HEAT SHOCK PROTEIN (HSP) EXPRESSION IN THE SHIONOGI MOUSE MAMMARY CARCINOMA (SCI15) IN RESPONSE TO STEROID HORMONE TREATMENT IN VITRO AND A CHANGE IN SOCIAL HOUSING CONDITIONS IN VIVO

by

HEATHER NICOLA ANDREWS
(B.Sc. Animal Biology, University of British Columbia, 1994)

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The University of British Columbia
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ABSTRACT:

Previous studies in our laboratory have shown that social housing conditions can significantly alter the growth rate of the Shionogi mammary carcinoma (SC115). The present study extended investigations of mechanisms underlying the effects of housing condition on tumor growth rate. We focused on changes occurring at the molecular level, on the expression of a group of stress-responsive proteins, the heat shock proteins (HSPs). HSP25, 70 and 90 were examined in SC115 cells in vitro, following exposure to SC115 growth regulatory hormones, and in tumors grown in vivo in mice in different social housing conditions. In vitro, physiological levels of dihydrotestosterone (DHT, n=3) and pharmacological levels of hydrocortisone (HC, n=3) upregulated HSP25, whereas HSP70 and 90 remained unchanged. Conversely, physiological levels of β-estradiol (E₂, n=2) upregulated HSP90, whereas HSP25 and 70 remained unchanged. For the in vivo experiments, mice were reared in groups (G) or as individuals (I). Immediately following tumor cell injection, mice were rehoused from group to individual (GI, n=36), from individual to group (IG, n=40) or they remained in groups (GG, n=36). All animals (GI, GG, IG, n=112) were exposed to a daily novelty stressor, shown to increase the differences in tumor growth rates among mice in the different social housing conditions. In addition, mice in a control condition were maintained under standard housing conditions (no housing change or daily novelty stress, GGNS, n=12). Tumor tissue was resected for HSP examination when the average weight of tumors reached 0.8 g (n=56) or 3 g (n=56). In mice subjected to daily novelty stress, HSP25, 70 and 90 was increased in tumors from IG mice compared to GG and GI mice (0.8 g and 3 g
tumors). HSP90 was greater in 3 g tumors compared to 0.8 g tumors from mice in the IG condition. In group housed mice (GGNS and GGS), daily novelty stress decreased HSP70 (0.8 g and 3 g tumors), decreased HSP90 (3 g tumors), and did not alter HSP25. This study has shown that HSP expression is altered in SC115 cells by steroid hormones \textit{in vitro} and this is the first study to report that psychosocial stressors, a change in social housing condition, can also induce differences in HSP expression \textit{in vivo}. 
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CHAPTER 1: BACKGROUND:

A) Definition of Stress

The notion that stress can play a role in human disease is not new. For example, Galen observed that “melancholy women” were more prone to breast cancer than “sanguine women” (LeShan, 1959). Since then many scientists have conducted studies to examine this question. Of particular interest to this thesis are the studies which focus on the association of such psychological factors as affective state, coping style and stressful life events with the occurrence, progression and/or prognosis of cancer.

Before focusing on how stress can affect disease, and in particular cancer, the term stress needs to be discussed and defined. An organism’s stress system consists of two main components: the sympatho-adrenomedullary system and hypothalamic-pituitary-adrenal (HPA) axis. The sympatho-adrenomedullary system is responsible for the immediate response to stress, termed the “fight or flight” reaction by Walter Cannon (reviewed in Johnson, 1992). When stimulated, catecholamine (norepinephrine and epinephrine) release leads to arousal and sympathetic activation within an organism. The HPA axis response follows the sympatho-adrenomedullary response to stress and in time a cascade of hormones are released from the hypothalamus, anterior pituitary and the adrenal cortex. Corticotrophic releasing hormone (CRH) is released from the hypothalamus and stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH, in turn, is released into the general circulation and acts on the adrenal cortex to cause release of glucocorticoids (cortisol in humans and corticosterone in rodents). Finally, elevated levels of plasma glucocorticoids feed
back on the hypothalamus and anterior pituitary to inhibit the release of the hormones CRH and ACTH. These two systems work together and result in physiological and behavioral changes which allow an organism to respond and adapt to the stressful situation. For the purpose of our research, we will focus primarily on the responsiveness of the HPA axis and the definition of stress by Selye will be used. According to Selye, stress is “an alteration in the body’s hormonal and neuronal secretions caused by the central nervous system in response to a perceived threat” (Selye, 1975). A stressor is then defined as “a stimulus that changes an organism’s internal or external environment which is perceived by the organism as threatening” (Selye, 1975).

Stressors may be physical, examples of which include disturbances of the internal environment (anoxia and hypoglycemia), changes in external temperatures (heat and cold), and multifaceted stressors (noxious stimuli and physical strain such as exercise), or psychological, examples of which include stimuli that affect emotion and result in fear, anxiety or frustration (reviewed in Johnson, 1992). Data have demonstrated that psychological stimuli may be as effective as noxious physical stimuli in eliciting a stress response (Mason, 1975, Mason, 1975 and Weinberg and Levine, 1980). Psychological stressors also include situations that result from social interactions, such as the death of a friend or family member, divorce, or the lack of social support (Asterita, 1985) and in these instances are called psychosocial stressors. These latter type of psychosocial stressors are often referred to as stressful life events in literature. It has been suggested that both physical and psychological stressors act through the HPA axis and increase emotional arousal (Mason, 1975). Thus, HPA activity is often used as a measure of the level of emotional arousal as an indicator of the magnitude of stress response to certain stimuli (Hennessey and Levine, 1979).
B) Human Studies of Stress and Cancer

Many studies using human subjects have found relationships between stress and the incidence and/or progression of human cancer (reviewed in Jensen, 1991, Hilakivi-Clarke et al., 1993 and Spiegel, 1997). For example, stressful life events such as death of a close friend, personal illness and nursing responsibility of a relative, are correlated to increased breast cancer incidence (Cooper et al., 1989 and Gehde and Baltrusch, 1990, Scherg et al., 1981, Scherg and Blohmke, 1988) and a shorter disease-free survival period after surgery (Ramirez et al., 1989), whereas perception of good social support, the ability to cope with breast cancer and group therapy have been correlated to increased disease-free survival (Hislop, 1987, Spiegel et al., 1989 and Waxler-Morrison et al., 1991).

In contrast, some studies have shown no correlation between stress and human cancer. For example, there was no increased incidence of cancer in prisoners of war after World War II and the Korean War compared to cancer incidence in the normal population (Keehn et al., 1974 and 1980). Also, a study by Tross et al. (1996), failed to provide evidence that psychological factors contributed to the length of disease-free or overall survival of women with breast cancer. Inherent problems in human studies, such as the perception of stressors after one has been diagnosed with cancer and the timing of reported stressful events relative to the initiation of cancer may have led to these inconsistent data (reviewed in Irvine et al., 1991).
C) Animal Models of Stress and Cancer

Animal models investigating relationships between stressors and tumor growth can generate data under more controlled conditions. However, even in animal models the data are often complex; variables such as tumor type, type and timing of stressors, coping ability and social housing condition make the interpretation of the results difficult and can influence whether an increase or decrease in tumor growth in response to stressors is observed (Sklar and Anisman, 1980, 1981, Justice, 1985, Amkraut and Solomon, 1972, Burchfield, 1978 and Riley, 1981). A few examples of how these animal-model variables can interact and influence tumor growth results illustrate this point.

Stressors such as restraint, sound, crowding, cold and electric shock have all been found to decrease tumor growth. For example, restraint stressors were found to decrease the growth of Walker carcinosarcoma tumors (a transplantable mammary tumor) (Newton, 1964), chronic exposure to sound in crowded housing condition was found to decrease the size of tumors induced by the chemical carcinogen 7, 12 dimethylbenz[a]anthracene (DMBA; Bhattacharyya and Pradhan, 1979), exposure to 2°C for 24 h was found to decrease the size of transplantable gliosarcommas (Baker, 1977) and electric footshock was found to decrease the growth of DMBA tumors (Newberry et al., 1972 and Ray and Pradhan, 1974).

In contrast, stressors such as rotation, electric shock, inescapable electric shock and isolated housing have been found to increase the incidence and growth of tumors. For example, rotational stress (created by placing an animals cage on a turntable rotating at 45 revolutions/min) was found to increase the size of 6C3HED lymphosarcomas (Riley, 1981), single, electric shocks at the time of mastocytoma injection into mice have been found to
increase tumor growth (Sklar and Anisman, 1981), yoked mice exposed to inescapable electric shock had increased tumor growth compared to mice that had escapable electric shock treatment (Sklar and Anisman, 1981) and mice housed individually were found to have increased tumor growth of transplanted P815 mastocytomas (Sklar and Anisman, 1980).

It is impossible to determine whether it was the type of tumor, the type of stressor, the duration of the stressor, the timing of application of the stressor, or an interaction of variables that was of key importance in altering the tumor growth rates from these studies. For this reason, the interpretation of animal-model data and the comparison of data between studies should be done with caution and consideration of the variables within each animal model.

Another important element to consider when interpreting data from animal models is the animal’s ability to cope with an experimental stressor. A relevant quote from Anisman and Sklar (1981) reads, “...stress may either exacerbate or inhibit tumor development; the effect manifested, however, was dependent on the availability of coping responses, premorbid stress history, stress type, stress chronicity and social conditions.”. As mentioned earlier, Sklar and Anisman (1981) found that animals exposed to escapable electric shock treatment had a reduced tumor growth rate compared to animals exposed to an inescapable electric shock treatment (Sklar and Anisman, 1981). The ability to avoid the shock may have provided a coping response. Another example of an animal’s coping response is fighting, which is known to reduce HPA activation (Conner et al., 1971, Weiss et al., 1976 and Williams and Eichelman, 1971), and tumor growth (Amkraut and Solomon, 1972).

