# Putative role of cocaine- and amphetamine-regulated transcript (*CARTPT*) in dominant follicle selection in cattle

GW Smith[1, 2, 3], A Sen[1, 2], JK Folger[1, 2] and JJ Ireland [2,3]

*Laboratory of Mammalian Reproductive Biology and Genomics[1], Departments of Animal Science[2] and Physiology[3], Michigan State University, East Lansing, MI 48824-1225*

The mechanisms regulating development of a single (dominant) follicle capable of ovulation during each follicular wave in cattle and atresia of remaining follicles (dominant follicle selection) are not well understood. FSH and IGF1 are known regulators of follicle growth and granulosa cell estradiol production during follicular waves. Recent evidence indicates cocaine and amphetamine regulated transcript (*CARTPT*), with intraovarian expression only in single-ovulating species, is a novel regulator of follicular development. The mature CARTPT peptide (CART) is a potent negative regulator of FSH and IGF1 action on granulosa cells in vitro and can inhibit follicular estradiol production in vivo. Follicular fluid CART concentrations in healthy follicles decrease after dominant follicle selection and *CARTPT* mRNA is lower in healthy versus atretic follicles collected prior to and early after initiation of follicle dominance, suggestive of a regulatory role in the selection process. The inhibitory actions of CART on FSH signaling and estradiol production are dependent on the $G_{o/i}$-subclass of inhibitory G proteins and linked to multiple components of the FSH signal transduction pathway resulting in reduced *CYP19A1* mRNA and estradiol production. Evidence to date supports a potential important functional role for CART in regulation of dominant follicle selection and the species-specific ovulatory quota in monotocous species.

## Introduction

The ovarian cycle is central to the reproductive process, because only mature ovarian follicles release oocytes to be fertilized. However, > 99.9% of follicles die via atresia at various stages of folliculogenesis and never ovulate. Antral follicle development occurs in a characteristic wave like pattern in monotocous species including cattle and humans (Fortune 1994, Ginther *et al.* 2001, Baerwald *et al.* 2003). A transient increase in serum FSH precedes the onset of a follicular wave and stimulates emergence of a cohort of small antral follicles. Typically, in the face of declining FSH concentrations, a single dominant follicle out of this cohort is selected to continue to grow to ovulatory size (Fortune *et al.* 2004), and produces increased amounts of estradiol. The remaining smaller "subordinate" follicles rapidly lose their capacity to produce estradiol and die via atresia (Richards & Hedin 1988, Sunderland *et al.* 1994, Mihm *et al.* 1997, Baerwald *et al.* 2003). The process whereby development of a single (dominant) follicle capable of ovulation during each

Corresponding author E-mail: smithge7@msu.edu

follicular wave and atresia of remaining follicles occurs is referred to as dominant follicle selection. Selection of a single dominant follicle is an evolutionarily conserved mechanism critical to control of number of offspring per pregnancy in monotocous species. Production of estradiol by the dominant follicle is essential for follicular growth and triggers the preovulatory gonadotropin surge to promote resumption of meiosis and ovulation (Fortune 1994, Greenwald & Roy 1994, Vander *et al.* 1994). Estradiol producing capacity is lost in ovarian follicles before apoptosis and morphological signs of atresia (Sunderland *et al.* 1994, Austin *et al.* 2001). While the key role of pituitary gonadotropins in mediating the wave like pattern of follicular development is well established, the intrafollicular mechanisms and regulatory molecules that are obligatory for selection of a single dominant follicle during each follicular wave have not been fully established.

Differences in the milieu of intrafollicular factors between the future dominant and subordinate follicles are believed to have a role in selection (Beg *et al.* 2002, Beg & Ginther 2006). A prominent local role for multiple growth factor systems, including the IGFs, in selection has been proposed (Fortune *et al.* 2001, Knight & Glister 2003, Webb *et al.* 2003, Fortune *et al.* 2004). The IGFs increase granulosa cell proliferation, synergize with gonadotropins to promote granulosa cell differentiation (Spicer & Echternkamp 1995), stimulate estradiol production (Spicer *et al.* 1993, Gutierrez *et al.* 1997) and enhance sensitivity to FSH (Monget & Monniaux 1995). Concentrations of free IGF1 diverge in the largest versus second largest follicle within a cohort of growing follicles during follicular waves (Beg *et al.* 2002, Rivera & Fortune 2003b). These results support a functional role for increased IGF1 bioavailability in future dominant follicles during selection. Intrafollicular IGF1 bioavailability is regulated by the low molecular weight IGF binding proteins (IGFBP) and IGFBP4 and IGFBP5 levels are regulated by IGFBP proteases (Rivera & Fortune 2003a). Follicular fluid IGFBP levels are greater in atretic versus healthy follicles (Echternkamp *et al.* 1994, de la Sota *et al.* 1996, Stewart *et al.* 1996, Mihm *et al.* 1997). Lower levels of IGFBP4 and IGFBP5 are present in the largest growing follicles during selection and in the dominant follicle compared with smaller (subordinate) follicles (Rivera & Fortune 2003a). Follicles with reduced IGFBP4 and IGFBP5 also displayed higher levels of proteolytic activity for IGFBP4 and IGFBP5 (Rivera & Fortune 2003a), activity attributed to PAPPA (Mazerbourg *et al.* 2001, Rivera & Fortune 2003b, Spicer 2004). Furthermore, studies have shown that the follicle with the lowest follicular fluid IGFBP4 levels in a cohort prior to selection is destined to become the dominant follicle (Mihm *et al.* 2000).

