Animal Model

Expression of a K48R Mutant Ubiquitin Protects Mouse Testis from Cryptorchid Injury and Aging

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Testis injury models can be useful for determining the *in vivo* function of genes. In this study, ubiquitin, a tag for 26S-proteasome degradation, was mutated at lysine 48 (K48R) to inhibit ubiquitin chain assembly. K48R transgenic mice had testes with delayed germ cell loss following the acute injury of experimental cryptorchidism, and were resistant to the chronic injury of aging-associated testicular atrophy. After 4 days of cryptorchid-mediated heat stress, the average weight of cryptorchid testes in wild-type ubiquitin mice was significantly lower ($P < 0.05$) than in K48R mutant ubiquitin mice, indicating that altered ubiquitination delayed germ cell death. Light microscopy confirmed that the testicular injury, in both wild-type and K48R ubiquitin mice, was due to germ cell death. In addition, wild-type ubiquitin mice aged 19 to 22 months showed greater testicular atrophy and decreased average seminiferous tubule diameter when compared with K48R-aged testes. These results demonstrate a resistance to testicular injury conferred by the K48R mutation, suggesting that ubiquitin-mediated protein degradation is involved in the processing or modulation of testicular insults. (*Am J Pathol* 2003, 163:2595–2603)

Stressing cells via injury is a useful approach to elucidating the *in vivo* roles of genetic mutations. The adult testis is unique in maintaining a continuously high level of germ cell proliferation, balancing germ cell production with germ cell culling via apoptosis and managing the complex morphogenetic process of spermatogenesis. Germ cells are completely dependent on their intimate association with Sertoli cells, which provide both structural and paracrine support.^{1,2} Because of this dependent relationship and the highly proliferative nature of germ cells,

inducing cell injury in the testis has the potential to reveal subtle defects in homeostasis induced by genetic mutations.^{3,4} Therefore, we examined two systems of testicular injury in our mutant mouse model, experimental cryptorchidism as an acute insult and testicular aging as a model of chronic injury.

The testis is sensitive to a variety of damaging agents, including heat stress.^{5,6} Normally, scrotal testes are maintained at a lower temperature than the rest of the body, and raising the testis temperature via experimental cryptorchidism induces a germ cell heat-stress response and apoptosis.^{6,7} Within a few days of heat-induced stress, cryptorchid testes undergo atrophy characterized by a progressive germ cell loss. During the course of this germ cell loss, multinucleated giant cells and Sertoli cell vacuolization are observed.^{8,9} Although the precise mechanism by which normal body temperature induces germ cell loss is not known, protein and lipid oxidation along with p53 are involved.^{4,9–13} Heat shock is known to cause alterations in protein architecture, and ubiquitin is required for the tagging and subsequent degradation of altered proteins,^{14–16} suggesting a role for ubiquitin in the mechanism of injury following experimental cryptorchidism.

The chronic effect of aging is a complicated model of testicular injury. Studies in the Brown Norway rat have shown that serum testosterone levels decrease significantly at 18 to 21 months of age. This decrease in testosterone may result from dysregulation at the hypothalamic-pituitary axis, in addition to age-related changes in Leydig cells.^{17–19} Along with hormonal changes, alterations in testis vasculature may contribute to injury. In Sprague Dawley rats, aged testes have a significant reduction in blood vessel volume; 6-month and 24-month-

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old testes had 71% and 31% of the blood vessel volume, respectively, when compared to 3-month-old controls.²⁰

Ubiquitin is a highly conserved and essential protein in all eukaryotes and tags proteins for degradation by the 26S proteasome. Along with the removal of misfolded or adducted proteins, control over short-lived signaling proteins is an important regulatory function of ubiquitin.²¹ For example, cyclins require ubiquitination to signal degradation, and therefore proteasome inhibitors can cause cell cycle arrest and subsequent apoptosis of gastric cancer cells.²² The universal tag for protein degradation is the chain assembly of numerous ubiquitin proteins to one another, namely polyubiquitination.

