u>Figure 14. RNF149 are co-localized with ER by PA domain but not mitochondria in F9 cells.</u>

PA type RNF149 isoform are co-transfected with Blue ER mark and Red Mito mark through Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol 24 hours before observation.

stained with blue lysotracker, PA-EGFP RNF149 did not co-localized with lysosome in F9 cells, which corresponds to the expression pattern seen in C18-4 cells (Fig 15A). However, RING-EGFP RNF149 did not co-localize with ER, but was highly aggregated in lysosomes (Fig 15B). These results suggest that in both C18-4 and F9 cells, the PA-domain of RNF149 is a key factor for RNF149 localization in the ER, and that the PA variant protein might have a role in ER, while the RING-domain variant protein might be an essential element for RNF149 localisation in lysosome, related protein degradation pathway.

Discussion

To date, the ubiquitin system has been widely studied due to its multiple functions in regulating protein degradation, kinase activation, DNA repair, trafficking, translation and signal pathway activation. In addition, among all three key enzymes of ubiquitin system, the E3 ligases probably play the most important role by giving specificity to the process. The RING-type E3 ligases have been reported to be important regulators in many diseases, such as Mdm2 that can ubiquitinate P53 (Haupt et al., 1997), and Skp2 that can degrade c-Myc (Kim et al., 2003). However, until now, seldom studies have been conducted on transmembrane RING-type E3s such as RNF128 and RNF5, which were reported to participate in cell proliferation and differentiation processes. Little is known about RNF149, except that it might be a directly involved in cell proliferation via degrading CD9 (Li et al., 2013), or indirectly regulating cell differentiation by reducing BRAF, which has pro-proliferation function (Hong et al., 2012). But what role RNF149 plays in proliferation and differentiation, especially in gonocyte is still unclear.

Our previous results showed that RNF149 is strongly expressed in PND3 gonocytes but not in PND8 spermatogonia (Manku et al., 2012c), which indicates that RNF149 is expressed