A combination of FSH and IGF1 is well established to regulate estradiol production and growth of follicles during follicular waves in cattle (Beg & Ginther 2006). While the role of FSH in initiating emergence of a follicular wave is well established, dominant follicle selection occurs as FSH concentrations are declining (Adams *et al.* 1992, Sunderland *et al.* 1994). A combination of follicle ablations and experimental suppression of FSH concentrations below normal levels at onset of diameter deviation has been used to functionally demonstrate greater FSH responsiveness of the future dominant follicle versus future subordinate follicles in cattle (Ginther *et al.* 2003). However, *FSHR* mRNA (Xu *et al.* 1995, Bao *et al.* 1997, Evans & Fortune 1997) and FSHR binding (Ireland & Roche 1982, Ireland & Roche 1983a, Ireland & Roche 1983b, Braden *et al.* 1986) do not increase coincident with selection. Hence, rather than enhanced FSH action, it is plausible that local (intrafollicular) inhibition of FSH action could be functionally linked to selection. Observed temporal regulation of cocaine- and amphetamine– regulated transcript (*CARTPT*) expression during a follicular wave and observed negative effects of mature form of the CARTPT peptide (CART) on FSH and IGF1 action, also suggest a potential functional role for *CARTPT* as a mediator of dominant follicle selection. The focus of this review is on intrafollicular expression, actions and mechanism of action of *CARTPT*; evidence supportive of a functional role for *CARTPT* in regulation of dominant follicle selection.

## Discovery and intrafollicular expression of *CARTPT*

The dawning of the genomics and genome sequencing era in livestock has provided tremendous potential for advancement in understanding of the molecular mechanisms involved in regulation of ovarian follicular development. During experiments reported in 2004 designed to characterize the transcriptome of bovine oocytes and generate an oocyte cDNA microarray for physiological studies (Yao *et al.* 2004), we identified five expressed sequence tags with similarity to *CARTPT*. Expression of *CARTPT* mRNA in the mammalian ovary had not been reported previously. The presence of *CARTPT* mRNA in the bovine oocyte was of considerable interest because CART is a well characterized neuropeptide with documented biological activity. Numerous pleiotropic actions of CART, including anorexigenic (Kristensen *et al.* 1998, Lambert *et al.* 1998, Vrang *et al.* 1999, Hunter *et al.* 2004), neuroendocrine (Vrang *et al.* 2000, Baranowska *et al.* 2003, Kuriyama *et al.* 2004, Raptis *et al.* 2004, Smith *et al.* 2004) and anti-psychostimulant effects (Jaworski *et al.* 2003, Kim *et al.* 2003, Kuhar *et al.* 2005) have been described in the brain. To date, the gastrointestinal tract (Ekblad *et al.* 2003), pancreatic cells (Wierup *et al.* 2005, Wierup *et al.* 2006) and ovarian granulosa cells (Sen *et al.* 2007, Sen *et al.* 2008, Lv *et al.* 2009) are the prominent non-neural sites of *CARTPT* action described. Furthermore, *in vitro* model systems for investigation of *CARTPT* mechanism of action linked to a downstream physiological response had not been described at the time of the serendipitous discovery of *CARTPT* expression in the bovine ovary. Thus, CART met initial criteria for a potential novel intrafollicular regulatory molecule of interest and further experiments were conducted to characterize bovine ovarian *CARTPT* expression and investigate its intrafollicular localization and potential regulatory role.

While intraovarian *CARTPT* expression was initially documented in the oocyte, the granulosa cell layer is the prominent source of *CARTPT* expression in the bovine ovary. To date, the function of CART of oocyte origin is unclear. In initial studies, *CARTPT* mRNA and peptide localization was observed specifically in the granulosa cell layer of some but not all antral follicles and not in preantral follicles (Kobayashi *et al.* 2004). The temporal expression of *CARTPT* mRNA in granulosa cells of antral, but not preantral follicles suggests that local CART action is presumably restricted to follicles that have undergone advanced stages of differentiation.