Polyubiquitination is a covalent linkage of the ubiquitin C-terminal glycine to a lysine (K) residue on either a target protein or another ubiquitin. Polyubiquitination occurs at ubiquitin residues K6, K11, K29, K48, and K63. The role of K6, K11, and K29 polyubiquitination points are not fully understood; however, it is clear that K48 and K63 play important roles in the cell.^{23–25} K63 polyubiquitination is involved in DNA repair and degradation of cell surface receptors, whereas chain assembly at K48 signals for 26S-proteasome degradation.²⁶ Mutation of the ubiquitin lysine 48 residue to an arginine (K48R) results in the termination of polyubiquitination. Since polyubiquitination targets the protein for 26S-proteasome degradation, this branch ending K48R mutation has dominant-negative effects regarding proteasome degradation *in vitro*.²⁷ Previous studies have shown that transient transfection of the K48R ubiquitin mutant gene into mouse HT4 neuroblastoma cells causes sensitivity to canavanine,²⁸ an amino acid analog that leads to misfolding of proteins, as quantified by live and dead detached cells. This work led to the construction of transgenic mice expressing K48R ubiquitin to study this system *in vivo*.²⁹

Using these K48R ubiquitin transgenic mice, we tested the hypothesis that suppression of the polyubiquitination signal for 26S-proteasome degradation modulates the normal testicular injury response. Our results show that the K48R mutation produces a resistance in germ cells to cell death induced by both experimental cryptorchidism and aging. This implicates ubiquitin as a protein important in the regulation of cell death in the testis.

Materials and Methods

Animals

Mice were maintained on a 12-hour light-dark cycle and housed in 35% to 70% humidity and 74 ± 2°F temperature controlled rooms with access to Purina Rodent Chow 5001 (Farmer's Exchange, Framingham, MA) and water *ad libitum*. All procedures involving animals were performed in accordance with the guidelines of Brown University's Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines.³⁰

The generation and characterization of mice expressing K48R mutant ubiquitin used previously described methods.²⁹ These mice were created on a FVB/N back-

ground, and therefore non-transgenic controls of FVB/N wild-type mice are used where possible. The transgenes used a human ubiquitin promoter to drive human ubiquitin fused to a 6X-His epitope and EGFP. Human ubiquitin is identical in amino acid sequence to mouse ubiquitin. These transgenes were fused to enhanced green fluorescent protein (EGFP) to facilitate the evaluation of transgene temporal and spatial expression. Expression was found to be highest in the testes, brain, and eyes while lower in blood-rich organs such as liver and kidney. The K48R mutant ubiquitin transgene contains the same ubiquitin sequence as wildtype with a K48R mutation, thereby inhibiting polyubiquitination at this lysine.

These transgenic mice were characterized and are described in a paper by Zhang et al.³¹ Western blots of brain lysates from K48R ubiquitin mice probed with an antibody specific for the hexahistidine epitope tag on the transgene showed high molecular weight proteins corresponding to K48R ubiquitin conjugated to other proteins. These proteins were not present in Wt ubiquitin brain lysates, providing evidence that protein degradation is inhibited in these animals.

Spermatid Head Counts

Testes were stored at –80°C until analysis. Both testes from each animal were homogenized separately, and sperm heads were counted on a hemocytometer using previously described methods.³ The counts from the two testes of each animal were averaged for statistical analysis.

Experimental Cryptorchidism

Eight-week-old mice were anesthetized with isoflurane during the procedure. The right testis was made cryptorchid by first cutting the gubernaculum and then tying the cranial aspect of the caput epididymis and testis to the lowermost rib of the mouse. The rib was chosen as an anchor for the cryptorchid testis instead of abdominal muscle because over the course of several days, muscle stretching causes the testis to return to the scrotum. Each testis made experimentally cryptorchid to induce heat stress had a contralateral scrotal control within the same animal.

Light Microscopy

Testes were embedded in Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim, Germany) after 24 to 48 hours of fixation in 10% neutral-buffered formalin per the manufacturer's instructions. Plastic-embedded testes were sectioned (2 µm) and stained with periodic acid Schiff's reagent, and hematoxylin (PASH).

Twenty seminiferous tubule cross-sections per stained slide were assessed for total germ cell content, the number of multinucleated giant cells, and seminiferous tubule diameter. Seminiferous tubule cross-sections were chosen by overlaying a virtual grid using the vernier scales on the X and Y axes of the microscope. Elongated seminiferous tubules were defined as those whose major and

minor axes differed by more than approximately 1.5 and were not counted. All counts and measurements were performed blind and two slides were quantified for each testis.