Of particular interest to this thesis is how housing condition, a psychosocial variable, can affect tumor growth. Studies have shown that both individual housing and a change in housing condition can increase the growth of tumors (Dechambre and Gosse, 1973, Sklar and
Anisman, 1980 and Steplewski et al., 1987). For example, there was an increased number of ascites tumor cells in mice that were isolated immediately after weaning compared to mice that were housed in groups (Dechambre and Gosse, 1973) and in mice that were moved from group to individual (GI) housing compared to mice that did not experience a change in housing condition (Dechambre and Gosse, 1973). Similarly, there was an increased tumor weight in DMBA-induced adenocarcinomas in rats that were changed from a group to individual housing condition (GI) compared to tumor weights in rats that did not experience a change in housing condition (Steplewski et al., 1987). Our recent data have shown that both a change in housing condition and the direction of change are critical in altering tumor growth rates (Weinberg and Emerman, 1989 and Emerman and Weinberg, 1989) (discussed in detail below). The mechanisms underlying the alterations in tumor growth rate induced by differential housing conditions are not known at present. This area is the focus of much research in our laboratory.

D) SC115 Animal-Tumor Model to Study the Effects of Psychosocial Stressors

The Shionogi Mammary Carcinoma (SC115)

The tumor utilized in our laboratory to examine the effects of psychosocial stressors on tumor growth is the Shionogi mouse mammary tumor (SC115). This tumor arose spontaneously from mammary epithelium in a female mouse of the D/DS strain. After 19 passages in male mice, a subline arose that grows more rapidly in males than in females. In our laboratory, the subline that is used for all experiments is the androgen-responsive (AR) SC115, referred to in this thesis as the SC115. Following injection of $2 \times 10^6$ SC115 cells
subcutaneously (s.c.) in the interscapular region, a palpable tumor mass will arise in approximately 8-10 days and this will develop into a 2 to 3 g mass in approximately 3 weeks. When SC115 cells are injected into a female mouse, a 1g tumor will develop in approximately 40 days.

The SC115 tumor is similar to many human endocrine-responsive tumors such as breast and prostate cancer (Uchida et al., 1981) in its sensitivity to steroid hormones including androgens (King and Yates, 1980), estrogens (Noguchi et al., 1987) and glucocorticoids (Watanabe et al., 1982). Cells of the SC115 tumor have been shown to possess functional androgen receptors and physiological concentrations of testosterone have been shown to stimulate SC115 growth in vitro and in vivo (Bruchovsky and Rennie, 1978, Emerman and Siemiatkowski, 1984 and Jiang et al., 1993). In addition, castration of male mice to remove endogenous androgens results in the outgrowth of a slower growing androgen-independent tumor (Bruchovsky and Rennie, 1978, Emerman and Worth, 1985 and Kitamura et al., 1979). Pharmacological concentrations of glucocorticoids also stimulate growth of SC115 cells both in vitro (Darbe and King, 1987 and Jiang et al., 1993) and in vivo (Watanabe et al., 1982). Similarly, SC115 cells have been shown to possess estrogen receptors, and they are stimulated to grow by pharmacological concentrations of estrogen in vivo (Nohno et al., 1982 and Noguchi et al., 1985) and are inhibited by both physiological and pharmacological doses of estrogen in vitro (Noguchi et al., 1987). It is thought that the stimulatory effect of estrogen in vivo is acting through the androgen receptor as there is cross-reactivity of estrogen with the androgen receptor and estrogen-induced growth stimulation only occurs at suboptimal levels of androgens (Nohno et al., 1982, and Noguchi et al., 1987). Finally, SC115 cells have been shown to be stimulated to grow by a growth factor, basic fibroblast growth factor or (bFGF)
(Tanaka et al., 1990 and Jiang et al., 1993). Thus, a number of hormones can stimulate the growth of these cells, but androgens appear to be the primary growth stimulators of these cells.

The Animal-Tumor Model

Our animal-tumor model is similar to a model developed by Sklar and Anisman (1980). Their model suggested that both individual housing and a change in housing condition can alter the growth rate of the P815 mastocytoma tumor. Mice were group housed and following tumor cell injection, they either remained group housed or were placed in individual cages. After 14 days, it was found that there was an increased tumor weight in the animals that were individually housed compared to the animals that were housed in groups of 5. From these data they concluded that social isolation exacerbated tumor growth. However, in their next set of studies, the data suggested that it was the change in housing condition and not the isolation per se that caused the increase in tumor growth. That is animals that experienced a change in housing condition, from group to individual housing (GI) had a significantly increased tumor weight on days 10 and 16 compared to animals that did not experience a change in social housing conditions [animals that were in groups remained in groups (GG) and animals that were individually housed remained individually housed (II)].

A series of studies in our laboratory investigated the effects of different social housing conditions on the growth rate of the SC115 tumor (Weinberg and Emerman, 1989, Emerman and Weinberg, 1989). Male mice of the DD/S strain were housed either individually (I) or in sibling groups of 3 (G) at the time of weaning (3 weeks of age). When mice were 2-4 months of age, they were injected with tumor cells and experimental housing conditions formed. Mice
raised as individuals (I) either remained as individuals (II) or were rehoused in non-sibling groups of 5 (IG). Mice raised in sibling groups (G) either remained in sibling groups (GG) or were rehoused as individuals (GI) (Figure 1). In addition to housing condition, all animals were exposed to an acute daily novelty stressor consisting of exposure for 15 min/day, 5 days/week to one of 5 novel environments, a treatment that we had previously shown enhances tumor growth rate differences among mice in experimental housing conditions (Weinberg and Emerman, 1989). Tumors from mice in the GG and II housing conditions had similar tumor growth rates and grew to a mass of about 2 g by 21 days. In contrast, mice of the GI group had significantly increased tumor growth rates, whereas mice of the IG group had significantly decreased tumor growth rates compared with those of mice in the GG group (Figure 2).
Figure 1. *Experimental Design of Animal-Tumor Model.*
Mice at Time of Weaning
(3 weeks)

Housed in Sibling
Groups of 3
(G)

Remain as
Groups of 3
(GG)

Rehoused as
Individuals
(GI)

Housed as Individuals
(I)

Remain as
Individuals
(II)

Rehoused as
Non-Sibling
Groups of 5
(IG)

All Mice Exposed to Acute Daily Novelty Stress
(15 min/day, 5 d/wk)
Figure 2. Tumor Growth in Mice in the Four Experimental Housing Groups. Points represent mean ± SEM. GG, raised and maintained in sibling groups of three, GI, raised in sibling groups of three, then separated and housed singly, IG, raised singly housed, then rehoused in non-sibling groups of five, II, raised and maintained singly housed. All mice were injected with $2 \times 10^6$ SC115 tumor cells. At 18 d post tumor cell-injection, GI=II>GG>IG, $p$'s<0.05 (Rowse, 1993).
Studies then examined basal plasma levels of testosterone and corticosterone at 1, 3, 7 and 21 days post tumor cell injection and group formation (Weinberg and Emerman, 1989 and Rowse et al., 1992). Previous data from our laboratory demonstrate that up to 7 d post tumor cell injection and housing condition change, mice in the GI condition, which have the fastest tumor growth rates, have high basal testosterone levels and low basal corticosterone levels. In contrast, mice in the IG condition, which have the slowest tumor growth rates, exhibit low basal testosterone levels and high basal corticosterone levels. However, no significant differences in the basal plasma levels of testosterone or corticosterone among mice from the 4 housing groups were found 21 days post tumor cell injection and housing condition change. Thus, steroid hormone-induced alterations in SC115 tumor growth rates in our experimental housing conditions are likely occurring within the first week post tumor cell injection and housing condition change.

Possible involvement of the immune system in mediating tumor growth was also investigated. A series of studies examined natural killer (NK) cell activity in mice in the different social housing conditions of our model. NK cells are thought to be one of the body's first lines of defense against foreign antigens, particularly tumor cells (Haller et al., 1977). Splenic NK cell activity was examined in mice in our experimental housing conditions at 1, 3, 7 days post tumor cell injection and housing condition change (Rowse et al., 1995). It was hypothesized that animals with the smallest tumors (IG) would show the greatest NK cell activity, and that animals with the largest tumors (GI) would show the lowest levels of NK cell activity. The data indicated that the SC115 tumor significantly stimulates splenic NK cell activity, with the greatest stimulation occurring at 3 days and activity decreasing by 7 days post tumor cell injection. Contrary to what was predicted however, stimulation of NK cell
activity was greater in animals in the GI condition compared to that in mice in all other experimental groups. If NK cells do in fact play a role in containing tumor growth, one would expect the greatest NK cell activity in IG animals that have the smallest tumors. Instead, the greatest activity was found in GI mice that have the largest tumors. These data suggested two possibilities: 1) that NK cells might not be an important regulator of SC115 tumor growth or 2) that there may be a redistribution of NK cells between compartments, i.e. from spleen to tumor site.

A second set of studies was then undertaken to examine NK activity at the tumor site at 1, 3 and 7 days post tumor cell injection and housing condition change. It was found that NK cell activity was significantly greater in mice of the GI group than in mice of all other groups at both 3 and 7 days. These results are similar to what was observed in the spleen; that is, NK cell activity was once again greatest in GI mice who have the largest tumors. Other mechanisms must clearly be involved in mediating tumor growth in our model.

Thus to date, differences have been found in both the endocrine and immune systems in response to a psychosocial stressor, a change in housing condition. These changes may in part be responsible for the observed tumor growth rate differences in our model, but it is likely there are other important changes which are yet to be discovered. This thesis was undertaken to extend investigations to the molecular level, focusing on the expression of a group of stress-responsive proteins, the heat shock proteins (HSPs) in the SC115 tumor cells.