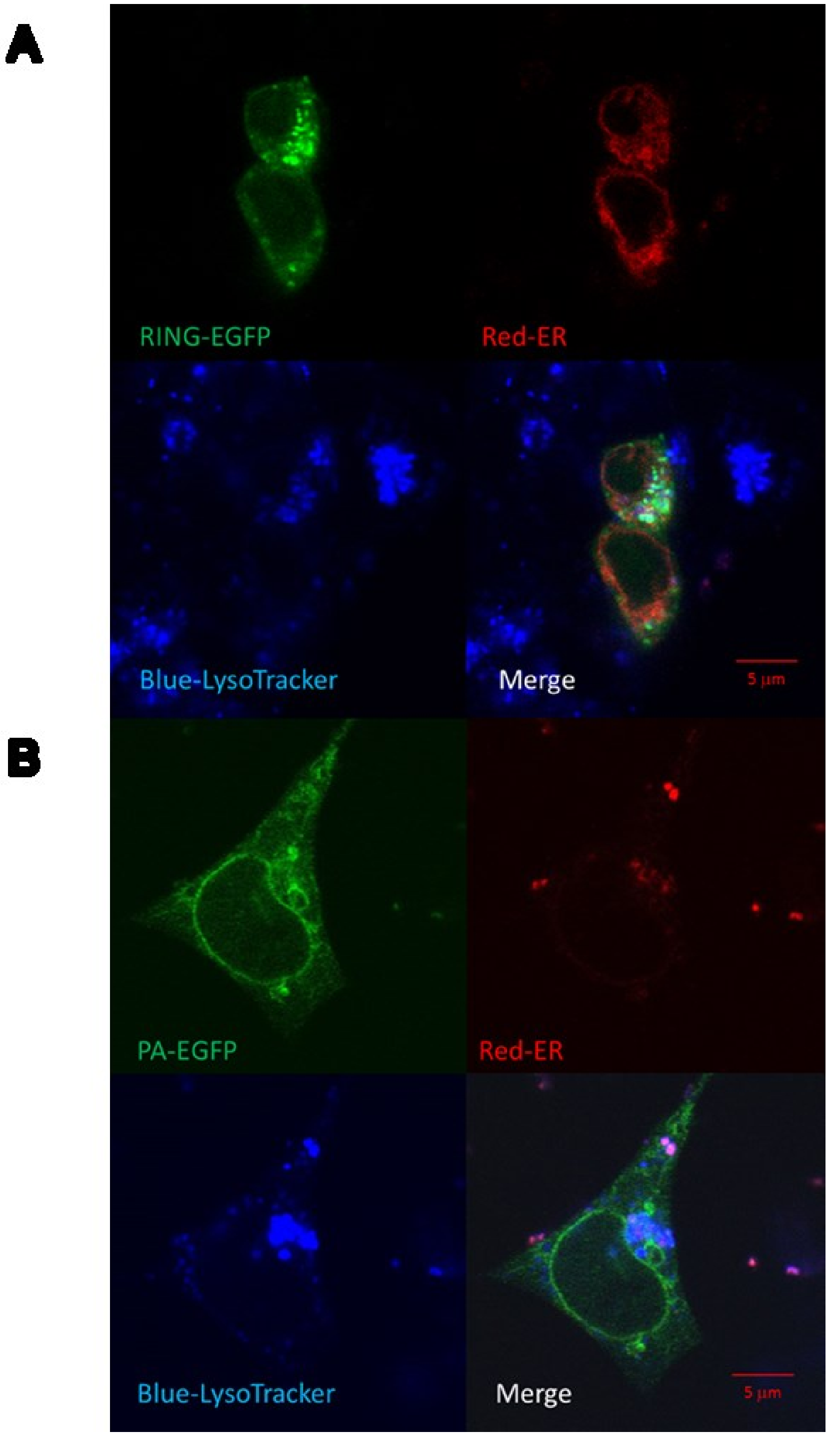


Figure 15. PA-domain potentially co-localized with ER while RING-domain is co-localized with lysosome in F9 cells.

(A) RING-EGFP vector are co-transfected with mitochondria mark and ER mark in F9 cell. (B) PA-EGFP vector are co-transfected with mitochondria mark and ER mark in F9 cell. Both co-transfection are performed with Lipofectamine™ 3000 (Invitrogen) 24 hours before observation according to the manufacturer's protocol before observation.

mainly during the phase of gonocyte proliferation, and it might need to be removed before gonocytes can undergo differentiation to form spermatogonia. This trend is also observed in testes section samples, where, RNF149 have the highest protein expression in PND2 and PND3 gonocytes, especially in the nucleus, in contrast to its expression in Sertoli cell cytosol in pubertal testis. In addition, RNF149 is found highly expressed in certain cell types in kidney and other organs from PND2 to 35 (puberty), the strongest being in cells from kidney tubules. It is interesting to note that RNF149 profiles reported in the Human Protein Atlas public website were similar to our findings at younger ages in rat (http://www.proteinatlas.org/ENSG00000163162-RNF149/tissue). Indeed, RNF149 was found to be strongly expressed at the surface of spermatocytes and in Sertoli cell cytoplasm in adult human testis, in kidney tubule cells, and in bile duct cells. Results from one of our lab member also showed strong expression of RNF149 in adult (PND120) Sertoli cell cytoplasm as well as in the cytoplasm of elongated spermatids (G. Manku; data not shown). Thus, our results indicate that RNF149 might be an essential regulator for the postnatal development of different organs. Moreover, its expression varies in testicular germ cells from a nuclear expression in neonatal gonocytes to a cytoplasmic localisation in spermatogonia and elongated spermatids, suggesting specific roles in restricted phases of germ cell development. Also of interest is its presence in the cytoplasm of pubertal to adult Sertoli cells, suggesting a potential role in differentiated, but not immature, Sertoli cells.

The finding that silencing RNF149 with low siRNA concentration on GSM cell mixture leads to reduced proliferation marker PCNA mRNA levels suggests that RNF149 may be involved in gonocyte proliferation. The comparison of the effects of high and low siRNA concentrations shows that even at 48 hrs treatment, the higher siRNA concentration seems to give better silencing efficiency in enriched gonocytes (Fig 7 and 8). Morever, the experiment using 300 nM siRNA showed good silencing at the protein level for PE-treated gonocytes, with nearly 80% of gonocytes no longer expressing RNF149, correlating with a good decrease in PCNA mRNA expression. The last experiment used very high siRNA concentrations (1800 nM), as suggested when using an electroporation device, which opens pores in the plasma membrane only for a short time, limiting the penetration time of siRNA to few seconds, by contrast to the long penetration times (24-72 hours) used with lipofectamin or similar detergent approaches. The results showed efficient silencing at 24 hours, but variable changes at 48 hours, possibly due to off-targeting effect caused by the high concentrations of siRNA duplexes used.