To further define the window of follicular development when biological actions of CART are most likely manifest, temporal changes in granulosa cell *CARTPT* mRNA and follicular fluid CART concentrations were determined in bovine follicles collected at the following specific stages of the first follicular wave: 1) predeviation (d 1.5 after emergence of the first follicular wave), 2) early dominance (first day in wave when one follicle in cohort is 2 mm larger than others), 3) mid dominance (second day in wave that dominant follicle does not increase in size), 4) loss of dominance (first day that a new cohort in second follicular wave appears) and 5) preovulatory (1 d after $PGF_{2\alpha}$ injection on d 7 post estrus). Health status of each follicle was determined by calculating follicular fluid estradiol: progesterone ratios. Follicles with an estradiol: progesterone ratio $\geq 1$ are estrogen-active and healthy and follicles with an estradiol: progesterone ratio $< 1$ are estrogen-inactive and atretic (Ireland & Roche 1982, Ireland & Roche 1983a, Sunderland *et al.* 1994). Results revealed granulosa cell *CARTPT* mRNA and follicular fluid CART concentrations are higher in estrogen inactive atretic follicles versus estrogen active healthy follicles collected during (predeviation stage) and immediately after selection (early dominance stage) and relatively low in follicles collected at remaining stages of a follicular wave (Lv *et al.* 2009). Furthermore, follicular fluid CART levels are decreased in estrogen-active healthy follicles coincident with dominant follicle selection (Lv *et al.* 2009). Observed elevated granulosa cell *CARTPT* mRNA and follicular fluid CART concentrations in estrogen-inactive subordinate compared with estrogen-active potential dominant follicles, coupled with the higher follicular fluid CART concentrations in healthy follicles collected before versus immediately after selection (Lv *et al.* 2009) support a potential functional role for *CARTPT* in the process of dominant follicle selection.

### *CARTPT* regulation of estradiol production and mechanism of action

The observed negative relationship of *CARTPT* expression with follicle health status and temporal regulation of granulosa cell *CARTPT* expression during follicular waves in cattle is suggestive of a functional role in dominant follicle selection. Hence the ability of CART to inhibit granulosa cell estradiol production in vitro was investigated using a long term culture system where cells gain significant estradiol producing capacity with time in culture and respond in a dose-dependent fashion to FSH (Sen *et al.* 2007) and IGF1 (Gutierrez *et al.* 1997) with an increase in estradiol production. Treatment with CART inhibits (in a dose-dependent fashion) FSH-stimulated estradiol production and *CYP19A1* mRNA expression by bovine granulosa cells (Sen *et al.* 2007). Treatment with CART also suppresses IGF1-stimulated estradiol production in this culture system (Fig. 1). Collectively, results demonstrate potent inhibitory effects of CART on actions of two of the major tropic hormones for estradiol production during the selection process. Furthermore, CART treatment can also reduce LH-induced production of androstenedione, the androgen precursor for estradiol synthesis, by theca tissue collected prior to (pre deviation stage) and early post selection (early dominance stage), but not at later stages of a follicular wave (Lv *et al.* 2009). Thus, the inhibitory effects of CART on estradiol production in vivo during selection could be mediated indirectly by inhibition of production of theca cell androgen precursor for estradiol biosynthesis and well as directly through actions on FSH- and IGF1-stimulated granulosa cell estradiol production.

