Chapter 6

Regulation of lactation-induced MSP expression in female SMG
6.1 INTRODUCTION

Lactation is one of the important phases of the female reproductive cycle, during which time the female acquires maternal behavior resulting in the mother-pup bonding[235,236]. The parturient animal's maternal care is synchronized with the developmental demands of her litter and this decreases as the pups grow and find their own food [235][236]. In the early lactation period, when the pups are most dependent on her care, the female hamster displays high levels of licking, nest building, nursing and retrieving all collectively called as maternal behavior [235][236]. Although the onset of maternal behavior is believed to be due to hormonal stimulation initiated pre-partum, its maintenance is thought to be due to interaction between mother and pup [235-238].

An important component of pup's interaction with mother is suckling. Suckling by pups provides an important stimulus for the mother [239]. It induces oxytocinergic neurons in the hypothalamus to release massive amounts of oxytocin into blood stream which in turn act on breast tissue alveoli resulting in milk ejection[240,241]. Suckling is also known to change hormonal levels in mothers, maintain the lactational acyclic state in mammals including hamsters and humans [235,236,239,240,242,243] and also in maintaining maternal behavior [235,236,241].

Chemical signals emanating from the mother and/or pups also play an important role in maintenance of interaction between mother and pup [235,236,244-246]. In rats, it has been shown that a pheromone, dodecyl propionate secreted by pup's preputial gland stimulates the mother to lick the pup's anogenital region. This licking behavior of the mother is required for the initiation of urination and defecation response in pups [247]. Additionally, feces of lactating rats are shown to contain a pheromone which promotes ingestion of the feces by its pups, which is required for pup's normal development [248-250]. In rabbits, a nipple search pheromone, 2-metylbut-2-enal (2MB2), has been suggested to be present in the milk of the mother which attracts the new born pup and helps it to locate the nipple [251-253]. Finally, maternal pheromones are also believed to be present in humans, which can affect the infant as well as the behavior and menstrual cycle of other females[254,255].

Lactation-induced gene expression in mammary glands, ovaries, uterus, pituitary and hypothalamus are reported [256]. Other than the brain, there are few studies on the lactation induced gene-expression changes in adipose tissue and skeletal muscle [257-259] and apart from these, very few reports are available on gene-expression in other non-
reproductive tissues. Lactational influences on gene expression could be mediated by the different hormonal milieu present in lactating females compared to normal females[235,236,238,260,261]. Of the different hormonal changes occurring post-partum in the hamster, the most important ones are the increase in prolactin levels and the establishment of a temporary anestrus state of the ovaries (cessation of estrus cycles) resulting in little or no circulating estrogen levels [206,236,260-262]. The increase in prolactin is thought to be required for maintaining lactation. Interestingly, the anestrus state prevailing in lactating hamsters is similar to the anestrus state induced by prolonged exposure to short-photoperiod, constant darkness, blinding or prolonged late-afternoon melatonin treatment of female hamsters [263-271].

In the previous Chapters it was seen that, upon gonadectomy in female hamsters, MSP expression was induced in SMG. This was shown to be due to the low-estrogen state created artificially by gonadectomy. Lactation is a known natural low-estrogen state[234,236,260,261] where it was earlier found that expression of LG lipocalins (FLP and MSP) were increased [91,108,176] and interestingly, MSP was expressed in SMG [109] of lactating female hamsters. Further characterization of the lactational induction of MSP in SMG is reported in this chapter. In the beginning, the protein profiles of SMG of lactating hamster, rat and mouse are compared with their normal female counterparts to know whether major/detectable changes in expression of proteins in SMG during lactation is a common feature in all species. The time-dependant effect of lactation and subsequent weaning of pups on the levels of the MSP in SMG of hamster dams are studied. The effect of presence of pups on the lactational expression of MSP in SMG of hamster dams and the effect of treatments with sex hormones, bromocriptine (a dopamine agonist and a prolactin release inhibitor), oxytocin and melatonin on the expression of these lipocalins during lactation and after weaning were also studied.