Light and fluorescent microscopic images were obtained on a Zeiss Axiovert 35 microscope (Carl Zeiss, New York, NY) connected to a Spot RT camera (Diagnostic Instruments Inc., Sterling Heights, MI). Images were downloaded into Photoshop 6.0 imaging software (Adobe Systems Inc., San Jose, CA) for resizing and fluorescent layering. Final figures were assembled using Canvas 8.0 software (Deneba Systems Inc., Miami, FL).

Testosterone Measurements

Eighteen-month-old mice were anesthetized under isoflurane and serum collected by orbital bleed. Serum was isolated from blood using a microhematocrit centrifuge and frozen for later assay. Testosterone was measured using a DSL4000 (Diagnostic Systems Laboratory, Webster, TX) kit with standards and samples dissolved in phosphate-buffered saline with 0.1% gelatin, as described previously.^{32,33}

Statistics

The mean and SEM (SEM) were calculated for each data point and represented as mean $\pm$ SEM. One way analysis of variance followed by the Tukey test was used for all statistical analyses with significance at $P < 0.05$.

Results

Testis Postnatal Development

Testicular development in the K48R ubiquitin mice was compared to that of wild-type (Wt) ubiquitin mice. Testis weights were the same at 7, 14, 21, 28, or 40 days (Figure 1). Moreover, body weights, seminal vesicles, epididymis, brain, spleen, heart, liver, and kidney weights were the same in the K48R mutant and Wt ubiquitin mice (data not shown). Spermatogenesis began normally and continued at similar rates, as assessed by light microscopy. To investigate sperm production, homogenization-resistant spermatid heads were counted in 40- and 56-day-old testis, with no significant difference between the K48R mutant and Wt ubiquitin mice (data not shown). Spermatogenesis continued normally in young adulthood in both strains as assessed by light microscopy (Figure 2).

Ubiquitin Transgene Expression Profile

In the transgenic mice, ubiquitin-EGFP fusions were co-translationally processed to provide free ubiquitin and EGFP; EGFP therefore serves as a marker of protein expression that can be assessed by fluorescence microscopy.²⁹ Since the transgene was expressed as a fusion with EGFP, its expression could be assessed by fluorescence microscopy. Both K48R and Wt ubiquitin

Figure 1. Combined testes and epididymal weights of Wt ubiquitin mice (open circle) were not significantly different from those of K48R mutant ubiquitin mice (closed circle) at 7, 14, 21, 28, and 40 day time points ($n \geq 3$).

genotypes had similar expression profiles in the testis (Figure 3). Expression was low in spermatogonia, increased in spermatocytes, and was most intense in

Figure 2. Light microscopy of non-stressed Wt (A) and K48R (B) 8-week-old adult testis. Seminiferous tubules contain a full complement of germ cell types (spermatogonia, spermatocytes, and spermatids) with open lumens. (PASH, Bar, 50 μ m).

Figure 3. Testicular expression of K48R ubiquitin transgene as assessed by EGFP in cross-sections co-stained with Hoechst. The Wt ubiquitin transgene (not illustrated) showed the same expression pattern. **A:** Transgene expression varied depending on germ cell type throughout spermatogenesis. **B:** Spermatocytes had a moderate level of expression (arrowhead), while step 12 to 16 spermatids showed strong transgene expression in the cytoplasmic lobes (arrow). Interstitial expression was high in blood vessels (open arrow). **C:** Magnification of boxed area in **B** shows expression of the transgene in Leydig cells (asterisk). (Bar, 50 μ m).

round and early elongate spermatids (Figure 3B). No expression was observed in Sertoli cells. In the interstitium relatively high transgene expression was observed in blood vessels, and low-level expression was observed in Leydig cells.