E) Heat Shock Proteins

Heat shock proteins (HSPs) were first discovered in *Drosophila* in response to
treatments of elevated temperatures or heat-shock (Ritossa, 1962). At this time they were named heat-shock proteins, but today the HSPs belong to a larger group of "stress proteins" which also include the glucose-regulated proteins (GRPs) (Welch, 1992). Together the HSPs and GRPs function to help cells survive stressful events and are often referred to as "chaperones" as they bind to and protect denatured proteins during the stress event and help them refold when the stress is over. Both groups of proteins are functionally and structurally related, but they are located in different areas of the cell and are uniquely induced by specific cellular stressors. For example, excessive heat and exposure to heavy metals and steroid hormones induce mainly the HSPs, whereas, anoxia, glucose deprivation, inhibitors of glycosylation, calcium ionophores and acidification induce mainly the GRPs. In general, most of the HSPs are located within the cytoplasm, nucleus and nucleolus and the GRPs are located in the endoplasmic reticulum (ER). One exception to this are the stress proteins, HSP 58 and GRP 75 kDa, located in mitochondria.

HSPs are grouped into families according to their molecular weight (Welch, 1992). There are four families with both HSP and GRP members: the HSP 27 kDa family, the HSP 60 kDa family, the HSP 70 kDa family and the HSP 90 kDa family. Families of similar molecular weight are often alike in structure and perform similar functions. For example, the HSP 70 kDa family includes HSP 72 kDa, HSP 73 kDa, GRP 75 kDa and GRP 78 kDa, and their overall function is to bind ATP and assist in refolding proteins.

HSP25, 70 and 90 are of particular interest to this thesis because of their known functions in normal cells. HSP25 is located in the cytoplasm and is involved in thermotolerance, actin polymer stabilization, and possibly signal transduction cascades (HSP25 functions reviewed in Welch, 1992 and Ciocca et al., 1993). The HSP70 protein that
will be examined is HSP73 kDa, the inducible form of HSP70, and will be referred to as HSP70 in this thesis. HSP70 is a cytoplasmic protein involved in thermotolerance, antigen processing, translocation of antigens to the major histocompatibility complex and chaperoning proteins such as p53 (Fourie et al., 1997) and steroid hormone receptors (HSP70 functions reviewed in Welch, 1992, Jaattela and Wissing, 1992 and Schlesinger, 1994). HSP70 itself can also translocate to the nucleus and nucleolus and chaperone unfolded nuclear proteins during stressful events. Similarly, HSP90 is primarily a cytoplasmic protein. It has a high constitutive level and is involved in thermotolerance and binding to proteins such as pp60-src, a viral oncogene, (Opperman et al., 1981) and inactive steroid receptors along with HSP70 (HSP90 functions reviewed in Welch, 1992 and Schlesinger, 1994). HSP25, 70 and 90 all associate with and may regulate the activity of proteins that may be involved with cell proliferation.

HSP expression is regulated mainly by exposure to acute stressors such as heat and chemicals (Welch, 1992 and Morimoto, 1993), but is also regulated by exposure to steroid hormones (Edwards et al., 1981, Ramachandran et al., 1988 and Shyamala et al., 1989). For example, estrogen is known to regulate levels of HSP27 in human endometrial cells throughout the estrous cycle (Tang et al., 1995) as well as HSP90 in murine uterine cells (Ramachandran et al., 1988 and Shyamala et al., 1989). Steroid hormone regulation of HSPs is of interest as the SC115 cells are hormone-responsive and are stimulated to proliferate by steroid hormones. There is a possibility that steroid hormones may also regulate HSP expression in SC115 cells and this may in turn alter the activity of the steroid hormone receptors and perhaps the responsiveness of SC115 cells to growth stimulatory steroid hormones.
In addition to the function of HSPs in normal cells, they have been observed to be overexpressed in tumor cells (Ferrarini et al., 1992, Fuller et al., 1994 and Conroy and Latchman, 1996); however, their role in tumor development and progression is unclear. It is possible that overexpression of HSPs may alter the activity of HSP-associated proteins such as steroid hormone receptors (Catelli et al., 1985, Sanchez et al., 1987, Kost et al., 1989, Picard et al., 1990 and Edwards et al., 1992), p53 (Finlay et al., 1988 and Fourie et al., 1997), pp60src (Operman et al., 1981 and Xu and Lindquist, 1993) and other cellular proteins. For example, studies using normal cells have found that upregulation of HSPs by heat shock can alter the activity of steroid hormone receptors (Sanchez et al., 1987, Kost et al., 1989, Picard et al., 1990 and Edwards et al., 1992), hence altered levels of HSPs in tumor cells may play a role in regulating the growth of hormone-responsive tumors. HSP70 may also alter tumor immunogenicity through aiding in tumor-antigen processing and presentation at the cell surface (Ferrarini et al., 1992, Srivastava, 1993, Srivastava et al., 1994, Multhoff et al., 1995 and 1997). Overexpression of HSP25 is known to increase tumor cell resistance to chemotherapy (Ciocca et al., 1992, Oesterrich et al., 1993 and Fuqua et al., 1994). Thus, altered expression of HSPs in tumor cells could affect tumor cell interactions and responses to the endocrine and/or immune systems, resulting in altered tumor growth rates.
CHAPTER 2: INTRODUCTION AND HYPOTHESIS:

To further our investigations of changes occurring in mice exposed to psychosocial stressors resulting in altered SC115 tumor growth rates, there are two sets of experiments in this thesis. The first set of in vitro experiments will examine if steroid hormones known to alter SC115 growth rate both in vitro and in vivo can also alter levels of HSP25, 70 and 90. The second set of in vivo experiments will focus on the expression of HSP25, 70 and 90 in SC115 tumors in mice in different housing conditions.

A) In Vitro Experiments

The SC115 tumor has been considered an excellent model to study the proliferative effects of steroid hormones as it contains several steroid hormone receptors, including androgen receptors (Bruchovsky and Rennie, 1978), glucocorticoid receptors (Watanabe et al., 1982) and estrogen receptors (Noguchi et al., 1985) and has been shown to be sensitive to androgens (King and Yates, 1980, Darbe and King, 1987 and Jung-Testae et al., 1988), glucocorticoids (Watanabe et al., 1982 and Omukai et al., 1987) and estrogens (Noguchi et al., 1985 and 1987). The SC115 tumor has maintained its responsiveness to physiological concentrations of androgens in vivo (Matsumoto et al., 1982) and in vitro (Desmond et al., 1976 and Jang et al., 1993). The growth of the SC115 tumor is stimulated by pharmacological doses of glucocorticoids both in vivo (Watanabe et al., 1982) and in vitro (Darbe and King, 1987 and Jang et al., 1993) and by pharmacological doses of estrogen in vivo (Noguchi et al., 1987). The growth-stimulatory effects of androgens, estrogens, and
glucocorticoids are mediated through their receptors (Jung-Testae et al., 1985, 1988).

Evidence suggests that effects of androgens and glucocorticoids on SC-3 cell proliferation (an SC115 cell line derived from the SC115 tumor) may be mediated by the synthesis of a fibroblast growth factor-like polypeptide that acts on the cells in an autocrine and/or paracrine manner (Lu et al., 1989, Nakamura et al., 1989, Nonomura et al., 1988). Thus, local factors are also likely playing a role in SC115 growth regulation.

As stated earlier, HSP25, 70 and 90 may be involved in cell growth through chaperoning cellular proteins that regulate the cell cycle. For example, HSP90 is known to bind to and help translocate pp60-src, a protein involved in signal transduction cascades (Opperman et al., 1981 and Xu and Lindquist, 1993). HSP70 binds to p53, a regulator of apoptosis (Finlay et al., 1988 and Fourie et al., 1997). HSP25 is known to associate with actin and help stabilize the polymers and thus, may play a role in cytoskeletal maintenance and how a cell interacts with its surroundings (Huot et al., 1995 and Guay et al., 1997). Thus, altered HSP levels may alter the functions of any proteins they associate with, including proteins involved in cell cycle regulation.

The purpose of this set of experiments was to examine levels of HSP25, 70 and 90 in cultured SC115 tumor cells in response to direct exposure to the SC115 growth stimulatory steroid hormones and growth factor, dihydrotestosterone (DHT), hydrocortisone (HC) and basic fibroblast growth factor (bFGF), respectively, and the growth inhibitory steroid hormone estradiol (E₂).
B) In Vivo Experiments

Using our animal-tumor model we have demonstrated the effects of social housing condition on SC115 tumor growth rate (Emerman and Weinberg, 1989, Weinberg and Emerman, 1989 and Grimm et al., 1996). Briefly, being reared in a group and then individually housed (GI) following tumor cell injection increases tumor growth rate, whereas being reared individually and then group housed (IG) following tumor cell injection decreases tumor growth rate, compared to mice that remain in their rearing conditions (II and GG). In addition, tumor growth rate differences between mice in the GI and IG conditions are increased if animals are subjected to acute daily novelty stress. Steroid hormone studies have revealed that up to 7 d post tumor cell injection and housing condition change, basal levels of plasma testosterone and corticosterone are altered in GI and IG mice (Rowse, 1992). Basal levels of testosterone are greater in GI mice compared with IG mice, whereas basal levels of corticosterone are greater in IG mice compared to GI mice. These data suggest that testosterone and corticosterone are likely playing key roles in differentially altering SC115 growth rates following housing condition change during the initial phase of tumor development. Furthermore, we have also demonstrated that SC115 cells are immunogenic, natural killer (NK) cell activity is increased differentially in mice in the GI and IG conditions (Rowse, 1995).