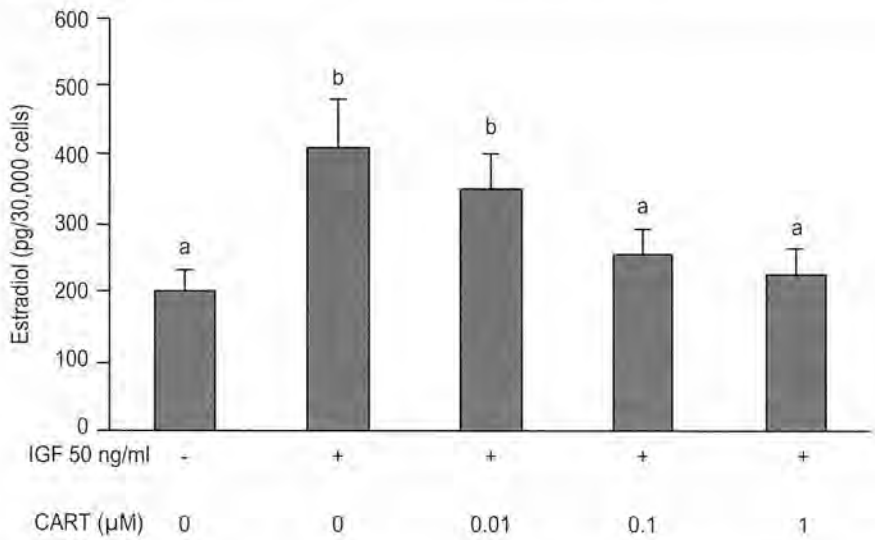


**Fig. 1.** Dose dependent inhibitory effect of CART on IGF1-induced granulosa cell estradiol production in vitro. Serum-free long-term granulosa cell culture was performed using previously described procedures (Sen *et al.* 2007, Sen *et al.* 2008). Cells were cultured for 7 d in the presence or absence of 50 ng/ml LR3 IGF1. Every 48 h, 75% of media was replaced with fresh medium containing appropriate treatments. CART treatments (0, 0.01, 0.1 and 1 μM) were initiated on d 6 of culture and continued for 24 h. On d 7, media was removed for estradiol assay and the cells were washed 2X, trypsinized and cell number determined. Data (pg estradiol) were normalized to 30,000 cells and bars (mean +/- SEM of four replicate experiments) with different superscripts denote significant differences across treatments ($P < 0.05$). Note dose dependent (> 90%) inhibition of IGF1-induced estradiol production in response to CART treatment.

To confirm physiological relevance of inhibitory effects of CART on estradiol production obtained in vitro, the effects of intrafollicular injection of CART (into early dominant follicles) on follicular estradiol production and granulosa cell *CYP19A1* mRNA in vivo was performed (Lv *et al.* 2009) using our previously validated ultrasound mediated intrafollicular injection procedures (Peters *et al.* 2004, Li *et al.* 2006, Li *et al.* 2007). Ultrasound mediated intrafollicular injection provides a powerful tool for administration of ligands, antagonists, inhibitors etc into follicles at specific stages of a wave to test gene function/contribution to mechanisms associated with selection.

In above described studies, elevation of concentrations of CART in follicular fluid approximately 3-fold resulted in a 54% reduction in follicular fluid estradiol and a 96% decrease in granulosa cell *CYP19A1* mRNA 24 h post injection, with no effect on follicle size (Lv *et al.* 2009). Concentrations of androstenedione were not affected, but this was not unexpected as injection of CART occurred after the selection process was complete. Thus, CART can negatively regulate follicular estradiol production and *CYP19A1* mRNA in vivo supporting a potential physiological role for CART in negative regulation of granulosa cell estradiol production during selection of the dominant follicle.

To further dissect the mechanisms involved in *CARTPT* regulation of granulosa cell estradiol production, effects of CART treatment on specific components of the FSH signal transduction cascade were determined using a combination of pharmacological inhibitors, siRNA mediated gene knockdown strategies and biochemical assays. FSH-induced granulosa cell cAMP accumulation and estradiol production is $Ca^{2+}$ dependent and CART can inhibit FSH-stimulated $Ca^{2+}$ influx (Sen *et al.* 2007). Furthermore, it is well known that FSH activates the cAMP-protein kinase A pathway leading to *CYP19A1* expression and estradiol production (Richards 2001, Richards *et al.* 2002). Treatment with CART induces a dose dependent decrease in cAMP accumulation in bovine granulosa cells in response to FSH or forskolin treatment (Sen *et al.* 2007). However, inhibitory effects of CART on FSH-stimulated estradiol production are not mediated solely by inhibition of cAMP accumulation, as CART also inhibits estradiol production in response to treatment of granulosa cells with 8-Br-cAMP (Sen *et al.* 2007). The ability of CART to inhibit 8-Br-cAMP-induced estradiol production clearly indicates that cAMP is not the sole limiting factor involved in the negative effects of CART on FSH signaling. Thus, CART likely regulates other FSH-induced signaling proteins obligatory for estradiol production that are downstream of cAMP.

Bovine granulosa cell estradiol production in response to FSH is also Erk 1/2 (MAPK 3/1) and Akt (AKT1) dependent (Sen *et al.* 2007) and a growing body of evidence supports a prominent role for regulation of MAPK3/1 and AKT1 pathways in the selection process. Subordinate follicles collected near the time of selection of the dominant follicle had reduced levels of phosphorylated AKT1 in granulosa cells and phosphorylated MAPK3/1 and AKT1 in theca cells when compared to bovine dominant follicles (Ryan *et al.* 2007). In addition, intrafollicular administration of MAPK3/1 and AKT1 inhibitors into growing sheep follicles disrupted growth and estradiol production (Ryan *et al.* 2008). Evidence suggests that CART may function as an endogenous negative regulator of MAPK3/1 and AKT1 signaling associated with dominant follicle selection. Transient CART stimulation of bovine granulosa cells shortens the duration of FSH-induced MAPK3/1 and AKT1 signaling while a prolonged CART treatment completely blocks FSH-induced MAPK3/1 and AKT1 activation (Sen *et al.* 2008). The CART-induced accelerated termination of MAPK3/1 and AKT1 signaling is mediated both by induced expression and impaired ubiquitin-mediated proteasome degradation of the mitogen-activated protein kinase (MAPK) phosphatases dual specific phosphatase 5 (DUSP5) and protein phosphatase 2A (PP2A). Stimulation of granulosa cells with CART alone induces MAPK3/1 activation