Experimental Cryptorchidism

Compared to Wt ubiquitin and FVB/N wild-type mice, the K48R ubiquitin mice showed a delayed response to testicular injury caused by experimental cryptorchidism. Wt and K48R ubiquitin testes were made cryptorchid and evaluated after 0, 2, 3, 4, 5 (K48R only), 6, 7 (K48R only), and 9 days (Figure 4). As a control, background strain, FVB/N, mice testes were made cryptorchid for 4 and 6 days (Figure 4). The ratio of cryptorchid to scrotal testis weights increased during the first few days of injury, as previously demonstrated.⁹ After 4 days of experimental cryptorchidism, the cryptorchid testis/scrotal testis weight ratios of Wt ubiquitin and FVB/N testes were significantly lower than that of K48R ubiquitin mutant testes (FVB/N, $n = 5$; Wt, K48R, $n = 6$; $P < 0.05$). By 6 days after

Figure 4. The ratio of cryptorchid to scrotal testis weights are evaluated after experimental cryptorchid surgery. Cryptorchid testes from Wt ubiquitin (open circle) and FVB/N wild-type mice (open triangle) underwent a weight reduction at 4 days while those from K48R mutant mice (closed circle) remained elevated ($n \geq 3$ for each data point; $n = 5$ for FVB/N; *, $P < 0.05$, $n = 6$ at 4 days). Cryptorchid testes weights from K48R mutant ubiquitin mice became similar to those from Wt ubiquitin and FVB/N mice again after 6 days.

heat stress, the K48R ubiquitin mutant cryptorchid testes had decreased in weight, a delay of 2 days compared to the FVB/N control and Wt ubiquitin mice (Figure 4).

The differences in the dynamics of testis weight loss were mirrored by morphological changes (Figure 5). After 4 days, in the K48R ubiquitin cryptorchid testes, all cell types were present, and there were no multinucleated giant cells or Sertoli cell vacuolization. In comparison, these hallmarks of testicular injury were observed in Wt ubiquitin and FVB/N cryptorchid testes at this time point. Light microscopy revealed a progressive germ cell loss in Wt and FVB/N cryptorchid testes beginning at 4 days and in the K48R mutant testes beginning at 6 days. After 9 days of heat shock, both K48R and Wt ubiquitin cryptorchid testes were similar in histopathology and roughly 50% of their original weights.

Testis weight ratios of 4-day experimentally cryptorchid Wt ubiquitin and FVB/N testes were significantly different from K48R ubiquitin, consistent with a quantitative germ cell loss, as revealed by light microscopy. The average number of germ cells per seminiferous tubule cross-section in K48R ubiquitin testes (Figure 6A) was significantly reduced ($P < 0.05$) between 4 days (209 to 225 average germ cells/seminiferous tubule cross-sections/testis) to 6 days (75 to 98 average germ cells/seminiferous tubule cross-sections/testis) days of experimental cryptorchidism with a concomitant significant ($P < 0.05$) increase in the number of multinucleated giant cells (Figure 6B). Moreover, the average number of germ cells per seminiferous tubule cross-section was significantly reduced ($P < 0.05$) in Wt ubiquitin testes (87 to 103 average germ cells/seminiferous tubule cross-sections/testis) and FVB/N testes (57 to 105 average germ cells/seminiferous tubule cross-sections/testis) compared to K48R ubiquitin testes (209 to 225 average germ

Figure 5. Shown are the testes of Wt ubiquitin mice 2 days (**A**) and 4 days (**B**) after experimental cryptorchidism compared to those of K48R mutant mice at 4 days (**C**) and 6 days (**D**). Note the similarity of germ cell content and degree of atrophy when comparing **A** to **C** and **B** to **D**, highlighting the delayed response of the K48R ubiquitin mouse to injury. The presence of multinucleated giant cells (arrow) and germ cell loss in **B** and **D** are indicative of this injury. (PASH, Bar, 50 μ m).

cells/seminiferous tubule cross-sections/testis) at 4 days corresponding to testis weights at this time point. Quantitation of germ cell content and multinucleated giant cells also revealed that values for K48R ubiquitin testes at 6 days corresponded to values for Wt ubiquitin and FVB/N testes at 4 days, paralleling testis weight ratios.