In the following studies, expression of MSP in SMG was analyzed in both SDS-PAGE protein-stained profiles and Western blots using MSP antiserum. However, for a simple presentation of results, protein-stained profiles of SMG extracts are shown in the following Results section. Moreover, the two forms of MSP lipocalins in SMG will be at times (for simplicity sake), together referred to as the SMG lipocalins.
6.2 RESULTS

6.2.1 Comparison of SMG profiles of normal and lactating mouse, rat and hamsters

Lactating females are well known to have a different hormonal milieu than normal females [236,260] [234]. To check how this affects the expression of the MSP in SMG of hamster, tissue extracts from 20-day lactating female hamsters were checked on SDS-PAGE and compared with that of age-matched virgin normal females. Additionally, tissue extracts from SMG of 20-day lactating and normal female rat and mouse were also compared for any detectable protein profile differences. Figure 6.1 shows that SMG of 20-day lactating female hamsters show abundant expression of MSP lipocalins which are undetectable in normal female. Interestingly, almost no protein profile differences are detectable between SMG of normal and lactating in mouse or rat. Thus, the abundant expression of MSP lipocalins in SMG of 20-day lactating hamsters at levels easily detectable by a commassie-stained protein profiles was unique. This prompted further studies on the lactational regulation of these MSP lipocalins in hamster.

![Figure 6.1. Comparison of protein profiles of SMG between normal and lactating females of mouse (M), rat (R) and hamster (H). Protein profiles of SMG extracts of age-matched normal-virgin and 20-day lactating (L) females are shown. Lactation-specific induction of MSP in SMG is seen in Syrian hamsters. No major difference between protein profiles of normal and lactating mouse or rat is detectable. Equal volumes of tissue extracts (2.5% w/v) were loaded on to 11% SDS-PAGE gels and stained with Coomassie Blue R-250. Representative profiles are shown.](image)

6.2.2 Time-dependent effect of lactation and weaning on expression of SMG lipocalins

The abundant expression of MSP seen in SMG of lactating hamster should be due to the pre or post-partum changes in hormonal milieu of the females. Thus, the expression of these lipocalins in SMG of late-pregnant hamsters, which are known to have an extremely high-estrogen state [260,272], and at different time-periods during lactation and post-weaning were compared. Figure 6.2 shows that, normal and late-pregnant females do not express any MSP in SMG. Till 5 days of lactation (i.e. 5-day post-partum) MSP is almost undetectable in SMG. Low levels of MSP expression was detected in SMG at 7-day and
thereafter a rapid increase is seen which peaks at 17-day lactation. The very high level of MSP expression seen at 17-20 days lactation is similar to that in SMG of adult gonadectomized females. MSP expression in SMG of lactating hamsters was temporary and after weaning at 20-day post-partum, a rapid fall is seen resulting in obliteration of expression after 1 week.

![Figure 6.2 Time-dependent effect of lactation and weaning on expression of MSP in SMG of hamsters.](image)

6.2.3 Both estrogen and androgen repress the lactation-induced lipocalins in SMG

Estrogen and prolactin are the two important hormones, the levels of which decrease and increase respectively during lactation [236,260,262,273]. To investigate whether the changes in levels of estrogen and prolactin are responsible for the up-regulation of the SMG lipocalins, lactating dams were treated with estrogen or bromocriptine (a dopamine agonist and prolactin release inhibitor) [178,262] from 5-day post-partum till 20-day post-partum and expression of MSP in SMG was checked. Since, treatment with androgens have no effect on MSP expression in males, while they repress MSP expression in SMG of ovariectomized females, their effect on the expression of these lipocalins in lactating female was also checked to know whether MSP expression in this (female-specific) state was also androgen-sensitive or not.