and CART-induced expression of DUSP5 is MAPK3/1 dependent. Ablation (using siRNA) of DUSP5 and (or) PP2A in cultured granulosa prevents the CART-induced early termination of MAPK3/1 and AKT1 signaling (Sen *et al.* 2008). Results support CART-induced suppression of MAPK3/1 and AKT1 signaling as an important component of its inhibitory actions on FSH-stimulated estradiol production.

Despite pleiotropic actions of CART on bovine granulosa cells and described anorexigenic (Kristensen *et al.* 1998, Lambert *et al.* 1998, Vrang *et al.* 1999, Hunter *et al.* 2004), neuroendocrine (Vrang *et al.* 2000, Baranowska *et al.* 2003, Kuriyama *et al.* 2004, Raptis *et al.* 2004, Smith *et al.* 2004) and anti-psychostimulant (Jaworski *et al.* 2003, Kim *et al.* 2003, Kuhar *et al.* 2005) effects of CART in the brain, the molecular identity of the receptor mediating biological actions of CART remains undetermined. Binding studies using the AtT20 cell line (Vicentic *et al.* 2005), PC12 cells (Maletinska *et al.* 2007) and primary neural cell cultures (Jones & Kuhar 2008) provide direct evidence for existence of a putative CART receptor. Specific, saturable, high affinity binding (kD 0.468 nM) of CART to bovine granulosa cells has been detected and granulosa cell CART binding is increased in response to FSH treatment in vitro (Folger *et al.* 2009), suggesting that the transient increase in FSH preceding initiation of each follicular wave may be important for conferring granulosa cell CART responsiveness to the recruited cohort of follicles. Furthermore, previously described actions of CART on bovine granulosa cells are dependent on activity of the o/i subclass ($G_{o/i}$) of inhibitory G proteins and blocked by pretreatment with a $G_{o/i}$ inhibitor. Thus, results suggest that CART actions are presumably mediated by binding to a G-protein ($G_{o/i}$) coupled receptor of unknown identity.

Collectively, results of above described studies support the following model (Fig. 2) depicting the intracellular mechanism of action of CART in inhibition of FSH-induced MAPK3/1 and AKT1 signaling in bovine granulosa cells associated with dominant follicle selection. Upon binding to its receptor ($G_{o/i}$ linked), CART inhibits FSH-stimulated $Ca^{2+}$ influx, cAMP accumulation and MAPK3/1 (Erk 1/2) and AKT1 (Akt) signaling resulting in reduced *CYP19A1* mRNA and estradiol production. Inhibitory effects of CART on MAPK3/1 and AKT1 signaling are mediated by accelerated dephosphorylation of phosphorylated MAPK3/1 (pErk1/2) and AKT1 (pAkt). CART-induced early termination of the FSH-induced MAPK3/1 and AKT1 signaling pathways are mediated via induced expression and decreased proteasome degradation of DUSP5 and PP2A. CART-induced expression of DUSP5 is dependent on MAPK3/1 thereby exhibiting a negative feedback loop where activated MAPK3/1 induces DUSP5 expression which in turn terminates MAPK3/1 activation.

## Proposed functional role for *CARTPT* in dominant follicle selection

Follicular waves in cattle can be divided chronologically into two distinct phases, the predominance phase and the dominance phase. Events occurring during the predominance phase are obligatory for selection of a single dominant follicle and culminate with dominant follicle development during the dominance phase. A transient increase in FSH at the beginning of the predominance phase triggers emergence of each follicular wave (Fortune 1994, Ireland *et al.* 2000, Fortune *et al.* 2001). After emergence, follicles in the cohort initially grow at a similar rate (common growth phase) (Beg & Ginther 2006). Molecular determinants predictive of the future subordinate follicles, such as high IGFBP4 concentrations, have been identified as early as the predeviation stage (Mihm *et al.* 2000), but follicle ablation studies have demonstrated that all follicles in the cohort retain the capacity to become the dominant follicle during this common growth phase (Beg & Ginther 2006). Diameter deviation is defined as the divergence in growth rates between the two largest follicles in a follicular wave, during which time the