Aging

Testes from aged Wt ubiquitin and K48R ubiquitin mutant 18.69- to 24.69-month-old mice were examined histopathologically for alterations in spermatogenesis. Like the experimental cryptorchidism model, K48R ubiquitin testes were more resistant to injury induced by aging. Testis weights of aged Wt ubiquitin mice were significantly less than those of K48R mice (52.2 ± 3.06 mg compared to 66.4 ± 3.59 mg; $P < 0.015$). Almost all K48R ubiquitin mutant mice ($n = 12$) displayed normal spermatogenesis in most seminiferous tubules as assessed by light microscopy (Figure 7). In contrast, most aged Wt ubiquitin mice ($n = 8$) had seminiferous tubules devoid of most germ cells, and large Sertoli cell vacuoles were present throughout the sections. Overall, $33.12 \pm$

7.46% of Wt ubiquitin seminiferous tubules versus $89.44 \pm 4.72\%$ of K48R ubiquitin seminiferous tubules had >50 germ cells ($P < 0.001$), illustrating a protective effect of the K48R mutation (Table 1). Moreover, average seminiferous tubule diameters for Wt ubiquitin testes (156.8 ± 7.23 μ m) were significantly ($P < 0.001$) smaller than those of K48R ubiquitin testes (200.4 ± 6.29 μ m).

Levels of serum testosterone were measured in 18-month-old K48R mutant and Wt ubiquitin mice. Although there was no significant difference between K48R mutant ubiquitin (5.0 ± 2.2 ng/ml, $n = 6$) and Wt ubiquitin (3.2 ± 0.91 ng/ml, $n = 6$) testosterone levels, there was a trend toward higher serum testosterone in the K48R ubiquitin mutant mice.

Interestingly, blood vessels within the Wt ubiquitin animals were enlarged with accumulations of hyaline material (Figure 7). Seminiferous tubules depleted of germ cells sometimes surrounded these hyalinized blood vessels.

Discussion

Ubiquitination targets proteins for degradation by the 26S proteasome, and modulates the turnover of short-lived

Figure 6. Average germ cells (A) and multinucleated giant cells (B) per seminiferous tubule (ST) for Wt ubiquitin (open circle, $n = 3$), FVB/N wild-type (open triangle, $n = 5$) and K48R ubiquitin (closed circle, $n = 3$) mouse testes 4 and 6 days after experimental cryptorchidism. Note that K48R ubiquitin testis 6 days after cryptorchid surgery had statistically equivalent numbers of germ cells (GC) and multinucleated giant cells (MGC) as 4-day Wt ubiquitin and FVB/N testes. Different letters designate statistically significant differences within each group ($P < 0.05$).

proteins involved in signal transduction. Transgenic mice expressing K48R mutant ubiquitin were viable and displayed no obvious phenotype under basal conditions, suggesting that in the dynamic assembly and disassembly of ubiquitin chains, endogenous ubiquitin was able to compete with mutant ubiquitin and effectively mask any deleterious effects. Postnatal developmental characterization of the testes showed no differences between the Wt ubiquitin and K48R ubiquitin animals. The K48R ubiquitin mutant transgenic mouse testes developed normally and had a normal young adult phenotype in comparison to Wt ubiquitin transgenic mice. This, in itself, is informative about this model system, indicating that basal expression of the transgene is innocuous or readily compensated.

To unmask a phenotype associated with the K48R mutation, the testis was examined after injury. Testicular injury in the form of heat stress, performed by experimental unilateral cryptorchidism, showed a time-dependent resistance to injury in the K48R mutant. Why internal body temperature is a heat stress to the testis is not fully understood; however, it is clear that several days of experimental cryptorchidism leads to testicular atrophy.⁶⁻⁹ The initial increase in cryptorchid testis weight is a phenomenon that occurs with cryptorchid surgery. This initial

Figure 7. Histopathology of 19 month-aged testes. The K48R mutant ubiquitin-aged testis (A) has normal seminiferous tubule architecture and contains all types of germ cells while the Wt ubiquitin-aged testis (B) is almost devoid of germ cells and manifests Sertoli cell vacuolization (arrowhead). Hyalinized blood vessels (arrow) are also prominent in the Wt ubiquitin-aged testis. (PASH, Bar, 50 μ m).

weight increase is explained by an accumulation of fluid due to inflammation of the injured testis. Subsequent germ cell depletion leads to significant loss in testis weight since germ cells are the most abundant cell type in the testis. The timing of germ cell loss is consistent within a species and strain. Therefore, non-transgenic controls of the background strain, FVB/N, were used in the experimental cryptorchidism experiments in addition to the Wt ubiquitin transgenic mouse.