As shown in Figure 6.3, estrogen administration resulted in complete repression of MSP expression whereas bromocriptine administration had little or no effect. Treatment with androgens also markedly repressed MSP, which was similar to the effect of androgens in ovariectomized females. Overall, the results strongly indicate that the high expression of MSP in SMG of lactating hamsters was similar to the expression seen in gonadectomized adult females and could be due to the prevailing low-estrogen state. Moreover, it seemed
unlikely that, the high prolactin levels (prevailing during lactation) were inducing the high expression of MSP lipocalins in SMG of lactating females.

6.2.4 The lactational induction of the lipocalins in SMG is dependent on presence and number of pups

The temporary and dramatic lactational induction of MSP in SMG and their marked decline post-weaning suggested that their expression is maintained by the lactational status of the mother. Thus, interruption of lactation by removal of pups might affect the expression of these lipocalins. As shown in Figure 6.4, early pup-deprivation completely prevented the dramatic post-partum induction of MSP when checked at both 9 and 20-day post-partum.

Alternately, prolonging the lactational period by postponing the weaning of pups (normally done at 20-day post-partum), might prevent or delay the marked decline in expression of MSP, which is seen after normal weaning. To investigate this possibility, hamster dams were maintained with their pups for 30 and 45-days post-partum, which were longer than the
usual (20 days) period. As shown in Figure 6.5, postponing the normal weaning and maintaining the dam with its pups till 30-days post-partum (lane 2) results in a delay in the decline of MSP levels in SMG but at 45-days post-partum (lane 3) MSP expression is almost obliterated even though the dam was maintained with its litter.

![Figure 6.5 Post-weaning decline of MSP in SMG of hamster dams can be delayed by prolonging their stay with pups.](image)

Results shown in Figures 6.4 and 6.5 suggest that the presence of suckling pups or the suckling stimulus itself may be required for inducing and maintaining the expression of MSP in SMG. If suckling has an effect on the expression of MSP, then the number of suckling pups may also affect their expression. To investigate this, dams with 6 or more healthy pups were divided into two groups within few hours after parturition. For dams of one group, all but one pup were removed and dams in the other group served as controls. Dams of both groups were sacrificed at 20-day post-partum (normal weaning day) and SMG extracts of the dams were compared between the groups for expression of MSP. Figure 6.6 shows that expression of MSP is considerably reduced in dams which nursed only one pup till normal weaning day.

![Figure 6.6 Post-partum induction of MSP in SMG of hamster dam is dependent on number of suckling pups.](image)
6.2.5 Effect of oxytocin treatment
Oxytocin is secreted in response to the suckling of pups and it is believed to be important for mother-pup bonding and could also be responsible for prolonging the anestrus state of lactating hamsters [235,241,242,273,274]. Since it was also found that the post-partum induction of MSP expression in SMG depends on the presence and number of suckling pups, it was felt that oxytocin secretion in the dam in response to suckling by its pups may have a role in the induction or in maintaining the expression of MSP in SMG of the nursing dam. To investigate this, daily administration of exogenous oxytocin (three times a day) was given to dams after they were pup-deprived on 10-day post-partum and expression of MSP checked after sacrifice at 20-day post-partum. As shown in Figure 6.7, such oxytocin treatment of pup-deprived dams (lane 2) could not maintain the high expression of MSP, which is seen in untreated dams maintained with their pups (lane 1) and the levels of MSP in oxytocin treated dams fell to negligible levels.

![Figure 6.7 Oxytocin treatment of dams deprived of pups on 10th day post-partum is unable to maintain high expression of MSP in SMG. SMG of 20-day post-partum dams maintained untreated with pups (lane 1) and dams which were deprived of their pups on 10th day post-partum and then treated with oxytocin (10 IU, three times a day) till 20-day post-partum (lane 2) are shown. Equal volumes of SMG extracts (2.5% w/v) were resolved in 10.5% SDS-PAGE gels and stained with Coomassie Blue R-250. Representative SDS-PAGE profiles are shown.](image)

