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Differential regulation of kit ligand A (*kitlga*) expression in the zebrafish ovarian follicle cells – Evidence for the existence of a cyclic adenosine 3′, 5′ monophosphate-mediated binary regulatory system during folliculogenesis

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ABSTRACT

Kit ligand (*Kitl*) is an important paracrine factor involved in the activation of primordial follicles from the quiescent pool and in the maintenance of meiotic arrest before germinal vesicle breakdown (GVBD). It has been reported that follicle-stimulating hormone (FSH) stimulates but luteinizing hormone (LH) suppresses the expression of *Kitl* in the granulosa cells in mammals. Considering that both gonadotropins signal in the follicle cells mainly by activating cyclic adenosine 3′, 5′-monophosphate (cAMP) pathway, we are intrigued by how cAMP differentially regulates *Kitl* expression. In the present study, we demonstrated that both human chorionic gonadotropin (hCG) and pituitary adenylate cyclase activating polypeptide (PACAP) inhibited insulin-like growth factor I (IGF-I)-induced Akt phosphorylation and *kitlga* expression in the zebrafish follicle cells. Further experiments showed that cAMP was involved in regulating the expression of *kitlga*. However, two cAMP-activated effectors, protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac), had converse effects. PKA promoted whereas Epac inhibited the expression of *kitlga*, as demonstrated by the respective activators. Interestingly, cAMP also appeared to exert differential effects on *kitlga* expression at different stages of follicle development during folliculogenesis, significantly stimulating *kitlga* expression at the early growth stage but suppressing it at the full-grown stage before final oocyte maturation, implying a potential mechanism for differential effects of the same pathway at different stages. The inhibitory effect of forskolin (activator of adenylate cyclase) and H89 (inhibitor of PKA) on IGF-I-induced expression of *kitlga* suggested cross-talk between the cAMP and IGF-I-activated PI3K-Akt pathways. This study, together with our previous findings on IGF-I regulation of *kitlga* expression, provides important clues to the underlying mechanism that regulates Kit ligand expression during folliculogenesis in the ovary.

1. Introduction

In mammalian ovaries, folliculogenesis begins from the quiescent primordial follicles, which are comprised of central oocytes arrested at the diplotene stage of the first meiosis, and surrounding pregranulosa cells. When the follicles leave the resting primordial pool, the oocytes grow and the granulosa cells proliferate to form primary and preantral follicles. Later, the follicles become acutely dependent on gonadotropins from the pituitary for further growth and development until the oocytes resume and complete meiosis and ovulate. The dynamic changes associated with ovarian folliculogenesis are closely regulated by both endocrine hormones and paracrine factors within the follicle, especially those involved in communications between the oocyte and the surrounding follicle layer, as has been shown in both mammals (Canipari, 2000; Eppig, 2001; Gilchrist et al., 2004; Matzuk et al., 2002; Moley and Schreiber, 1995) and fish (Clelland and Peng, 2009; Ge, 2005). The Kit system is now believed to be an important member of the complex regulatory network in the follicle, and is widely involved in various folliculogenic processes, including the proliferation of granulosa cells (Otsuka and Shimasaki, 2002; Reynaud et al., 2000; Yoshida et al., 1997), the growth and survival of oocytes (Reynaud et al., 2000; Thomas et al., 2007; Yoshida et al., 1997), and final oocyte maturation (Ismail et al., 1996, 1997; Reynaud et al., 2000; Thomas et al., 2007; Thommes et al., 1999; Yao and Ge, 2013; Yoshida et al., 1997). As an important paracrine factor responsible for signaling

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from the follicle cells to the oocyte, the Kit ligand (*KITL/Kitl*) is probably subject to regulation by various systemic and local factors; however, the available information on its regulation is very limited.