There are several possible mechanistic explanations for the germ cell loss during experimental cryptorchidism, including induction of heat-shock proteins, p53 induction, and generation of reactive oxygen species.^{4,7-11,34,35} The role of heat-shock proteins in cryptorchidism is not clear, since the physiological temperature attained when the testis is elevated into the body cavity is not sufficient to induce many heat-shock proteins. Indeed, testis-specific heat-shock proteins, such as Hsp70-2, do not increase their activity after experimental cryptorchidism.^{34,35} A role for p53 in experimental cryptorchidism has been more convincingly characterized. Cryptorchid-induced germ cell loss and morphological alterations are delayed by several days in p53 knockout mice.^{4,9} It has recently

Table 1. Comparison of Aged Wt and K48R Mutant Ubiquitin Mouse Testes

Genotype	N (mice)	Age (months)		Testes weight (mg)		ST diameter (μ m)		GC > 50 per ST (%)	
		Mean (SEM)	Range	Mean (SEM)	Range	Mean (SEM)	Range	Mean (SEM)	Range
Wt Ub	8	21.20 (0.319)	19.11–22.03	52.2 (3.06)*	39.1–67.4	156.8 (7.23) [†]	123.0–183.2	33.12 (7.46) [†]	0–57.5
K48R Ub	12	21.55 (0.586)	18.69–24.69	66.4 (3.59)	42.5–85.2	200.4 (6.29)	150.4–223.5	89.44 (4.72)	48.8–100

Note: ST = seminiferous tubule; GC = germ cells. Statistical comparisons within categories are significant as shown: *, $p < 0.015$; [†], $p < 0.001$.

been shown, however, that ubiquitination and exportation of p53 from the nucleus does not require lysine residues K48 or K63 to be present on the ubiquitin molecule, indicating that altered p53 function is not the explanation for our results.^{36–38}

A final mechanism we consider for germ cell loss during cryptorchid injury is the role of reactive oxygen species. Rat testes made surgically cryptorchid have higher amounts of reactive oxygen species as assessed by increases in lipid peroxidation products.¹¹ Moreover, inhibiting normal testicular descent, which occurs between postnatal days 18 to 21, also causes increases in diene conjugates and fluorescent chromolipids (indicators of lipid peroxidation) when compared to contralateral scrotal controls evaluated at postnatal day 30.¹⁰ Further evidence for the role of reactive oxygen species in the pathogenesis of cryptorchid injury involved using allopurinol, a xanthine oxidase inhibitor, to reduce production of reactive oxygen species and decrease germ cell loss.¹³ One source of increased reactive oxygen species is a decrease in the activity of superoxide dismutase in the cryptorchid testis.¹¹ In addition, high levels of superoxide dismutase activity are normally found in the cell types targeted for cell death during experimental cryptorchidism, namely pachytene spermatocytes and round spermatids.³⁷ Given the role of ubiquitin in degradation of altered proteins, which result from oxidative stress, a role for reactive oxygen species in the resistance of K48R mutant ubiquitin testes to injury is plausible. There is, however, controversy as to the requirement of ubiquitination in degradation of oxidized proteins, and it has been shown *in vivo* that oxidized proteins can be degraded without a fully competent ubiquitin system.³⁸ Although the essential role of ubiquitin in degradation of oxidized proteins is up for debate, experiments demonstrating that ubiquitin is not essential have been performed in cell culture and not in animals. Therefore we will assume that oxidatively damaged proteins are normally polyubiquitinated and degraded; logically, if these proteins were not degraded as easily due to altered ubiquitination, then the resulting germ cell death could be delayed assuming that a ubiquitin-bound protein is still functional. Moreover, expression of the transgene was observed in the testicular cells most sensitive to experimental cryptorchidism, namely germ cells and not the Sertoli cells.