Interestingly, adding PDGF-BB and E_2, which induce gonocyte proliferation (Li et al., 1997; Thuillier et al., 2010), increased the expression levels of RNF149 in mock cells, whereas the blocking effects of siRNA in P+E treated cells were consistent with the silencing efficiency, with PCNA induction only prevented when RNF149 was successfully silenced (Figures 7 and 8). Thus, one observation that seemed to hold between experiments is that the expression levels of RNF149 and PCNA showed similar trends, being both high and both low at the same time. Together, these studies imply that RNF149 might positively regulate proliferation. The strategy of using mixed somatic-germ cells for electroporation is the best option for gene silencing considering the very small numbers of gonocytes. Additional experiments will be needed to reduce potential off-targeting effects and confirm the usefulness of isolating gonocytes after electroporation instead of treating mixed somatic germ cells, considering that most somatic cells die in the absence of serum, providing an adding level of gonocyte enrichment that might be similar to the enrichment using a BSA gradient.

RNF149 has been reported to be a transmembrane protein mostly expressed on ER membrane and lysosome in one study on cell line. Thus, it will be important to find out where it is located in gonocytes. Our preliminary data suggest that RNF149 is a pro-proliferation regulator. One study proposed that RNF149 promotes proliferation by degrading CD9. However, other studies have linked CD9 to the maintenance of stemness in spermatogonia. So, it is possible that the target of RNF149 in gonocytes is different from CD9, or that it requires the recruitment of other proteins to affect CD9. In addition, F9 might serve as a good model to study gonocyte development, based on studies conducted by our lab (Manku et al., 2015; Wang & Culty, 2007). Moreover, our data suggest that the spermatogonial C18-4 cell line is a good model for comparing RNF149 changes of subcellular localization and function between gonocytes and spermatogonia.

Besides its potential role in cell proliferation, RNF149 might also participates in the regulation of recycling endosome trafficking. Goliath and Godzilla, two Drosophila members of the PA-TM-RING RNF proteins family and their human homologue RNF167 are described to regulate recycling endosome trafficking via ubiquitylation of the VAMP3 (vesicle-associated membrane protein 3) SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein, and induce enlargement of EEA1 (early endosome antigen 1)/Rab5-positive early endosomes both in vitro and in vivo (Yamazaki et al., 2013). Moreover, a recent study conducted on LGR5+ stem cells, demonstrated that two other PA-TM-RING family proteins RNF43 and ZNRF3 are able to reduce Wnt signals by enhancing endocytosis of Frizzled receptors via its ubiquitylation, hence arrest the growth of the cells (Koo et al., 2012). As a matter of fact, massive activation of Wnt signalling are found in either mice lacking these genes or cancer cells harbouring loss-of-function mutations of RNF43. These results implicate a shared regulatory function for the PA-TM-RING ubiquitin ligases in intracellular trafficking/sorting, and suggest that abrogation of their function may lead to cellular signalling disorder which can eventually cause cancer.

Because little is known regarding RNF149, and its gene sequence was only confirmed in human and mice, the function of RNF149 in rat remains unclear. Thus, it was critical to obtain information on RNF149 transcripts expressed in rats, in order to generate overexpression vectors coding for existing mRNAs and to produce a recombinant protein corresponding to the testicular rat protein to be used as bait for substrate proteins. In order to do so, I cloned the gene from rat testes and other tissue samples. Interestingly, two variant mRNA forms of RNF149, *VA1* and *VA2*, were found rat testes, liver, and kidney cDNA libraries. Sequence analysis and the positions of start and stop codons showed that *VA1* includes the sequence of PA domain, but lacks the RING domain. On the other hand, the start codon and stop codon of *VA2* defined a sequence including TM and RING domains. Thus, *VA2* was referred as RING form. These two constructs were then ligated to N1/C2 EGFP vector for mammalian expression of RNF149, to examine their subcellular localization in F9 and C18-4 cells.