Because the *KITL/Kitl* is expressed in the follicle cells (Manova et al., 1993; Yao and Ge, 2013), it is probably regulated by pituitary gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on the follicle cells to control follicle growth and maturation, respectively (Burns and Matzuk, 2002; Nagahama, 1994; Prat et al., 1996; Richards, 2001; Swanson et al., 1991; Weil et al., 1995). In mammals, FSH has been reported to increase the steady-state *Kitl* messenger RNA (mRNA) level in both cultured preantral granulosa cells (Joyce et al., 1999) and oocyte–granulosa cell complexes (OGCs) from murine preantral follicles (Thomas et al., 2005). LH, however, decreases the level of *KITL* mRNA in both human granulosa–luteal cells (Laitinen et al., 1995) and rat cumulus granulosa cells (Ismail et al., 1996). FSH and LH activate their cognate receptors (FSHR and LHCGR) on granulosa and theca cells (Hillier, 2001; Richards, 1994; Themmen and Huhtaniemi, 2000; Zhong and Kasson, 1994), and both receptors belong to the large superfamily of G protein-coupled receptors (Bogerd, 2007; Braun et al., 1991; Dias et al., 2002; Vassart et al., 2004). FSHR and LHCGR signal in the follicle cells mainly by activating adenylate cyclase (AC) to increase intracellular cyclic adenosine 3′, 5′-monophosphate (cAMP) levels (Sunahara et al., 1996). It is generally accepted that protein kinase A (PKA) is the major effector of cAMP signaling in various cellular systems (Kopperud et al., 2003). In mammals, cAMP has been demonstrated to induce *Kitl* expression, which can be blocked by H89, a potent inhibitor of protein kinase A (PKA) (Packer et al., 1994), suggesting that cAMP–PKA could be a potential downstream pathway to control *Kitl* expression. However, there is also evidence that PKA cannot mediate all actions of cAMP (Cass et al., 1999; Dremier et al., 1997). Increasing evidence has accumulated for the existence of cAMP-dependent but PKA-independent pathways in different cell types, one of which involves Epac (exchange protein directly activated by cAMP, including Epac1 and Epac2), a guanine nucleotide exchange factor (GEF) for the small G protein Rap (de Rooij et al., 1998; Kawasaki et al., 1998). It has been documented that the two cAMP-activated effectors can act synergistically (Hochbaum et al., 2008), independently (Cass et al., 1999) and even conversely (Gonzalez-Robayna et al., 2000). How these pathways are involved in the regulation of *Kitl* expression in the ovary is completely unknown, in particular with regard to their roles in the actions of gonadotropins.

One major function of the Kit ligand in the ovary is to suppress the maturation of oocytes in both mammals (Ismail et al., 1996, 1997) and teleosts (Yao and Ge, 2013). KITL treatment of full-grown rat oocytes resulted in a significant delay in the resumption of meiosis, which could be reversed by an antibody against the KIT receptor (Ismail et al., 1996, 1997). In agreement with the effects in mammals, recombinant zebrafish Kit ligand A (Kitlga) also significantly reduced the rate of spontaneous oocyte maturation or germinal vesicle breakdown (GVBD) in full-grown immature follicles (Yao and Ge, 2013). The physiological relevance of the inhibition of oocyte maturation by Kitlga is supported by its decreased expression in the late stages of follicle development prior to maturation (Yao and Ge, 2010). Because this occurs against a rising expression of LH receptor (*lhcgr*) (Kwok et al., 2005; Tse and Ge, 2009), which is known to promote oocyte maturation (Richards, 2001), it is conceivable that *kitlga* could be negatively regulated by LH from the pituitary. This is supported by several studies in mammals. In cultured human granulosa–luteal cells, the steady-state level of *KITL* mRNA was rapidly decreased by human chorionic gonadotropin (hCG, an LH analog) in a time- and dose-dependent manner (Laitinen et al., 1995). Consistently, hCG-induced meiotic resumption in oocytes was accompanied by a shift in *Kitl* expression from the membrane-bound form to the soluble form and a loss of expression of both forms in cumulus granulosa cells (Ismail et al., 1996). In the zebrafish, we recently showed that IGF-I was a potent regulatory factor that up-regulated the expression of *kitlga* in the follicle cells. The effect of IGF-I on *kitlga* was mediated via PI3K-Akt but not the MEK-ERK pathway although both were activated by IGF-I. In contrast, the MEK-ERK pathway seemed to play a negative role in controlling *kitlga* expression. The regulation of *kitlga* expression by IGF-I appeared to be stage-dependent with a greater induction at early stage, which was likely due a decreased signaling intensity of the PI3K-Akt pathway and a relatively stable or increased activity of the MEK-ERK pathway during folliculogenesis (Yao et al., 2014). Considering that gonadotropins (FSH and LH) are primary hormones that control the reproductive axis and their major second messenger is cAMP that also has two downstream pathways (PKA and Epac), we were curious about how cAMP and its two pathways are involved in controlling *kitlga* expression in the zebrafish ovary.