The other protective effect of the K48R ubiquitin mutation in the testes observed in these studies was a resistance to atrophy during aging. It was not possible to obtain 21-month-old FVB/N mice as non-transgenic controls in addition to the Wt ubiquitin control mice; however, aging rats and mice show a similar testicular phenotype to that seen in our Wt ubiquitin mice, including low testis

weights, loss of germ cells, and hyalinized blood vessels.³⁹ Studies in the Brown Norway rat, a strain susceptible to testicular aging, have shown alterations in intratesticular testosterone concentrations in aged *versus* young animals.^{40,41} Aged Leydig cells in culture have a reduced ability to respond to leutinizing hormone (LH) and subsequently produce less testosterone due to impaired steroidogenesis.^{17,42,43} Suppression of steroidogenesis during adulthood protected the aged Leydig cells later in life.⁴⁴ According to this hypothesis, Leydig cell steroidogenesis leads to the production of mitochondrial-reactive oxygen species and thereby causes the Leydig cell damage. The protective effects of K48R ubiquitin mutant transgene expression in aged testes can be explained, similar to the cryptorchidism results, through a reactive oxygen species mechanism. We postulate that in the aging model, the production of reactive oxygen species from steroidogenesis causes oxidative stress and leads to damaged proteins. These oxidatively damaged proteins are normally polyubiquitinated and sent to the 26S proteasome for degradation. In the K48R ubiquitin mutant mice, the steroidogenic enzymes are altered, but not turned over as readily. Therefore, the K48R ubiquitin mice testes may have a slightly lowered level of steroidogenesis, and therefore less chronic damage to the Leydig cells. This slightly lowered level of steroidogenesis in K48R mutants could be such that spermatogenesis is sustained, and the androgen levels are not so low that there would be large increases in leutinizing hormone to compensate. According to this hypothesis, young Wt ubiquitin mice would have a higher testosterone concentration than their K48R mutant ubiquitin counterparts, while in older animals the reverse would be true. Our limited testosterone data indicate a nonsignificant trend toward higher testosterone levels in older K48R mutant ubiquitin mice compared with Wt ubiquitin mice.

In addition, the observation of occluded blood vessels within the testis of atrophic animals may be a consequence of reactive oxygen species accumulating in the blood vessels leading to arteriosclerotic damage. We do not believe that hyalinized blood vessels and subsequent hypoxia were the sole cause for testicular atrophy in the Wt ubiquitin transgenic mouse testes, since many atrophic sections analyzed had normal blood vessels. A question that needs to be answered in this model is whether this phenotype, and protection by the K48R ubiquitin mutant transgene, results from a molecular mechanism within the testis, or from a whole body alteration, such as altered endocrine levels or arteriosclerotic lesions.

While the precise mechanisms of experimental cryptorchid germ cell death and testicular atrophy with aging

Figure 8. Schematic representation postulating a role for reactive oxygen species in the ubiquitin-mediated response to experimental cryptorchidism and aging. Experimental cryptorchidism and steroidogenesis cause production of reactive oxygen species (ROS), which leads to protein damage and subsequent polyubiquitination. In the acute injury of experimental cryptorchidism (crypt) critical proteins are degraded leading to germ cell loss. In the chronic injury (aging), turnover of steroidogenic enzymes leads to their replacement by new enzymes, which results in higher production of ROS as a feedback loop. This chronic production of ROS leads to Leydig cell damage and testicular atrophy. The K48R ubiquitin mutation blocks the step of polyubiquitination therefore leading to retention of critical proteins in experimental cryptorchidism, allowing prolonged germ cell survival. Further, retention of altered and hindered steroidogenic enzymes prevents higher production of ROS and inhibits testicular atrophy during aging.

remain to be elucidated, it is clear from this work that ubiquitin plays a role in these processes. Based on what is known about testicular aging and experimental cryptorchidism, we have hypothesized a mechanistic model (Figure 8) connecting injuries resulting from reactive oxygen species generation to ubiquitin. An interesting conundrum that has arisen from this study is that these *in vivo* results contradict *in vitro* studies showing that expression of K48R ubiquitin sensitizes HT4 cells to the amino acid analog, canavanine, and an oxidizing agent, cadmium.²⁸ There are many possible explanations for why the K48R ubiquitin transgene is protective *in vivo* and sensitizing *in vitro* including: tissue specificity, duality in phenotype based on the stressor, and different levels of transgene expression. The cause for these different re-

sults *in vivo* and *in vitro* is an intriguing question that remains to be elucidated. Previous testis studies *in vivo* have shown that reactive oxygen species are critical in the pathogenesis of both the acute injury of experimental cryptorchidism and the chronic insult of testicular aging. Our results suggest a role for ubiquitin in mediating injuries dependent on degradation of oxidatively altered proteins.

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