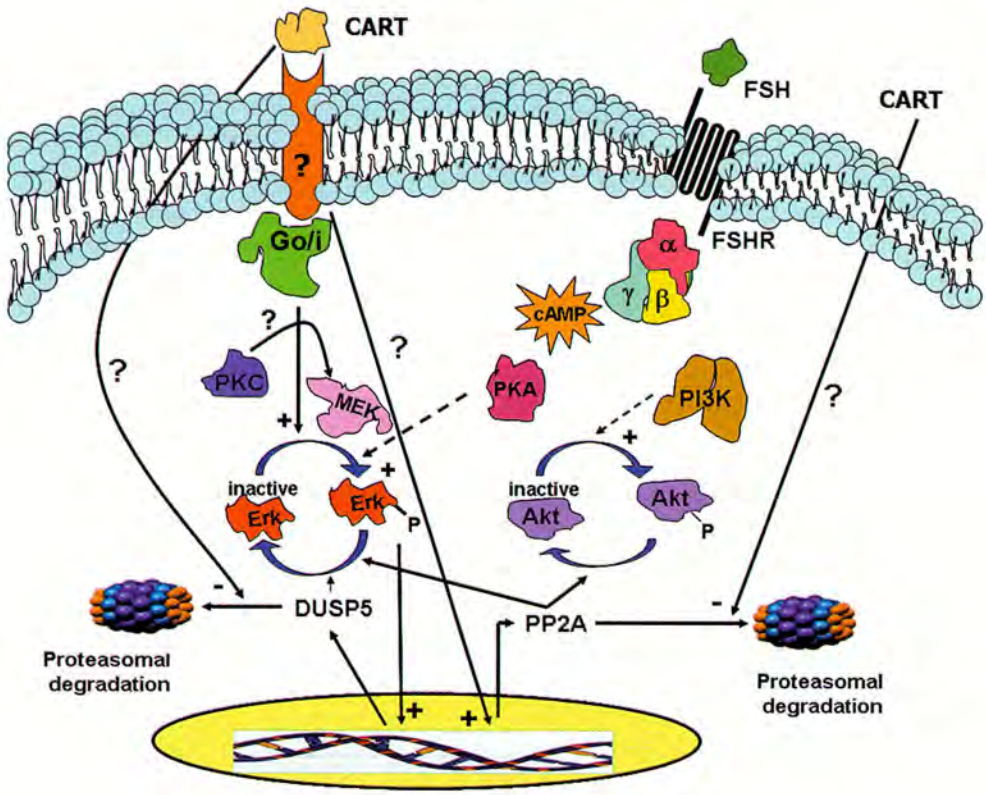


**Fig. 2.** Proposed model for the intracellular mechanism of CART action in inhibition of FSH stimulated MAPK 3/1 and AKT1 signaling in bovine granulosa cells. FSH stimulates MAPK3/1 (Erk1/2) and AKT1 (Akt) activation while CART treatment accelerates the termination of FSH-induced Erk1/2 and Akt signaling. CART stimulation alone also induces Erk1/2 activation in bovine granulosa cells. Expression of DUSP5 is dependent on Erk1/2 thereby exhibiting a negative feedback loop where activated Erk1/2 induces DUSP5 expression which in turn terminates Erk1/2 activation. CART-induced DUSP5 expression is functionally required for CART-induced termination of Erk 1/2 signaling and is mediated by Erk1/2 and transcription dependent mechanisms. CART increases PP2A expression which is also functionally required for CART-induced termination of Erk 1/2 and Akt activation and CART regulation of PP2A expression is mediated by transcription dependent and independent mechanisms. CART treatment also impairs the proteasomal degradation of both DUSP5 and PP2A resulting in their accumulation (Reprinted from Sen *et al.* 2008).

largest (future dominant follicle) continues to grow and the future subordinate follicle experiences a reduced growth rate and diminished estradiol production (Beg & Ginther 2006). The onset of diameter deviation occurs when the largest follicle reaches 8.5 mm in dairy cattle (Ginther *et al.* 2001, Beg *et al.* 2002) and marks initiation of divergence in growth rate and estradiol producing capacity between the F1 or largest (future dominant) and F2 or second largest (future subordinate) growing follicles resulting in acquisition of dominance and completion of the predominance phase and selection. While the role of FSH in initiating emergence of a follicular wave is well established, dominant follicle selection occurs as FSH concentrations are declining (Adams *et al.* 1992, Sunderland *et al.* 1994).