6.2.6 Anestrus state of lactation is different from photo-period induced anestrus state
Ovariectomized female hamsters are estrogen-deficient and express high levels of MSP in SMG due to absence of any repressive effects of estrogens. Prolonged exposure to constant darkness or prolonged maintenance of female hamsters in 10:14::light:dark cycle, results in anestrus ovaries [263,269] and also in an increase in expression of MSP in SMG [109]. In such light-deprived females, marked changes in hormonal milieu occur, with estrogen levels being grossly reduced due to the anestrus state of the ovaries [234,263,269]. Melatonin (the hormone of darkness) [270], is secreted by the pineal gland during the dark phase and is believed to have profound effects on hamster physiology [270,271]. The establishment of the anestrus state during constant darkness or short-photoperiod exposure of female hamsters is believed to be actually induced by altered melatonin secretion pattern by the pineal gland [270,271]. In fact, it has been shown that
timed-melatonin injection 2 hours before the start of the dark cycle for 45 days to female hamsters maintained in normal light cycle (14:10:light:dark), mimics all other hormonal changes induced by short-photoperiod or constant darkness exposure including the anestrus state [264,267]. Lactating hamsters are also known to be in a temporary anestrus state (with negligible endogenous estrogens) [242,261] and they also express high levels of MSP in SMG. This anestrus state is known to be reversed after normal weaning or early pup-deprivation when estrus cycles again resume [235,236].

It is thus possible that both the melatonin-induced and the lactational anestrus states are mechanistically established in the female hamster by similar pathways. If this is so, melatonin injection to lactating females should be able continue/prolong their anestrus state, and maintain the high expression of MSP lipocalins, even in the absence of pups. To check this, timed (late-afternoon) melatonin injections were given daily to hamster dams starting from 5 days before weaning of pups (15 days post-partum), and continued after normal weaning for additional 20 days. As shown in Figure 6.8, expression of MSP in SMG declined to levels seen in normal females, despite the 25 days melatonin treatment. These results suggest that the anestrus state achieved during lactation is different from that of the photo-period induced anestrus state and they are most likely achieved by different pathways, which may although converge and bring about the same final effect.

6.2.7 Effect of lactation and various manipulations on the uterus weights of hamsters

The levels of all the major hormones during different days of gestation, lactation and the estrous cycle of female hamsters are well documented in literature [235,260]. Uterus is an extremely estrogen responsive tissue which shows dramatic changes (including weight change) in response to estrogen levels. Wet weights of uteri from 20-day lactating females

![Figure 6.8 Lactation-induced expression of MSP in SMG could not be maintained post-weaning by melatonin treatment.](image)

Lanes 1 and 2 show protein profiles of SMG of untreated and melatonin-treated dams respectively, at 20-day post-weaning. Lane 3 shows SMG of untreated dams at weaning. Pups of all dams were weaned at 20-day post-partum and some untreated dams were sacrificed immediately and some at 20-day post-weaning along with melatonin-treated dams. Melatonin (100 µg/day) was administered daily at late-afternoon to a group of nursing dams, from 15-day post-partum till 20-day post-weaning. Equal volumes of SMG extracts (2.5% w/v) were resolved in 10.5% SDS-PAGE gels and stained with Coomassie Blue R-250. Representative profiles are shown.

6.2.7 Effect of lactation and various manipulations on the uterus weights of hamsters
and various other treated and untreated females were taken at sacrifice and used as an indicator of estrogen levels. Figure 6.9 is a histogram comparing the mean uterus to body weight ratios of different experimental groups of hamsters. The results suggest that high MSP expression in SMG is seen only in those states where the ratio is low. These results indicate that differences in the expression of MSP in SMG of normal lactating hamsters and after different manipulations must be due to the differences in their circulating estrogen levels.

Figure 6.9 Histogram showing uterus weights of female hamsters in different hormonal states. Ratio of uterus weight to body weight ratio (+/- SD) are shown. Low ratio and high expression of MSP in SMG appear to be co-related. Gx=gonadectomized; P=pups; +E=estrogen treated.