The purpose of the present *in vivo* experiments was to examine HSP25, 70 and 90 expression in 0.8 g and 3 g SC115 tumors (an early and late time point in tumor development) in mice exposed to a psychosocial stressor, a change in housing condition and daily novelty stress. It is possible that changes in HSP expression may be involved in a mechanism
underlying the differential tumor growth rates observed in our model. A secondary
experiment investigated the effects of the daily novelty stressor on HSP expression in the
SC115 tumor from group housed mice. HSP expression was examined in 0.8 g and 3 g
SC115 tumors from animals housed in the standard housing condition (no change in housing
condition and not subjected to daily novelty stress, GGNS) and compared to HSP expression
in 0.8 g and 3 g SC115 tumors from animals housed the that were group housed and subjected
to daily novelty stress (GGS).

These experiments were intended to extend our investigations of changes occurring at
the molecular level in SC115 cells in response to a change in housing condition and a daily
novelty stressor. The results may help to elucidate a molecular mechanism by which
psychosocial stressors may induce differential responses of SC115 cells to endocrine and
immune factors which may ultimately alter tumor growth rate.

C) Hypothesis

SC115 tumor cells have altered expression of HSP25, 70 and 90 in response to: a)
direct exposure to the steroid hormones DHT, HC and E₂ and the growth factor bFGF in
vitro and b) a change in social housing conditions and daily novelty stressors in vivo.

D) Specific Objectives

1. To examine levels of HSP25, 70 and 90 in SC115 cells in vitro when exposed to the
steroid hormones DHT, HC and E₂ and the growth factor bFGF.
2. To examine levels of HSP25, 70 and 90 in SC115 cells from mice exposed to different housing conditions (GI, GG and IG) and subjected to daily novelty stress.

3. To compare levels of HSP25, 70 and 90 in SC115 cells from mice in the standard housing conditions (group housed and not subjected to daily novelty stress, GGNS), with cells from mice that were group housed and subjected to daily novelty stress (GGS).
CHAPTER 3: METHODS AND MATERIALS:

A) Tumor Model

The androgen responsive SC115 mouse mammary carcinoma was maintained by serial transplantation in male mice of the DD/S strain. For propagation, tumors were dissociated to a single cell suspension (described below) and male mice (2-6 months old), housed in the standard housing condition (male mice raised and housed in sibling groups of 3) were injected with $2 \times 10^6$ cells/mouse.

1) Dissociation

Tumors weighing approximately 2 g were surgically removed using sterile technique and minced finely with scalpel blades. The tumor tissue was transferred to a dissociation flask and approximately 15 ml of Saline-Trypsin-Versine (STV) was added. STV consisted of 0.05% trypsin and 0.025% ethylenediamine tetraacetic acid (Sigma-Aldrich Canada Ltd., Oakville, Canada) in Ca$^{2+}$-Mg$^{2+}$-free Saline, pH 7.3. The flask was gently swirled for 2 min and the contents transferred to a centrifuge tube and spun at $80 \times g$ for 1 min in a bench top clinical centrifuge (International Equipment Company, Fischer Scientific, Vancouver, Canada). The supernatant was then transferred to a second centrifuge tube containing an equal volume of Dulbecco's Modified Eagle's Medium (DMEM, StemCell Technologies, Vancouver, Canada) and 5% Calf Serum (CS; Life Technologies, Burlington, Canada) to inactivate the trypsin. The tube was spun at $200 \times g$ for 4 min to enrich for epithelial cells. The pellet was then resuspended in DMEM and placed in a 37°C water bath.
Tissue in the original centrifuge tube was transferred back into the dissociation flask and 15 ml of STV added. The flask was left shaking at 100 rpm on a gyrator shaker (Junior Orbit Shaker, Lab-Line Instruments, Inc., Ill, USA) for 7 min in an incubator at 37°C. The contents of the flask were transferred to a centrifuge tube and centrifuged at 80 × g for 1 min. The supernatant was collected and combined with an equal volume of DMEM + 5% CS. This was spun at 400 × g, resuspended in DMEM and placed in a 37°C water bath. The remaining tissue was placed in the dissociation flask for the third and final dissociation with STV for 7 min. The supernatant was collected as described earlier, combined with an equal volume of DMEM + 5% CS and centrifuged. The pellet was then resuspended in DMEM. All 3 cell suspensions were pooled together and passed through a 50 μm Nitex filter (Tetko Inc., Elmsford, USA) to remove large cell aggregates and debris. The single cell suspension was centrifuged at 400 × g for 4 min and the pellet resuspended in 10 ml of DMEM. An aliquot was diluted 1:10 with DMEM and counted on a haemocytometer using trypan blue exclusion to determine viable cells. Trypan blue is able to penetrate the cell membranes of lysed cells and thus, the dead cells stain blue. The remaining cell suspension was then adjusted to the desired concentration for either freezing or injecting (both described below).

2) Freezing Tumor Cells

In addition to maintaining the SC115 in vivo, tumor cells were also stored in a liquid-nitrogen (LN2) tank (Locator Jr., Thermolyne, VWR/Canlab, Vancouver, Canada). For freezing, SC115 cells were adjusted to a concentration of 1 × 10^7 cells/ml freezing media [50% DMEM: 44% CS: 6% dimethylsulfoxide (DMSO, Sigma-Aldrich Canada Ltd., Oakville, Canada)]. One ml of cells (approximately 1 × 10^7 cells) in freezing medium were
then slowly frozen in cryotubes (Nalge Nunc International, Canadian Life Technologies, Vancouver, Canada) according to the protocol provided by the manufacturer of the freezing tank (Union Carbide Canada Ltd., Richmond, Canada). Once frozen, vials were transferred to the LN$_2$ storage tank.

3) Thawing Tumor Cells

Selected vials of tumor cells were removed from the LN$_2$ storage tank and quickly thawed in a water bath at 37°C. The tumor cell suspension was added to an equal volume of warmed DMEM to dilute the freezing media. This diluted suspension was spun at 400 x g for 4 min to wash the cells. The pellet was then resuspended, counted and suspended at 2 x 10$^6$ cells/ml for either tumor cell injection or for tissue culture experiments.

4) Transplanting Tumor Cells

For tumor cell injection, the cell suspension was adjusted to 2 x 10$^7$ cells/ml in DMEM. The total volume of tumor cell suspension required for injection of 2 x 10$^6$ tumors cells was 100 µl x the number of mice injected + 100 µl to allow for retention of fluid in the head of the syringe. The mice were lightly anesthetized with ether and then injected s.c. into the interscapular region with 100 µl of tumor cell suspension.

5) Monitoring Tumor Growth

For tumor propagation, mice were palpated after tumor injection, beginning on 8-10 d when a palpable tumor generally first appears and terminating at 18-21 days, when tumor size was between 2 and 3 g. When tumors were measurable, caliper measurements were taken
every second day and tumor weights calculated according to the formula (Simpson-Herrin and Lloyd, 1970):

\[
\frac{\text{length (cm)} \times [\text{width (cm)}]^2}{2} = g
\]

B. In Vitro Experiments

1) Culturing Tumor Cells

Selected vials of frozen SC115 cells were thawed and pooled together with an equal volume of warmed DMEM to dilute the freezing media. This diluted suspension was spun at 400 \( \times g \) for 4 min and the pellet was resuspended in 10 ml of DMEM. This wash step was repeated twice to rinse off freezing medium. Cells were then counted and resuspended in 10 ml of phenol red-free DMEM/Ham's Nutrient Mixture F12 (F12, 1:1, Sigma-Aldrich Canada Ltd.) in Hepes Buffer (DMEM/F12/H) medium containing 2% dextran charcoal-treated fetal bovine serum (DCTFBS, appendix I). The cells were plated overnight onto 10 cm\(^2\) non-tissue culture plastic Petri dishes (Sigma-Aldrich Canada Ltd., Oakville, Canada) in 25 ml of DMEM/F12/H + 2% DCTFBS medium at 37°C (95% air: 5% CO\(_2\)). The next day, the cell suspension was centrifuged and the supernatant decanted to eliminate non-viable cells. The cells were then counted and seeded at a density of \(2.7 \times 10^4\) cells/cm\(^2\) onto collagen-coated 75 cm\(^2\) tissue culture flasks (Corning, Fischer Scientific, Vancouver, Canada) in 10 ml of DMEM/F12/H + 2% DCTFBS medium. The following day, once cells were attached, 10 ml of test media consisting of serum-free (SF) medium [phenol red-free DMEM/F12/H medium with 1.0 mg/ml bovine serum albumin (BSA; Sigma-Aldrich Canada Ltd., Oakville, Canada)
and 0.1 μg/ml insulin (Sigma-Aldrich Canada Ltd., Oakville, Canada), with or without steroid hormones, were added.

2) Heat Shock Treatment

In each experiment, one flask of SC115 cells growing in SF medium were treated with heat shock (HS) to cause upregulation of HSP levels and serve as a positive control for HSP examination. HS consisted of treating the cells with a rise in temperature for 1 hour in a 42°C water bath and then allowing for a 5 hour recovery in the 37°C incubator (95% O₂:5% CO₂) for HSP production (Ritossa, 1951).

3) Steroid Hormones and Growth Factor

DHT, HC and E₂ (Sigma-Aldrich Canada Ltd., Oakville, Canada) were prepared by dissolving in 95% ethyl alcohol (EtOH, Stanchem Inc., Vancouver, Canada) in 10, 5 and 2 mg/ml stock solutions, respectively, serially diluted in DMEM/F12/H medium to working solutions of 100, 500 and 20 μg/ml and aliquoted at 1 ml/tube into polypropylene tubes (1.8 ml size; Falcon, Lincon Park, NJ, USA). Stock solutions were stored at 4°C in the dark and working solutions were made fresh for every media change of each experiment. The final concentration of EtOH in the culture medium was less than 0.05%. Basic fibroblast growth factor (bFGF, Sigma-Aldrich Canada Ltd., Oakville, Canada) was diluted to a 10 μg/ml stock solution in DMEM/F12/H medium containing 1% BSA (pH 7.0 -7.5), aliquotted to 100 μl/tube and stored at -20°C. One tube was used for each experiment and a fresh working solution of 1 μg/ml was prepared for every media change. Experiments were repeated in triplicate, except for E₂ experiments which were duplicated.
The hormone concentrations chosen were those shown previously to provide maximal stimulation or inhibition of SC115 growth (Jiang et al., 1995). The experimental concentrations of the steroid hormones and bFGF in the final volume of 10 ml of SF media in each 75 cm² tissue culture flask were as follows: 35 nM for DHT, 1 µM for HC, 10 nM for E₂ and 10 ng/ml for bFGF.

