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Retinoic acid regulates *Kit* translation during spermatogonial differentiation in the mouse

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ABSTRACT

In the testis, a subset of spermatogonia retains stem cell potential, while others differentiate to eventually become spermatozoa. This delicate balance must be maintained, as defects can result in testicular cancer or infertility. Currently, little is known about the gene products and signaling pathways directing these critical cell fate decisions. Retinoic acid (RA) is a requisite driver of spermatogonial differentiation and entry into meiosis, yet the mechanisms activated downstream are undefined. Here, we determined a requirement for RA in the expression of KIT, a receptor tyrosine kinase essential for spermatogonial differentiation. We found that RA signaling utilized the PI3K/AKT/mTOR signaling pathway to induce the efficient translation of mRNAs for *Kit*, which are present but not translated in undifferentiated spermatogonia. Our findings provide an important molecular link between a morphogen (RA) and the expression of KIT protein, which together direct the differentiation of spermatogonia throughout the male reproductive lifespan.

Introduction

Spermatogenesis begins in the neonatal mouse testis with the transition of quiescent prospermatogonia (also called gonocytes) into a heterogeneous population of undifferentiated and differentiated spermatogonia at approximately 3–4 days postpartum (dpp). A subset of undifferentiated spermatogonia retain stem cell potential, while the rest differentiate to eventually enter meiosis as leptotene spermatocytes by ~10 dpp (reviewed in de Rooij and Russell, 2000; Oatley and Brinster, 2012; Yoshida, 2012). It is unclear how spermatogonia make this critical cell fate decision, but afterwards there is a clear demarcation in the expression of specific proteins in undifferentiated (A_s–A_{al}) and differentiated (A_1-) spermatogonia. One critical cell surface marker that distinguishes differentiated spermatogonia is KIT (also called c-KIT). This receptor tyrosine kinase is required for fertility (Besmer et al., 1993; Loveland and Schlatt, 1997; Kissel et al., 2000), and has been particularly useful in the study of male germ cells; antibodies raised against KIT enable isolation of differentiated spermatogonia, and KIT expression is used to follow germ cell fate changes in transgenic and knockout mice or following various treatments (Prabhu et al., 2006; Mithraprabhu, and Loveland, 2009). KIT is bound by KIT ligand (KITL), which is expressed by neighboring Sertoli cells in the seminiferous epithelium (Loveland and Schlatt, 1997). This binding activates the PI3K intracellular signaling pathway in spermatogonia, which is required for entry into meiosis (Blume-Jensen et al., 2000; Kissel et al., 2000). KIT is also essential during differentiation of a number of stem cell populations (Edling and Hallberg, 2007), including embryonic stem cells (ESCs) (Bashamboo et al., 2006).

There is no consensus as to when KIT is first expressed in spermatogonia in the neonatal testis, although it is clear that *Kit* mRNA is present in undifferentiated spermatogonia and in prospermatogonia in the neonatal testis without detectable protein (Schrans-Stassen et al., 1999; Prabhu et al., 2006; Oatley et al., 2009; Yang et al., 2013b). The initiation of KIT protein expression in differentiating spermatogonia may involve a change in the utilization of *Kit* mRNAs. It has been clear for a long time that mRNAs are not all translated with equivalent efficiency (Lodish, 1976); those experiencing greater initiation activity recruit more ribosomes (polyribosomes, or polysomes) will be more efficiently

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translated, while those that initiate poorly lack ribosomes and will not produce protein. We recently assessed changes in mRNA translation efficiency during neonatal testis development using translation state array analysis (TSAA). This is a genome-wide microarray approach in which mRNAs are identified based on changes in their association with polysomes (described in Arava, 2003). We identified ~3000 mRNAs encoding both germ cell-specific and housekeeping proteins that become ≥2-fold enriched in polysomes in the neonatal testis from 1 to 4 dpp without a significant change in overall mRNA abundance (Chappell et al., 2013). We found that *Kit* mRNAs underwent significant movement (~5-fold) into heavy polysomes over that interval, and this provides a potential mechanism for the initial expression of KIT protein in spermatogonia.

The morphogen all-trans retinoic acid (ATRA, hereafter abbreviated as RA) regulates the sex-specific timing of meiotic initiation in mice, and has been implicated in spermatogonial differentiation in the testis (Koshimizu et al., 1995; Gaemers et al., 1998; Bowles et al., 2006; Koubova et al., 2006; Bowles and Koopman, 2007). Evidence supporting a role for RA in regulation of KIT expression has been largely gathered from *in vitro* experimentation. Although RA can increase *Kit* mRNA levels *in vitro* (Pellegrini et al., 2008; Zhou et al., 2008a; Raverdeau et al., 2012), this is not likely the primary mechanism regulating KIT protein production *in vivo*, as non-translating mRNA is already present in both prospermatogonia and in undifferentiated spermatogonia without detectable protein (Schrans-Stassen et al., 1999; Prabhu et al., 2006; Yang et al., 2013a, 2013b). We recently found that injection of RA into mice at 1 dpp (prior to endogenous exposure to RA at 3–4 dpp) induced precocious KIT protein expression in spermatogonia (Busada et al., 2014).