Greater FSH responsiveness of the future dominant follicle versus future subordinate follicles in cattle has been demonstrated previously (Ginther *et al.* 2003), but is not likely linked to differences in *FSHR* expression as *FSHR* mRNA (Xu *et al.* 1995, Bao *et al.* 1997, Evans & Fortune 1997) and FSHR binding (Ireland & Roche 1982, Ireland & Roche 1983a, Ireland & Roche 1983b, Braden *et al.* 1986) do not increase coincident with selection. Thus, local (intrafollicular) inhibition of FSH action in the future subordinate follicle could help mediate selection. A reduction in follicular fluid CART concentrations in the future dominant follicle occurs within this key time period (Lv *et al.* 2009). Therefore, it is plausible that CART functionally mediates reduced FSH responsiveness of future subordinate follicles during selection. CART is a potent inhibitor of multiple components of the granulosa cell FSH signaling pathway culminating in reduced estradiol production and *CYP19A1* mRNA (Sen *et al.* 2007, Sen *et al.* 2008). Greater intrafollicular CART concentrations and potentially greater CART responsiveness (granulosa cell CART binding sites) may target negative actions of CART specifically to the future subordinate follicles during selection.

Given the proposed role of CART in inhibition of FSH action in future subordinate follicles, what are the mechanisms that allow escape from such inhibitory effects in cases of co-dominant follicles or allow a future subordinate follicle (F2) to become dominant if the future dominant follicle (F1) is destroyed? Ultrasound mediated follicle ablation at the beginning of deviation has been utilized to gain further insight into the mechanisms associated with dominant follicle selection (Beg & Ginther 2006). Ablation of the largest follicle at the onset of deviation allows the second largest follicle to become dominant (Ko *et al.* 1991, Adams *et al.* 1993) and results in a transient increase in FSH (Ginther *et al.* 2002) which presumably helps promote continued growth of the second largest follicle. Furthermore, greater FSH concentrations prior to deviation (Kulick *et al.* 2001, Lopez *et al.* 2005) and a delay in the nadir of FSH concentrations (Lopez *et al.* 2005) are characteristic of follicular waves in which cattle display co-dominant follicles versus those in which a single dominant follicle develops. Our results indicate that granulosa cell *CARTPT* mRNA is reduced in vitro in response to FSH treatment (Lv *et al.* 2009). Hence it is possible that the higher FSH accompanying ablation of the largest follicle at deviation, or in waves in which co-dominant follicles develop, is sufficient to overcome the inhibitory effects of CART on FSH action and (or) to reduce *CARTPT* expression allowing the follicle in question to become dominant.

In addition to the regulatory role of FSH, differences in the milieu of intrafollicular factors between the future dominant and subordinate follicles are believed to have a role in selection (Beg *et al.* 2002, Beg & Ginther 2006). Concentrations of free IGF1 diverge in the largest (F1) versus second largest (F2) follicles within a cohort of growing follicles during follicular waves (Beg *et al.* 2002, Rivera & Fortune 2003b). Granulosa cell *CARTPT* mRNA is reduced in response to IGF1 treatment in vitro (Lv *et al.* 2009). Thus, it is possible that the enhanced IGF1 bioavailability in the future dominant follicle is responsible for observed reduction in follicular CART concentrations during dominant follicle selection. Furthermore, CART is a potent inhibitor of IGF1-stimulated estradiol production by bovine granulosa cells (Fig. 1). Thus, CART-induced inhibition of IGF1 action in future subordinate follicles may help promote dominant follicle selection. However, it is not yet known whether inhibitory effects of CART on IGF1 action are also mediated by stimulation of IGFBP expression.

Based on results described herein, the following model (Fig. 3) is proposed to explain the potential role of *CARTPT* in dominant follicle selection. This model is focused on the time period (onset of diameter deviation) where divergence in growth rate and steroidogenic capacity of the largest (F1; future dominant follicle) and second largest growing follicle (F2; future subordinate follicle) occurs during the first follicular wave, resulting in acquisition of dominance by the F1 follicle. Actions of FSH and IGF1 in the F1 follicle promote sustained

growth and estradiol production resulting in selection as the dominant follicle and such actions are mediated in part by enhanced FSH- and IGF1-stimulated MAPK3/1 and AKT1 signaling. Enhanced CART signaling in the F2 (future subordinate follicle), but not the F1 follicle, causes inhibition of FSH and IGF1 action resulting in inhibition of MAPK3/1 and AKT1 signaling, reduced estradiol production and *CYP19A1* mRNA, cessation of growth, and development as a subordinate follicle. It is also proposed that elevated CART and perhaps CART receptor expression in subordinate follicles, potentially due to negative regulation of *CARTPT* and CART receptor expression by IGF1 specifically in the F1 follicle, targets CART actions specifically to the F1 (subordinate follicle) at this key time point during follicular development.