4) The Ethanol Control

Although an EtOH control is always conducted, it was particularly important for these experiments since ethanol (EtOH) is a known inducer of HSPs at higher concentrations (Welch, 1992). A control examined for HSP induction contained the same amount of EtOH in the final volume of test medium (used to dilute steroid hormones). The same protocol to prepare the steroid hormones for experiments was followed for the EtOH control, except that no hormones were added. One hundred µl of 95% EtOH was added to 900 µl DMEM/F12/H medium for a stock solution and was stored at 4°C. A fresh stock solution was made for each experiment. A working solution was prepared by serial dilution to give a final EtOH concentration of 0.05% in 10 ml of SF medium. Experiments using medium with EtOH were initiated with experiments using media containing steroid hormones.

5) Harvesting Cultures

Once cultures reached approximately 80% confluence, they were terminated and cells were harvested for HSP examination. Test media from the 75 cm² culture flasks were decanted and cell monolayers were washed once with 10 ml of SF medium. A fresh 10 ml of SF medium was added into the 75 cm² flasks and cell monolayers were scraped with a rubber
policeman (Cell Scraper III, Costar Corporation, Cambridge, USA). The SF media containing the scraped cells were collected and spun at 400 × g to collect the cells. The supernatant was decanted and the pellets were placed on ice for cell lysis (described below) and HSP examination.

C) Animal Model

Mice were housed in polycarbonate cages (19 × 29 × 13 cm) with stainless steel lids and corn cob bedding and received food (bedding and food; Jamieson's Pet Food Distributors, Ladner, Canada) and water ad libitum. Cages were placed on stainless steel racks in a room with a 12 h dark/light cycle (0700 h - 1900 h). The room was relatively free of extraneous building noise and remained at a constant temperature of 22°C.

Following weaning at 3 weeks of age, male mice were housed individually (I) or in sibling groups of 3 (G). When the mice were 2 to 4 months of age, they were injected s.c. in the interscapular region with a single cell suspension of 2 × 10^6 cells in 100 μl of DMEM (tumor groups) and housed as follows:

1) GG - males raised in sibling groups of 3 remained in their sibling groups for the experiment.
2) GI - males raised in sibling groups of 3 were rehoused as individuals for the experiment.
3) IG - males raised individually housed were placed in non-sibling groups of 5 animals for the experiment.

Animals in all groups were subjected to a daily novelty stressor consisting of exposure, 15 min/d, 5 d/wk (prior to 1200 h), to 1 of 5 novel environments. The exposure to novel environments followed a set order of rotation as follows:
1) a round clear plastic jar 9 cm in diameter and 7 cm high with a white plastic screw top lid,
2) a polypropylene box $12 \times 10 \times 4$ cm with a lid,
3) a covered cardboard box with divisions forming compartments $7 \times 7 \times 14$ cm high,
4) a plastic cup (220 ml - 10 cm in height and top diameter of 6.5 cm, base diameter of 4.75 cm) with lid,
5) a clean cage (empty of bedding, food or water bottle) with a standard cage top.

All lids had holes punched in them for adequate ventilation.

D) In Vivo Experiments

1) Social Housing Condition Protocol

One hundred and twenty-four male DD/S mice were used in this experiment. One hundred and twelve mice (GI, n=36, GG, n=36, IG, n=40) received daily novelty stress beginning the day following tumor cell injection and a housing condition change and an additional 12 mice (GGNS, n=12) remained in the standard housing condition and did not receive daily novelty stress. When an experimental housing group was near the target tumor weight (either 0.8 g or 3 g), palpation and tumor weight calculation was done before the daily novelty stressor. Only groups that did not reach target weight were then stressed in the novel environments. The other groups were terminated so that tumor samples were taken at least 24 hours post daily novelty stress.

Mice were palpated twice weekly and once tumors were palpable, 8-10 d, caliper measurements were taken every other day. Tumor weights were calculated and when the average tumor weight of an experimental housing group reached either 0.8 g or 3 g (for GI
animals the average tumor weight of the housing group was calculated from the animals in the original rearing group of 3), the mice were terminated by over-halthanization and tumor tissue was removed. For the 3 g tumors, tumor tissue was minced, added to freezing medium, frozen and stored in LN$_2$. This procedure is known to keep cells viable and the frozen cells can be later thawed and either homogenized for stress protein examination or possibly used in future tissue culture experiments. After all the 3 g tumors were collected, 1 frozen vial from each tumor sample (which contained approximately 1g of tumor tissue) was thawed, washed twice in DMEM, the tumor tissue pelleted and DMEM decanted. The tumor tissue was then ready for homogenization and HSP25, 70 and 90 examination. For the 0.8 g tumors, all tumor tissue was frozen immediately in LN$_2$ and when all of the samples were collected, tumor tissue was thawed and homogenized for HSP25, 70 and 90 examination.

2) 3T3 Cells

3T3 cells (American Type Culture Collection, Rockville, USA) are a fibroblast cell line that grow easily and rapidly in culture. After thawing 1 vial (1 x 10$^7$ cells) of 3T3 cells, the pellet was washed twice in warmed DMEM and resuspended in 12 ml DMEM + 5% CS. A 1:6 dilution of one vial of 3T3 cells was made into 6, 75 cm$^2$ flasks, containing DMEM + 5% CS. Medium was changed every third day until cultures reached confluence at which time they were treated with HS and harvested for HSP examination. Heat shocked 3T3 (3T3/HS) samples were used as a positive control for HSPs in the in vivo SC115 studies. (Both SC115 and 3T3 samples showed upregulation of HSPs in response to HS treatment. Because 3T3 cells are easy to culture and can be processed in bulk, they were used as the positive control
for all of the in vivo experiments, whereas for the in vitro experiments, one extra flask of SC115 cells in SF medium was cultured for HS treatment and was used as the positive control.)

E) HSP Examination

Tumor samples were examined for HSP25, 70 and 90 expression by western blotting. As described above, samples were derived from both in vitro and in vivo experiments.

1) Cell Lysis and Protein Isolation

SC115 samples were lysed either by a freeze/thaw (F/T) or homogenization method. The F/T method was used for cell lysis in the in vitro experiments, which generated pellets of cells, and homogenization was used for cell lysis in the in vivo experiments, which generated tumor tissue. An experiment comparing HSP25, 70 and 90 expression in dissociated SC115 cells from animals in the standard housing condition lysed by the F/T or homogenization methods found no differences in expression of any HSP using either method.

Cell pellets from in vitro experiments were added 1:1 (volume) to a phosphate-buffered saline (PBS, Appendix II) buffer, pH 7.2, containing 2 mM phenylmethylsulfonylfluoride (PMSF, Sigma-Aldrich Canada Ltd., Oakville, Canada), frozen immediately in LN$_2$, thawed quickly in a 37°C water bath and vortexed at maximum speed for 3 bursts of 30 sec. This procedure was repeated 3 times and samples were either centrifuged
(described below) while thawed or stored frozen in a -70°C freezer until further HSP analysis.

Tumor tissue from *in vivo* experiments were homogenized on ice in a Hepes buffer containing 1% Triton X-100 (Sigma-Aldrich Canada Ltd., Oakville, Canada), 5 nM EDTA, and protease inhibitors (2 mM PMSF, 1 TIU/ml aprotinin, 60 µM pepstatin, 60 µM leupeptin; all purchased from Sigma-Aldrich Canada Ltd., Oakville, Canada). Homogenization (TRI-R STIR-R, gift from the late Dr. L. Jasche) was done on setting 5, for 3 bursts of 15 seconds. The homogenate was then centrifuged (RT 6000D Sorval Centrifuge, Mandel Scientific Co., Guelph, Canada) at 8000 × g force for 35 min at 4°C and supernatants were collected and stored in a -70°C freezer (Forma Scientific, Fischer Scientific, Vancouver, Canada).

2) Protein Assay

Total protein concentrations for each sample was determined by the Bio-Rad protein assay (Bio-Rad, Mississauga, Canada; Lowry Method, Lowry *et al.*, 1951). A set of known standards were made using concentrations of BSA diluted in distilled water at 0.1, 0.5, 1.0 and 2.0 mg/ml. Blanks containing homogenization buffer (Hepes buffer and protease inhibitors) were used to quantitate the protein contributed by the proteins in the homogenization buffer. Five µl of standards, blanks and samples were pipetted in duplicate into a 96-well tissue culture plate (Falcon, VWR/Canlab, Vancouver, Canada). Twenty-five µl of reagent A' (for detergent-based homogenization buffers) and 200 µl of reagent B from the kit were added to each sample as instructed. After 15 min, absorbances were read at a wavelength of 750 nm in a microplate reader (Bio-Tek Instruments, Mandel Scientific
Company Ltd., Guelf, Canada). A standard curve plotting the known concentrations of BSA against their absorbances was calculated and from the slope of the line and the y-intercept, a formula to calculate the concentrations of the samples was found. If any sample concentration lay outside the standard curve (0.1 - 2.0 mg/ml), all samples were diluted 1:10 in homogenization buffer and the protein assay was repeated.

The concentrations of the samples were calculated, an aliquot to deliver 300 µg of total protein was determined and added to 100 µl of sample buffer (SB, Appendix II) to give a 3 mg/ml stock for each sample. Experimental samples in SB were stored at -20°C or -70°C for future HSP examination.

3) SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Pilot gels with heat shocked 3T3 and SC115 cells grown in vitro were run on a mini-gel (Bio-Rad Laboratories, Mississauga, Canada) to determine the amount of total protein to load per well. Fifty µg and 30 µg of total protein for the in vitro or in vivo experiments, respectively, were found to be the optimal amounts to load in each well of a 10 or 15 well mini-gel. It was found that more total protein was needed from the in vitro samples to visualize the HSP bands. Equal amounts of total protein was then loaded into a 12.5% SDS-PAGE gel and transferred onto a supported nitrocellulose membrane with a pore size of 0.22 µM (NC; Micron Separations Inc., Westboro, MA). Protocols and recipes (stacking, separating, sample, running, transfer and washing buffers) for western blotting were taken from the Bio-Rad Instruction Manual (Appendix II).
Protein transfer from the gel to the NC membrane was done at a constant voltage (Bio-Rad Power Pac 1000, Bio-Rad, Mississauga, Canada) of 100 volts (V) for 1 hour at 4°C or 30 V overnight at 4°C (Bio-Rad manual). NC was then stained with ponceau S (Appendix II) to visualize protein transfer and destained during membrane blocking by a 3% BSA solution in tris-buffered saline and 0.05% Tween-20 (TBS-T, Appendix II) for a minimum of 1 hour at room temperature or up to overnight at 4°C. BSA binds to any protein-free area on the membrane and avoids non-specific antibody binding. The NC was washed in TBS-T for 15 min, 3 times (3 × 15 min), incubated with a specific HSP primary antibody diluted in TBS-T at concentrations of 1:5,000 for HSP25 and 70 and 1:10,000 for HSP90 (all purchased from StressGen, Victoria, Canada) for 1 hour at room temperature and washed 3 × 15 min in TBS-T. The NC was then incubated with secondary antibodies (HSP25 and 70, anti-rabbit and HSP90, anti-rat, Dako Diagnostics Canada Inc., Mississauga, Canada) again diluted in TBS-T to concentrations of 1:5,000 for 1 hour and washed 2 × 15 min in TBS-T and 1 × 15 min in TBS (no Tween). Finally, HSP bands were detected by enhanced chemiluminescence (ECL; Amersham Canada Ltd., Oakville, Canada) on X-Omat-AR film (Kodak, Vancouver, Canada). A volume of fresh ECL reagents was mixed in a 1:1 ratio, enough to cover the NC. After a 1 min exposure, the NC was drained of excess ECL solution, placed onto a glass plate and covered with plastic wrap for autoradiography. In a darkroom, the NC was put in a developing cassette and X-ray film was placed on top of the NC. Exposure times ranged from 1 min to 30 min.
4) HSP Qualitation

HSP bands on the X-ray films were scanned (Epson ES-1200C, Tracer Computers Center, Vancouver, Canada) into Adobe Photoshop (version 3). The area and density of each band were analyzed by NIH image densitometry (version 1.61).

5) Statistical Analysis

SC115 tumors growth rates for mice in GI, GG and IG housing conditions were analyzed separately for the 0.8 g and 3 g tumors by 2-way ANOVAs for the factors of Housing Condition and Day. Expression of HSP25, 70 and 90 in SC115 tumors all subjected to daily novelty stress were analyzed by 2-way ANOVAs for the factors of Tumor Size and Housing Condition. Expression of HSP25, 70 and 90 in SC115 tumors from group housed mice were analyzed by 2-way ANOVAs for the factors of Tumor Size and Daily Novelty Stress. For all ANOVAs, significant main effects and interactions were further analyzed by Tukey's post hoc tests.
CHAPTER 4: RESULTS:

A) SC115 Tumor Growth

SC115 tumor growth rates were examined in mice from different social housing conditions. Consistent with our previous data, for the 3 g tumors, the ANOVA revealed significant main effects of Housing Condition \[F(2,51)=117.686; \ p<0.001\] and Day \[F(3,153)=335.246; \ p<0.001\], and a significant Housing Condition by Day interaction \[F(6,153)=58.256; \ p<0.001; \text{Figure 3}\] on tumor growth rate. Post hoc analysis indicated that tumor growth rates on days 15-16 and 17-18 post tumor cell injection and housing condition change were significantly faster in GI compared to GG and IG mice, and were significantly faster in GG compared to IG mice (GI>GG>IG, \ p's<0.001). For days 11-12 and 13-14, tumor growth rates were significantly faster in GI and GG males compared to IG males (GI=GG>IG, \ p's<0.01).

Similarly, for the 0.8 g tumors, significant main effects of Housing Condition \[F(2,52)=44.345; \ p<0.001\] and Day \[F(1,52)=46.813; \ p<0.001\] and a significant Housing Condition by Day interaction \[F(2,52)=15.349; \ p<0.001; \text{Figure 4}\] were observed. Tumor growth rates on days 11-12 and 13-14 post tumor cell injection and housing condition change were significantly faster in GI mice compared to GG and IG mice, and were significantly faster in GG compared to IG mice (GI>GG>IG, \ p's<0.001).
Figure 3. *SCI15 Tumor Growth Rates in Mice in Different Social Housing Conditions (3 g Tumors).* Points represent mean ± SEM. For 3 g tumors, tumor growth rates were significantly slower in mice in the IG condition compared to mice in the GG and GI conditions on days 11-12 and 13-14 post tumor cell injection (GI=GG>IG, p's<0.01; * on graph) and tumor growth rates were significantly slower in mice in the IG condition compared to mice in the GG and GI conditions, and GG compared to GI conditions on days 15-16 and 17-18 post tumor cells injection (GI>GG>IG, p's<0.001; + on graph).
MEAN TUMOR WEIGHT (g)

DAYS POST TUMOR CELL INJECTION

- GI
- GG
- IG
Figure 4. *SC115 Tumor Growth Rates in Mice in Different Social Housing Conditions* (0.8 g Tumors). Points represent mean ± SEM. For the 0.8 g tumors, tumor growth rates were significantly slower in mice in the IG condition compared to mice in the GG and GI conditions, and GG compared to GI conditions on days 11-12 and 13-14 days post tumor cell injection (GI>GG>IG, p’s<0.001; + on graph).
B) *In Vitro* Experiments

This set of experiments examined whether the steroid hormones DHT, HC and E₂, and the growth factor bFGF would alter the levels of HSP25, 70 and 90 in SC115 cells *in vitro*. Neither bFGF nor the EtOH control had any effect on the levels of any HSP compared to the SF control (Figure 5A). The physiological concentration of DHT (35 nM) and the pharmacological concentration of HC (1 μM) upregulated HSP25 levels compared to a SF control without steroid hormones, whereas levels of HSP70 and 90 were unchanged (Figures 5B and 5C). Conversely, the physiological concentration of E₂ (10 nM) upregulated HSP90 levels compared to the control, whereas levels of HSP25 and 70 were unchanged (Figure 5D).
Figure 5. *Representative Western Blot of HSP25, 70 and 90 Expression in SC115 Cells In Vitro.* A) HSP expression in SC115 cells in serum-free medium (SF), in SF medium with <0.05 % EtOH, in SF medium with HS and in SF medium with 10 ng bFGF. There were no differences in HSP25, 70 and 90 expression in the EtOH and bFGF treated SC115 cells compared to the SF control. HS caused upregulation of HSP25 and 70, but not HSP90, compared to the SF control. B) HSP expression in SC115 cells in SF medium, in SF medium with HS and in SF medium with 35 nM DHT. DHT caused upregulation of HSP25 expression compared to the SF control, HSP70 and 90 were unchanged. C) HSP expression in SC115 cells in SF medium, in SF medium with HS and in SF medium with 1 μM HC. HC caused upregulation of HSP25 compared to the SF control, HSP70 and 90 were unchanged. D) HSP expression in SC115 cells in SF medium, in SF medium with HS and in SF medium with 10 nM E\textsubscript{2}. E\textsubscript{2} caused upregulation of HSP90 compared to the SF control, HSP25 and 70 were unchanged. Molecular weights (MW) of HSP bands were compared to the known MW standards (prestained rainbow MW markers, Bio-Rad, Mississauga, Canada). HSP70 blots were reprobed to determine HSP90 expression, and therefore, HSP90 blots have a second visible band at 70 kDa, which is HSP70 from the first immunolabeling.
C) *In Vivo* Experiments