Weak RNF149 protein expression was observed in spermatogonia cytoplasm in PND6-8, in agreement with its expression in C18-4 cells, an immortalized cell line that can be considered as type-A spermatogonia. Interestingly, its expression appeared to be stronger in some but not all C18-4 cells than in vivo PND8 spermatogonia, suggesting two subpopulations in growing C18-4 cells, in support of this cell line containing type A spermatogonia at different phase of differentiation, as observed with isolated spermatogonia from juvenile mice (Culty, 2009; Luo et al., 2009; Suzuki et al., 2009). As a type I transmembrane protein, RNF149 shares common features, such as N-terminal signal peptides (NS) and TM with other member, suggesting that a C-terminal fusion protein of EGFP and RNF149 should not disrupt the N-terminal signal peptides, allowing the fusion protein to remain in the cytoplasm, whereas the fusion of EGFP at the N-terminal might affect its PA-domain function. In RNF proteins related studies, due to the existence of predicted N-terminal signal peptides, EGFP are mostly conjugated to C-termini. In my results, EGFP ligated at the N-termini did change the localization of VA1 and VA2 RNF149, making them either widely spread in nuclear and cytoplasm, likely due to EGFP hindering the PA domain, resulting in the loss of ability to reside in ER and other potential sites in the cell, or leading to condensation into small dots as seen with VA2. This further suggests that the RING domain participates in intracellular trafficking/sorting. Therefore, in order to reduce the interference effect caused by EGFP, C-terminal EGFP tagged VA1 and VA2 RNF plasmid should be used in further studies.

In both C18-4 and F9 cell lines co-transfected with either ER, mitochondria marker or stained with lysotracker, VA1 RNF149 was localized in ER, suggesting a potential function of PA domain, in agreement with other studies. In contrast, VA2 is localized mainly to lysosomes in F9 cell, which was not observed in C18-4 cells. These results suggest that in both C18-4 and F9 cells, the PA-domain of RNF149 potentially exhibits its function in the ER, while in F9 but not C18-4, the RING-domain might be an essential element for RNF149 translocation to lysosome, in relation to protein degradation pathway. The apparent difference in RING domain localization between F9 cells and C18-4 cells is interesting, since F9 cells correspond to pluripotent embryonic stem cells with somatic and germ line potentials, whereas C18-4 cells represent more advanced undifferentiated spermatogonia. To date, only BRAF and CD9 are known targets for RNF149, and how they take part and react with both PA and RING domain of RNF149 remains unclear. Therefore, further studies focused on finding other potential substrates of RNF149 and its actual mechanism of action in these cell lines and gonocytes is required.

Interestingly, PCR cloning revealed that three PCR products at sizes ranging from 850bp to 1500bp were generated from neonatal ages to puberty in testis, kidney and liver. The largest product around 1500bp was excluded because it might have been generated from non-specific targeting, as the maximum length for predicted product is 1185bp. To confirm this result, a primer set was designed to examine whether there are more than one RNF149 isoforms. Based on the predicted gene sequence of RNF149, if there was only one RNF149 in normal rats, this primer set should only generate a single fragment at a size around 300bp. However, PCR results for testis and other organs still demonstrated three bands. Two of them were sequenced and confirmed to match the prediction that *VA1* and *VA2* are naturally existing transcripts in these organs, according to their sizes. The existence of the largest band stands from two possible causes, either as a non-specific product of these primers, or there is a third isoform that express PA, TM and RING domains at once, and an additional sequence such as an intronic retention product, as found in other variant transcripts in testis and cancer tissues. Yet this hypothesis needs more study to be confirmed.

In summary, my current work demonstrated the potential function of RNF149 in gonocytes development, highlighting the correlation between RNF149 expression and proliferation marker during PDGF/ estrogen co-treatment, the variant forms of RNF149 found in rat tissues, and the potential roles of PA and RING domain-containing variant proteins. Although these studies were not able to fully identify the role and substrates of RNF149 in response to proliferation or differential stimulation in gonocytes, C18-4 and F9 cells, , it revealed the possibility of RNF149 involvement in gonocyte proliferation in response to PDGF and estrogen stimulation, and tested the potential use of F9 and C18-4 cell lines as models for studying the function of RNF149. Finally, taken together with earlier studies in our laboratory, this work supports the hypothesis that RNF149 plays a role in gonocyte proliferation, and the perturbation of its function might lead to disorder in membrane protein trafficking and degradation.

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