6.2 DISCUSSION

SMG of only hamsters but not rat or mouse show abundant lactation-specific expression of proteins. These hamster proteins are the lipocalins (MSP) expressed in SMG. Thus, MSP must have a function in the female hamster, which is lactation-specific. As shown earlier, expression of MSP in SMG is markedly repressed by estrogens in females and they are abundantly expressed after ovariectomy (Chapter 5). Thus, expression of MSP in SMG in the female hamster is inversely dependant on the levels of estrogen. This explains the almost absence of expression of the MSP in SMG during late-pregnancy, a known high estrogen state [238,260,272]. Lactation in hamsters is an anestrus state with extremely low or negligible levels of estrogen [260,261]. The gradual increase in expression of the MSP in
the post-partum lactating-phase could be thus explained by the marked post-partum fall in circulating estrogen to negligible levels known to prevail in lactating hamsters, due to their anestrus state. Finally, during the post-weaning period, dramatic decrease of expression of MSP is observed, which must be due to the resumption of estrus cycle in the dam after weaning of pups and a consequent rise in circulating estrogens to normal female levels [234-236]. Thus, changes in estrogen levels could explain the expression pattern of MSP seen during late-pregnancy, throughout lactation and post-weaning. This is also supported by the observation that exogenous estrogen treatment resulted in complete obliteration of MSP in lactating dams (Figure 6.3). Finally, as shown in Figure 6.9, 20-day lactating dam (with pups) had a low ratio of uterine weight to body weight, which is indicative of their low-estrogen state and such dams expressed high levels of MSP in SMG.

In the early pup-deprived mothers, which had not received any suckling stimulus, a resumption of estrus cycles and rise of estrogen levels should be the reason for the absence (or grossly reduced) induction of MSP (Figure 6.4). The delay in the decline of MSP expression in SMG of dams, which were maintained with their pups for 30-days, could be due to the effect of some continued suckling stimulus from its pups (Figure 6.5), which may have somewhat prolonged the anestrus (low-estrogen) state. Extended suckling has been reported to delay the weaning related behavior and endocrine changes in dams [236]. When older pups prefer solid diet and stop suckling, the dam stops producing milk, mammary glands involute and the lactational anestrus ovary changes to a normal cycling ovary [234,236]. Thus, an eventual resumption of estrus cycles (loss of anestrus state) is inevitable even after prolonged maintainence of dam with its pups. The effect of suckling on the anestrus state and on expression of MSP was again apparent when the dams nursing 6 pups (with low uterine weight and high expression of lipocalins) were compared with the dams, which nursed a single pup (higher uterine weight and lower lipocalin expression) (Figure 6.6 and 6.9).

Suckling is known to induce a battery of changes in mother's neuroendocrine system, the most important of which is the release of the nanopeptide oxytocin which mediates the release of milk [240,242,275] and is also believed to be one of the main contributors for mother-pup bonding [240,241,274]. The results presented here indicate that oxytocin treatment in the absence of pups, could not maintain the expression of lipocalins suggesting that the lactational anestrus state was not maintained in the oxytocin-treated pup-deprived dams. However, it is possible that the systemic treatments with oxytocin could not substitute appropriately the natural release of oxytocin, which occurs in the brain of pup suckled dams.
Finally, results presented in this study show that melatonin administration (for 25 days) could not continue/prolong the lactational anestrus state (Figure 6.8). In other words, lactational anestrus state could not hasten the appearance of melatonin-induced anestrus state which normally occurs after 45 days of daily melatonin administration to normal females [264]. All these indicate that the melatonin-induced and lactation-induced anestrus states have some differences and are achieved by different pathways.

The temporary and massive lactational induction of MSP, which are secreted in saliva and have sequence similarities with odorant-/pheromone-binding lipocalins, suggests a possible role for these lipocalins in chemical communication in the hamster dam (e.g. in mother-pup communication). Moreover, the expression and secretion of the closely related lipocalin, FLP (and also MSP) by LG of female hamsters are also increased during lactation [91,108,176]. The possibility of a role in chemical communication for MSP and FLP lipocalins of SMG and LG of lactating female hamsters will be discussed in Chapter 8.