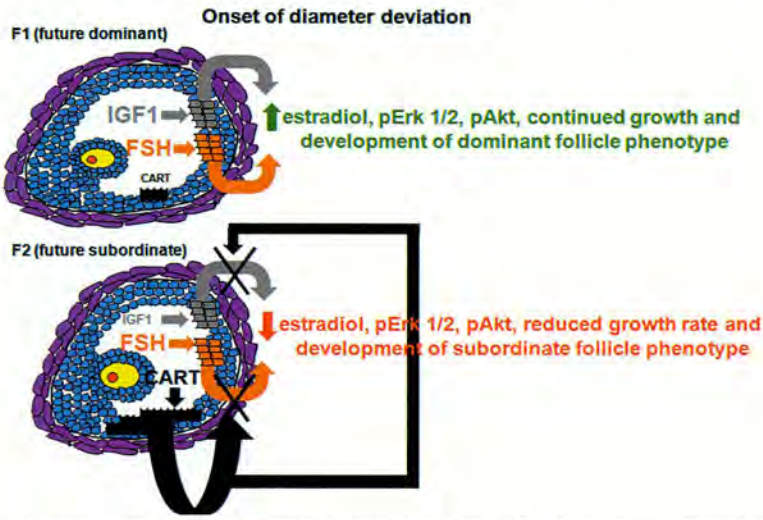


**Fig. 3.** Proposed model depicting differential actions of CART in F1 (future dominant) versus F2 (future subordinate) follicles at time of diameter deviation culminating in completion of dominant follicle selection. Actions of FSH (orange) and IGF1 (gray) in the F1 follicle promote activation of MAPK3/1 (Erk 1/2) and AKT1 (Akt) signaling (denoted by increased phosphorylated (p) Erk 1/2 and pAkt) and continued growth and estradiol production culminating in acquisition of dominant follicle phenotype. Enhanced CART signaling (black) in the F2 (future subordinate) but not the F1 follicle causes inhibition (X) of FSH (orange) and IGF1 action (gray) resulting in suppression of Erk 1/2 and Akt signaling, reduced estradiol production, cessation of follicular growth, and development of subordinate follicle phenotype. Elevated CART and perhaps CART receptor (■) expression in the F2 follicle, potentially due to negative regulation by IGF1 specifically in the F1 follicle, targets CART's actions specifically to the F2 (subordinate follicle) at this key time point during follicular development.

## Conclusions

Collectively, results described above support a novel local role for CART in regulation of follicular development in cattle. Expression of *CARTPT* is temporally regulated at specific stages of a follicular wave with greater *CARTPT* expression in atretic versus healthy follicles early in a wave and a pronounced decrease in intrafollicular CART concentrations in healthy follicles accompanying selection of the dominant follicle (Lv *et al.* 2009). Expression of *CARTPT* mRNA is negatively regulated by FSH and IGF1 (Lv *et al.* 2009), factors stimulatory to follicular growth and granulosa cell estradiol production. Results of in vitro culture experiments established CART as a potent negative regulator of IGF1-stimulated bovine granulosa cell estradiol

production (Fig. 1) and of multiple components of the FSH signal transduction cascade resulting in reduced estradiol production and *CYP19A1* mRNA (Sen *et al.* 2007, Sen *et al.* 2008). Similar effects of CART on estradiol production and *CYP19A1* mRNA were observed in vivo following ultrasound mediated intrafollicular injection of CART (Lv *et al.* 2009). Collectively, results presented support the proposed role for CART as a functional mediator of selection of a single dominant follicle during each follicular wave in cattle and perhaps in other mono-ovulatory species. In contrast, CART is not present in the ovaries of rats (Murphy *et al.* 2000) and *CARTPT* mutant mice are fertile (Asnicar *et al.* 2001). Furthermore, *CARTPT* expression is undetectable in ovaries of polytocous farm species such as the pig (Lv and Smith, unpublished observations) or in ovaries of sexually mature mice (Sen and Smith, unpublished observations). These observations suggest that poly-ovulatory species such as rodents may require a less stringent selection mechanism to ovulate multiple follicles thus providing an evolutionary explanation for the absence of *CARTPT* expression in the ovary of polytocous species. Hence, we hypothesize that *CARTPT* functions as a "gatekeeper" of the species-specific ovulatory quota in monotocous species such as cattle.

## Acknowledgements

Described studies of *CARTPT* expression and regulation and CART actions in the bovine ovary were supported by National Research Initiative Competitive Grant no. 2005-35203-16011 from the USDA National Institute of Food and Agriculture, Agriculture and Food Research Initiative Competitive Grant no. 2009-65203-05700 from the USDA National Institute of Food and Agriculture, and the Michigan Agricultural Experiment Station.

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