In this study, we identify a novel mechanism by which RA regulates KIT protein synthesis in differentiating spermatogonia. Our results indicate that *Kit* mRNA translates poorly in the absence of RA, but becomes recruited to polysomes in response to RA in the neonatal testis, resulting in the first appearance of KIT protein in neonatal spermatogonia. We show that the first population of KIT+ spermatogonia is also STRA8+, supporting the concept that RA exposure is required for KIT expression. However, KIT expression is not dependent upon STRA8, which suggests that there are independent RA-directed pathways regulating KIT and STRA8 expression. Importantly, we discovered that RA-activated PI3K and AKT signaling is required for induction of KIT but not STRA8. Taken together, these results suggest a novel non-genomic pathway in which RA activates the PI3K–AKT–mTOR signaling network to regulate KIT expression and spermatogonia differentiation in the neonatal testis.

Materials and methods

Animal treatments and tissue collection

All animal procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of East Carolina University (AUP #A178) and Washington State University (ASAF #1519). All analyses were performed using CD-1 mice (Charles River Laboratories), excepting *Stra8* knockout mice, which were congenic C57Bl/6 (Griswold Lab). Administration of exogenous RA was done as previously described (Busada et al., 2014). Briefly, neonatal mice received one subcutaneous injection of 100 μg all-trans RA (Cat# R2625, Sigma-Aldrich) dissolved in 10 μl dimethyl sulfoxide (DMSO) or DMSO alone at 1 dpp, and were euthanized by decapitation 24 h after injection (see Fig. S1A). To generate RA-deficient testes, neonatal mice received daily oral treatments of 100 μg/g body weight WIN 18,446, suspended in 5 μl 1% gum tragacanth, for 4 days, beginning at birth, and were euthanized by decapitation 24 h following the final treatment. Vehicle control animals received 5 μl 1% gum tragacanth alone.

Polysome gradient analysis

Resolution of polysomes, ribosomal subunits, and initiation complexes was performed with 15–45% sucrose gradients by centrifugation as previously described (Chappell et al., 2013). Testis lysates from 1 dpp and 4 dpp mice, and mice treated with DMSO and RA were prepared as previously described (Chappell et al., 2013).

Quantitative RT-PCR

Quantitative RT-PCR analyses were performed with RNA isolated whole testis lysate and with RNA from the heavy polysome fractions (fractions 9–14). RNA was isolated from 2 dpp prospermatogonia cultured in serum-free media for 24 h in a previous study (Hogarth et al., 2011). Fifty nanogram of RNA was subjected to reverse transcription and qPCR in the same reaction tube using iScript One-Step RT-PCR kit with SYBR green (BioRad). Amplification and detection of specific gene products were performed using the iCycler IQ real-time PCR detection system. Threshold temperatures were selected automatically, and all amplifications were followed by melt-curve analysis. Primers were designed on either side of exon junctions to avoid amplification of residual genomic DNA. Sequences were as follows: *Rpl19* (NM_009078, 5′-GAAATCGCCAATGCCAACTC and 5′-TCTTAGACCTGCGAGCCTCA), *Stra8* (NM_009292, 5′-GAGGTCAAGGAAGAATATGC and 5′-CAGAGACAATAGGAAGTGTC), *Kit* (NM_001122733, 5′-CATGGCGTTCCTCG CCT and 5′-GCCCGAAATCGCAAATCTTT), and *B2m* (NM_009735, 5′-CCGTGATCTTTCTGGTT and 5′-CGTAGCAGTTCAGTATGTTCG). The following comparative quantitation equations were used to calculate relative mRNA levels for pooled polysomal fractions: mRNA level=($2^{\Delta CT}$), where ΔCt=(Ct gene of interest)−(Ct min), where Ct min=the minimum Ct value within the gene set and controls. For qRT-PCR on total RNA, Ct values were normalized to either *B2m* (Figs. 1 and 2) or *Rpl19* (Figs. 4 and S1) and relative mRNA quantities were calculated by the $2^{\Delta\Delta Ct}$, with ΔCt=(Ct untreated gene)−(Ct treated gene).

Immunoblotting

Immunoblotting was performed using conventional methods. Blocking and antibody incubations were done in 1× PBST+3% BSA. Primary antibodies used were against phospho-mTOR (1:1000, #5536, Cell Signaling Technology), phospho-EIF4EBP1 (1:1000, #2855, Cell Signaling Technology), and RPS6 (1:2000, #2217S, Cell Signaling Technology), and were incubated overnight at 4 °C. Secondary anti-rabbit-HRP (1:3000, #7074S, Cell Signaling Technology) was incubated for 1 h at room temperature. Blots were developed using LumiGlo detection reagent (Cell Signaling Technology).

Indirect immunofluorescence

Sections from at least 4 testes from different mice were analyzed, and experiments performed at least thrice. Immunolabeling was done using conventional methods. Briefly, testes were fixed for 2 h in 4% PFA and then washed and incubated overnight in 30% sucrose prior to freezing in O.C.T. Five micrometer sections were blocked for 30 min at room temperature and then incubated in primary antibody for 1 h at room temperature or overnight at 4 °C. Primary antibodies used were against the following: KIT (1:1000, #3074, rabbit monoclonal, Cell