1) HSP Expression in SC115 Tumors in Mice in Different Housing Conditions

Figure 6 is a representative western blot with both 0.8 g and 3 g tumor samples from mice in different housing conditions (GI, GG and IG). In these experiments, all animals were subjected to daily novelty stress. Statistical analyses were done on HSP25, 70 and 90 levels in all tumor samples and these results, expressed as relative optical density units, are shown in Figure 7. Significant differences in the expression of HSP25, 70 and 90 in SC115 tumors taken from mice in GI, GG and IG housing conditions were found. Significant main effects of Housing Condition were observed for both HSP25 [F(2,68)=36.777; p<0.001; Figure 7A] and HSP70 [F(2,67)=30.336; p<0.001; Figure 7B] expression. Post hoc analyses for both the 0.8 g and 3 g tumors indicated that tumors from mice in the IG condition had higher expression of HSP25 and HSP70 (p’s<0.001) compared to tumors from mice in the GG and GI conditions. Analysis of HSP90 expression revealed significant main effects of Tumor Size [F(1,66)=9.076; p<0.01; Figure 7C] and Housing Condition [F(2,66)=16.463; p<0.001; Figure 7C] as well as a significant Tumor Size by Housing Condition interaction [F(2,66)=7.542; p<0.01; Figure 7C]. For both the 0.8 g and 3 g tumors, tumors from mice in the IG condition had higher expression of HSP90 compared to tumors from mice in the GG and GI conditions (p<0.001). Furthermore, HSP90 expression was significantly higher in 0.8 g tumors compared to 3 g tumors, in mice in the IG housing condition (p<0.001).
Figure 6. *Representative Western Blots of HSP25, 70 and 90 Expression in 0.8 g and 3 g SC115 Tumors in Mice in Different Housing Conditions.* These blots show representative SC115 tumor samples from mice in different housing conditions for both the 0.8 g and 3 g tumors. The housing conditions included a change in housing condition at the time of tumor cell injection, group to individual (GI), individual to group (IG) and group remaining in a group (GG). All animals were subjected to daily novelty stress. A) SC115 samples from 0.8 g tumors. Tumors from mice in the IG condition had greater expression of HSP25, 70 and 90 compared to tumors from mice in the GG and GI conditions. B) SC115 samples from 3 g tumors. Tumors from mice in the IG condition had greater expression of HSP25, 70 and 90 compared to tumors from mice in the GG and GI conditions. HSP90 expression was greater in 0.8 g tumors compared to 3 g tumors from IG mice. The positive control was a HS sample of 3T3 cells (3T3/HS). HSP70 blots were reprobed to determine HSP90 expression, and therefore, HSP90 blots have a second visible band at 70 kDa, which is HSP70 from the first immunolabeling.
Figure 7. HSP25, 70 and 90 Expression in 0.8 g and 3 g SC115 Tumors in Mice in Different Housing Conditions. A) Tumors from mice in the IG condition had greater expression of HSP25 compared to tumors from mice in the GG and GI conditions for both 0.8 g and 3 g tumors (p's<0.001). B) Tumors from mice in the IG condition had greater expression of HSP70 compared to tumors from mice in the GG and GI conditions for both 0.8 g and 3 g tumors (p's<0.001). C) Tumors from mice in the IG condition had greater expression of HSP90 compared to tumors from mice in the GG and GI conditions for both 0.8 g and 3 g tumors (p's<0.01). HSP90 expression was greater in 0.8 g tumors compared to 3 g tumors in mice in the IG housing condition (p<0.001).
2) HSP Expression in SC115 Tumors From Group Housed Mice Subjected to Daily Novelty Stress

Figure 8 is a representative western blot with both 0.8 g and 3 g tumor samples from mice housed in the standard housing condition (no change in housing condition and not subjected to daily novelty stress, GGNS) and mice that were group housed and subjected to daily novelty stress (GGS). Statistical analyses were done on HSP25, 70 and 90 levels in all tumor samples and these results, expressed as relative optical density units, are shown in Figure 9. The ANOVAs revealed a significant main effect of Daily Novelty Stress for HSP70 in both 0.8 g \([F(1,17)=14.700; p<0.01; \text{Figure 9B}]\) and 3 g \([F(1,17)=20.792; p<0.001; \text{Figure 9B}]\) tumors and for HSP90 in 3 g tumors only \([F(1,17)=8.430; p<0.01; \text{Figure 9C}]\). There were no significant differences for HSP90 in 0.8 g tumors or for HSP25 for either tumor weight. In general, daily novelty stress caused a decrease in HSP expression in tumors from group housed animals.
Figure 8. *Representative Western Blots of HSP25, 70 and 90 Expression in 0.8 g and 3 g SC115 Tumors from Group Housed Mice Subjected to Daily Novelty Stress.* These blots show representative SC115 samples from both 0.8 g and 3 g tumors from mice in the standard housing conditions (no change in housing condition and not subjected to daily novelty stress, GGNS) and mice that were group housed and subjected to daily novelty stress (GGS). A) SC115 samples from 0.8 g tumors. Tumors from mice in the GGNS condition had greater expression of HSP70 and 90 compared to tumors from mice in the GGS condition. There were no differences in HSP25 expression in tumors from mice in the GGNS and GGS conditions. B) SC115 samples from 3 g tumors. Tumors from mice in the GGNS condition had greater expression of HSP90 compared to tumors from mice in the GGS condition. There were no differences in HSP25 or 70 expression in tumors from mice in the GGNS and GGS conditions. The positive control was a HS sample of 3T3 cells (3T3/HS). HSP70 blots were reprobed to determine HSP90 expression, and therefore, HSP90 blots have a second visible band at 70 kDa, which is HSP70 from the first immunolabeling.
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<tr>
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<tr>
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<td></td>
<td>3T3/HS</td>
<td>GGNS</td>
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Figure 9. HSP25, 70 and 90 Expression in 0.8 g and 3 g SC115 Tumors from Group Housed Mice Subjected to Daily Novelty Stress. A) There were no differences in HSP25 expression in both 0.8 g and 3 g tumors from mice in the standard housing condition (no change in housing condition and not subjected to daily novelty stress, GGNS) compared to tumors from mice that were group housed and subjected to daily novelty stress (GGS). B) Tumors from mice in the GGNS condition had greater expression of HSP70 compared to tumors from mice in the GGS condition for both tumor weights examined, 0.8 g and 3 g (p<0.01). C) Tumors from mice in the GGNS condition had greater expression of HSP90 compared to tumors from mice in the GGS condition in 3 g tumors (p<0.01). No differences in HSP90 expression in the 0.8 g tumors from mice in GGNS and GGS conditions were found.
CHAPTER 5: DISCUSSION AND CONCLUSIONS:

Our previous data suggest that psychosocial stressor-induced alterations in both the endocrine and immune systems are likely playing a role in the differential SC115 tumor growth rates observed in mice in different housing conditions. The present study extended our investigations to the molecular level in order to identify a potential mechanism underlying these differential tumor growth rates, i.e. the altered expression of a group of stress-responsive proteins, the heat shock proteins (HSPs). We hypothesized that HSP expression in SC115 cells may be altered by: a) steroid hormone and growth factor exposure in vitro, and b) different social housing conditions in vivo. Altered levels of HSPs may in turn affect the way the endocrine and immune systems interact with the SC115 cells, resulting in the differential tumor growth rates observed in our animal-tumor model.

In vitro, physiological levels of DHT and pharmacological levels of HC were found to upregulate the expression of HSP25, whereas there were no changes in expression of HSP70 or 90. Conversely, physiological levels of E2 were found to upregulate expression of HSP90, whereas the expression of HSP25 and 70 remained unchanged. The growth factor, bFGF, did not alter expression of any HSP. In vivo, expression of HSP25, 70 and 90 were upregulated in tumors from IG animals compared to tumors from GG and GI animals at both tumor weights examined (0.8 g and 3 g). Interestingly, daily novelty stress in itself decreased expression of both HSP70 (0.8g and 3 g tumors) and HSP90 (3 g tumors only) in mice that were group housed (GGS) compared to that in mice in the standard housing conditions (GGNS). Although cellular HSP expression has been shown to change in response to acute and toxic stressors such as exposure to heat, chemicals and steroid hormones (reviewed in
Welch, 1992), this study is the first to report that a psychosocial stressor, a change in social housing condition, can induce differences in HSP expression.

Previous studies have shown that steroid hormones can regulate HSP expression in vitro. E₂, but not DHT or HC, was found to upregulate HSP25 in the MCF-7 breast cancer cell line (Edwards et al., 1981). In contrast, in SC115 cells, we found that DHT and HC upregulate HSP25, whereas E₂ upregulates HSP90. Interestingly, in both the Edwards et al., (1981) study and the present study, the steroid hormones that stimulate growth also induce HSP25 expression. However, steroid hormone-induced HSP25 expression is likely not due only to growth stimulation, as both studies demonstrated that other growth stimulators such as serum and bFGF do not induce HSP25.

In addition to the finding that steroid hormones regulate HSP expression in vitro, data have also shown that HSPs may regulate the activity of steroid hormone receptors (Edwards et al., 1992, Kost et al., 1989, Picard et al., 1990 and Sanchez et al., 1994). HSP70 and 90 bind to inactive steroid receptors (Catelli et al., 1985) and, following upregulation by heat shock, have been shown to alter the activity of the progesterone (Edwards et al., 1992 and Kost et al., 1989) and glucocorticoid receptors (Picard et al., 1990 and Sanchez et al., 1994). Thus, in the present study, stress-induced changes in HSPs could play a role in modulating differential tumor growth rates through an influence on steroid hormone receptor activity.

Previous studies have also found that in vivo both steroid hormones and various types of stressors can alter HSP expression (Ramachandran, 1988, Udelsman et al., 1994, Blake et al., 1995 and Fukudo et al., 1995). Increased HSP90 expression in murine uteri was found in response to treatment with E₂ after ovariectomy (Ramachandran, 1988). A decrease in
adrenal HSP70 expression was found after a 2 week dexamethasone (a synthetic glucocorticoid) treatment followed by an acute restraint stressor (Udelsman et al., 1994). A follow up to the Udelsman et al., 1994 experiment found that after an 8 week intermittent restraint stressor, there was a decrease in adrenal HSP70 expression in response to an acute restraint stressor (Blake et al., 1995). Finally, another study found increased cerebral and gastric HSP70 expression following a 12 hour exposure to a water-restraint stressor (Fukudo et al., 1995). Thus, these studies have all found associations between various types of in vivo stressors, steroid hormone levels and HSP expression.

The regulation of increased or decreased HSP levels in response changes in steroid hormone levels or different types of in vivo stressors is not clear. For example, activation of the heat shock transcription factor (HSF) is known to increase HSP expression (Morimoto, 1992, 1993 and Voellmy, 1994), but little is known about what may decrease HSP expression in vivo. Possible regulatory mechanisms by which HSP expression could be decreased are: a) an inhibitory transcription factor that is yet to be discovered, b) HSP mRNA stability or translation may be decreased or c) increased degradation of HSPs in the cytoplasm. Exact mechanisms leading to both increases and decreases in HSP expression remain to be elucidated.