To answer this question, we undertook this study with the aim to investigate the effect of hCG on *kitlga* expression in cultured zebrafish follicle cells. We also tested the effect of pituitary adenylate cyclase-activating polypeptide (PACAP) as previous studies have shown that PACAP is stimulated by gonadotropins in the ovary (Barberi et al., 2007; Lee et al., 1999; Sayasith et al., 2007; Wang et al., 2003) and both LH and PACAP promote final oocyte maturation in mammals and fish through cAMP pathways (Apa et al., 1997; Wang et al., 2003; Zhou et al., 2011). Afterwards, we went on to focus the study on the intracellular signal transduction mechanisms that underlie hCG and PACAP actions with particular emphasis on the pathways downstream of cAMP including PKA and Epac pathways. We also examined the influence of the developmental stage of the follicles on the response of *kitlga* to cAMP and the cross-talk between the cAMP- and IGF-I-activated pathways. Most experiments in the present study were carried out in the primary culture of zebrafish follicle cells where *kitlga* and receptors of gonadotropins (*fshr* and *lhcgr*) and PACAP (Barberi et al., 2007; Vaccari et al., 2006; Zhou et al., 2011) are co-expressed.

2. Materials and methods

2.1. Animals and chemicals

Zebrafish (*Danio rerio*) were obtained from a local tropical fish market and maintained in flow-through aquaria at 28 ± 1 °C on a photoperiod of 14L:10D. The fish were fed twice a day with the commercial tropical fish feed Otohime S1 (Marubeni Nisshin Feed Co., Tokyo, Japan) and once with artemia. All of the experiments performed were licensed by the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. Unless otherwise indicated, all common chemicals used were purchased from Sigma (St. Louis, MO), USB Corporation (Cleveland, OH), GE Healthcare (Waukesha, WI), or Merck (Whitehouse Station, NJ); enzymes from Promega (Madison, WI); and culture medium from Gibco Invitrogen (Carlsbad, CA). hCG, recombinant human IGF-I, and forskolin were purchased from Sigma, dibutyryl-cAMP (db-cAMP), H89, LY294002 and wortmannin from Calbiochem (La Jolla, CA), and cAMP analogs Sp-6-Phe-cAMPS (Cat No. P018) and Sp-8-pCPT-2′-O-Me-cAMPS (Cat No. C052) were from BIOLOG Life Science Institute (Bremen, Germany). Zebrafish PACAP38-2 was generously provided by Dr. Anderson O.L. Wong (School of Biological Sciences, the University of Hong Kong) and its synthesis and characterization have been described previously (Wang et al., 2003). hCG, IGF-I, PACAP, db-cAMP and Sp-6-Phe-cAMPS were first dissolved in water, and forskolin, H89, LY294002, wortmannin and Sp-8-pCPT-2′-O-Me-cAMPS in dimethylsulfoxide (DMSO). They were diluted to the desired concentrations with the medium before use. Antibodies for Akt (#9272) and phospho-Akt (#4060) were purchased from Cell