In the present study, we examined the expression of HSPs in vivo in SC115 tumor cells in animals that were exposed to a psychosocial stressor shown to alter plasma levels of steroid hormones, specifically testosterone and corticosterone. HSP25, 70 and 90 were elevated in tumor cells from mice in the IG condition compared to tumor cells from mice in the GG and GI conditions and thus, tumor cells from mice in the IG condition may be
considered to be more “stressed” at the cellular level. Considering that we found that specific steroid hormones upregulate specific HSPs in SC115 cells \textit{in vitro}, and that \textit{in vivo} all HSPs were upregulated in tumors in the IG condition, the induction of multiple HSPs \textit{in vivo} is more likely due to activation of the HSF, which binds to heat shock elements located in the promoter regions of all the HSP genes (Mirault \textit{et al.}, 1982, and reviewed in Morimoto, 1992, 1993 and Voellmy, 1994), than through specific steroid hormone regulation of HSPs. Additional studies are required to determine the regulation HSP expression in SC115 cells from mice in different social housing conditions.

In addition to understanding how HSPs are regulated in SC115 cells \textit{in vivo}, it is important to consider the consequences of elevated levels of HSPs in relation to tumor growth. To date, it is unclear how elevated levels of HSPs in human tumors relate to patient prognosis. High levels of HSP27 (human equivalent of murine HSP25) are correlated with a better prognosis in patients with malignant fibrous histocytoma (Fuller, 1994). In contrast, elevated levels of HSP70 are correlated with a shorter disease-free survival in breast cancer patients with negative auxiliary lymph nodes (Ciocca, 1993). In our model, mice in the IG condition, which have the slowest growing tumors, have the highest expression of HSPs, suggesting that a high level of HSPs in SC115 cells is related to a better outcome. This relationship between high HSP expression and slow tumor growth rate can be related to events at a cellular level. For example, stressed cells and tissues will upregulate levels of HSPs and stop production of cellular proteins concerned with cell division. Studies examining HSP expression and proliferation are needed.

Based on the assumption that high HSP expression is related to slow tumor growth, there are a number of ways different HSPs may be playing a role in altering tumor growth rate...
in our animal-tumor model. High levels of HSPs in SC115 cells from mice in the IG condition may be a result of an enhanced immune response to the housing condition, resulting in a greater cellular stress response and a slower tumor growth rate. High levels of HSP70 family members in SC115 cells from mice in the IG condition may present tumor antigens more efficiently, which would lead to greater SC115 tumor immunogenicity and a slower tumor growth rate in IG mice. Elevated HSP levels in tumors from mice in the IG condition, especially HSP70 and 90, could also result in increased binding to inactive steroid hormone receptors, and possibly decrease the growth stimulatory effect of steroid hormones. Finally, elevated levels of HSP25, which help to stabilize actin polymers and are important in cytoskeletal maintenance (Ciocca, 1993), may affect the way the SC115 tumor cells respond to their surrounding environment.

The relationship between high HSP expression and slow tumor growth rate is made more complex by examination of HSP expression and tumor growth rates in the other housing conditions (GI and GG) and in the GG condition in response to daily novelty stress. For example, tumors from mice in the GI condition grow at a faster rate than tumors from mice in the GG condition, yet no differences in HSP expression in tumors from the GI and GG conditions were found. In addition, mice in the standard housing condition (GGNS) and group housed mice subjected to daily novelty stress (GGS) have tumors growing at similar rates yet significant differences in HSP expression were found. Thus, our data indicate both that tumors growing at different rates may show no differences in HSP expression, and tumors growing at the same rates may show differential expression of HSPs. More studies are needed to examine these complex relationships among HSP expression, tumor growth rate and the effects of daily novelty stress in our animal-tumor model.
Other researchers have also studied the relationship between HSP expression and tumor growth rate. One study examined the expression of HSP27 and 70 in human breast tumors in relation to proliferating cell nuclear antigen (PCNA), silver staining nuclear organizer regions (AgNORs) and association with the mitotic spindle (Vargas-Riog et al., 1997). Using PCNA, and AgNOR staining, they found an inverse correlation between HSP27 expression and cell proliferation, a positive correlation between HSP70 expression and proliferation and that HSP70 was clearly associated with the mitotic spindle. Their conclusions from these data are that HSP27 is likely involved in cell growth arrest and differentiation while HSP70 is likely involved with cell proliferation.

A common assumption is that HSP expression is a good indicator of HSP function. However, this is not always the case. For example, HSP25 can be phosphorylated to alter its function (Huot et al., 1995 and Guay et al., 1997) and HSP70 in different cellular locations alters its function (Multhoff et al., 1997). The location and function of HSP70 may be of particular interest to our research. It has been found that the sensitivity of tumor cells to lysis mediated by a subset of NK cells correlates to HSP70 cell surface expression (Multhoff et al., 1997). Thus, it may be the that the location of HSP70 rather than its expression is altered by stress, resulting in a change of function.

Finally, it has been reported that two stress proteins, glucose-regulated proteins of 78 kDa and 94 kDa (GRP78 and 94), are upregulated in large murine sarcomas (Cai et al., 1993). GRP78 and 94 are upregulated when cells are stressed due primarily to metabolic stressors such as glucose and/or oxygen deprivation (Lee et al., 1984). As a tumor grows, inadequate delivery of nutrients and/or oxygen may occur in some areas within the tumor. We observed a higher expression of HSP90 in the 0.8 g tumors compared to the 3 g tumors and
no differences in HSP25 and 70 expression in 0.8 g and 3 g tumors. A possible role for altered GRP regulation in mediating differential SC115 growth rates in our animal-tumor model remains to be examined.

In summary, we found that HSP25, 70 and 90 expression can be altered by the steroid hormones DHT, HC and E2 in vitro and by social housing conditions in vivo. In particular; a) SC115 cells from mice in the IG condition, which have the slowest growing tumors, were found to have the highest level of expression of HSP25, 70 and 90 compared to mice in the GG and GI conditions, b) HSP90 expression was decreased in 3 g tumors compared to 0.8 g tumors in mice in the IG housing condition and c) daily novelty stress caused a decrease in HSP70 (in both 0.8 g and 3 g tumors) and HSP90 (in 3 g tumors only) expression in group housed mice. These data raise a number of interesting issues. Future studies will focus on possible mechanisms underlying the regulation of HSP expression in SC115 cells in vivo (heat shock factor expression), how HSPs responds to immune factors in our model, the possible role of GRPs (which may be more sensitive to stressors experienced in growing tumors) in SC115 tumor growth and, importantly, how these altered levels of HSPs may play a role in the modulation of different SC115 tumor growth rates in animals in different housing conditions (HSP cellular locations and states of phosphorylation).
CONCLUSIONS:

1) The steroid hormones DHT, HC and E₂, which are known to regulate SC115 growth, were found to alter the expression of HSP25, 70 and 90 following direct exposure in vitro, whereas, bFGF, which stimulates growth significantly, had no effect on expression of HSP25, 70 or 90 in vitro.

2) HSP expression was altered by exposure to different housing conditions in vivo. HSP25, 70 and 90 levels were increased in mice in the IG condition (slowest growing tumors), compared to mice in the GG and GI conditions (faster growing tumors). HSP90 expression was greater in 0.8 g tumors than 3 g tumors from mice in the IG condition.

3) HSP expression was altered in group housed mice subjected to daily novelty stress. HSP70 (both 0.8 g and 3 g tumors) and HSP90 (3 g tumors only) expression in tumors from group housed mice was decreased by exposure to daily novelty stress.
APPENDIX I: DEXTRAN CHARCOAL TREATED (DCT) SERUM:

1. To 100 ml serum, add:
   1 g activated charcoal
   100 mg dextran

2. Place in a shaking water bath at 37°C for 2 hours

3. Transfer to 50 ml centrifuge tube

4. Spin at 3000 RPM for 30 min at 4°C

5. Pre-filter (0.45 μm)

6. Filter sterilize (0.22μm)
   60 ml serum yields 45 ml DCT serum
APPENDIX II: BUFFER RECIPES:

1) Phosphate Buffered Saline, pH 7.2

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<tr>
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<tr>
<td>KCl</td>
<td>0.2 g</td>
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<tr>
<td>Na$_2$HPO$_4$</td>
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<tr>
<td>KH$_2$PO$_4$</td>
<td>0.24 g</td>
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<tr>
<td>deionized water</td>
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2) 4X Sample Buffer

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<tr>
<td>Glycerol</td>
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<td>10% (w/v) SDS</td>
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<td>2-mercaptoethanol</td>
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<tr>
<td>1% (w/v) bromophenol blue</td>
<td>0.4 ml</td>
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<td>-dilute 1:4 with samples</td>
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3) 4% Stacking Gel

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<tr>
<td>10% ammonium persulfate</td>
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<tr>
<td>TEMED</td>
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<td>-enough for 4 mini-gels</td>
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4) 12% Separating Gel

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</tr>
<tr>
<td>Acrylamide (30% stock)</td>
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<tr>
<td>10% ammonium persulfate</td>
<td>25 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.5 μl</td>
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<tr>
<td>-enough for 2 mini-gels</td>
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</table>
5) 5X Running Buffer

deionized water  to 600 ml
Tris-base  9 g
Glycine  43.2 g
SDS  3 g
-dilute 1:5 with deionized water

6) Transfer Buffer, pH 8.3

deionized water  800 ml
25 mM Tris-base  2.9 g
Glycine  14.5 g
20% (w/v) methanol  200 ml

6) 10X Washing Buffer (TBS-Tween), pH 7.6

deionized water  to 1000 ml
Tris-base  24.2 g
NaCl  80 g
1 M HCl  38 ml
Tween-20  500 μl
-dilute 1:10 with deionized water

7) Coomassie Blue Stain

deionized water  1000 ml
methanol  400 ml
acetic acid  100 ml
Coomassie Blue R250  1 gm

8) 10X Ponceau S Stain

deionized water  up to 100 ml
Ponceau S  2 g
Trichloroacetic acid  30 g
Sulfosalicylic acid  30 g
-dilute 1:10 with deionized water
REFERENCES:


