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Exogenous GDF9 but not Activin A, BMP15 or TGFβ alters tight junction protein transcript abundance in zebrafish ovarian follicles

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ABSTRACT

The tight junction (TJ) complex plays an important role in regulating paracellular permeability and provides mechanical stability in vertebrate epithelia and endothelia. In zebrafish ovarian follicles, TJ complexes in the follicular envelope degenerate as the follicles develop towards maturation. In the current study, transcript abundance of claudins (*cldn d, g, h, 1, and 12*) and occludins (*ocln, and ocln b*) were assessed in mid-vitellogenic follicles in response to treatment with exogenous growth factors that are reported to be involved in zebrafish follicle development (i.e. Activin A, BMP15, GDF9 and TGFβ). Exogenous GDF9 reduced the transcript abundance of *cldn g*, *ocln* and *ocln b* in mid-vitellogenic follicles, whereas Activin A, BMP15, and TGFβ had no effect. Subsequent studies with GDF9 revealed that this factor did not alter TJ protein transcript abundance in pre-vitellogenic follicles but did increase the abundance of *ocln b* in fully grown (maturing) follicles. GDF9 was also seen to increase the abundance of *StAR* mRNA in all but primary stage follicles. These data suggest a role for GDF9 in the regulation of TJ integrity in zebrafish ovarian follicles, perhaps in the facilitation of ovulation, and support a previously postulated role for GDF9 in zebrafish ovarian follicle development. In addition, data also support the idea that endocrine factors play an important role in the regulation of TJ proteins during ovarian follicle development.

1. Introduction

Tight junctions comprise the apical most elements of the intercellular junction complexes in epithelial and endothelial tissues [31] where they encircle the apicolateral surfaces of adjacent cells and form a continuous seal between apical (mucosal) and basolateral (serosal) compartments [43]. Functionally, the tight junction (TJ) behaves as "a barrier perforated by aqueous pores" [42] and by doing so, regulates the paracellular flow of water and solutes between apical and basolateral compartments (i.e. barrier function). TJs also restrict the distribution of elements within the plasma membrane to either the apical or basolateral region (i.e. fence function) [38]. The principal transmembrane barrier components of the TJ complex are members of the tetraspanin family of peptides, which include occludin and claudins, with claudins being recognized as the major constituent [3,31,43]. Occludin was the first tetraspan peptide to be described in TJs and its expression levels correlate with the number of TJ strands in an epithelium [10]. Occludin occurs as a single ortholog in mammals, however at least two isoforms are found in *Danio* and *Fugu* [4,26]. Although occludin is an important component of the TJ complex in vertebrates, in mammals at least, occludin does not appear to be essential for the formation of TJs but is likely to be an important mediator of intracellular signal transduction as evidenced for example, by its association with ALK5 (TGFβR1) and TGFβ, as well as its reported roles in epithelial to mesenchymal transformation (EMT) and epithelial cell migration [2,3,8,22]. Claudins comprise the principal components of the TJ barrier and are the primary regulators of paracellular transport. Multiple orthologs of claudin have been described, including some 24 in mammals [3,43] and 56 in the puffer fish *Fugu rubripes* [26]. The differential expression of claudin orthologs determines the characteristics of the TJ complex [23] and while some claudins exhibit tissue-specific expression patterns [37] others are more ubiquitously expressed and possess general sealing properties [30].

In zebrafish (*Danio rerio*) the majority of studies involving TJ proteins have been undertaken from a developmental perspective. Knockdown of zebrafish claudin 15 (*cldn 15*) in wild-type fish and strains containing mutations in the MOD5 and GCKD genes, produce experimental animals with multi-lumenate guts [1]. Similarly, knockdown of *cldn j* leads to the development of embryos with defective otoliths and dysfunctional hearing [13]. Other studies have implicated TJ proteins in axonal growth (claudin 1 and the scaffolding protein ZO-1) [28] and epiboly (claudin E) [40], while claudin 5 and ZO-1 play a role in the development of blood–brain and blood-retinal barriers in zebrafish [16,46,47]. The role of TJ

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proteins in pseudo epithelial tissues such as the somatic envelope surrounding the oocyte in fish ovarian follicles is not well understood. A recent study reports that TJ proteins are broadly expressed in the ovary of zebrafish [4]. Of the 20 TJ orthologs reported in the zebrafish ovary, the abundance of transcripts encoding for *cldn a, b, d, g, h* and *19* (recently reclassified as *cldn 1*) varied differentially throughout follicle development and a number of these orthologs appear to be sensitive to maturation-related endocrine factors. For example, *cldn h, 10 and 12* demonstrated stage specific sensitivity to E_2, whilst in mid-vitellogenic follicles *cldn d* and *cldn g* mRNA abundance increased in response to hCG treatment. In addition to these observations, it was also reported that MIH significantly reduced the abundance of *cldn h* and *cldn* 1 (formerly *cldn 19*). These observations suggested that (1) endocrine factors may play an important role in regulating the molecular machinery of the TJ complex in fish ovarian follicles, (2) select TJ proteins (i.e. *cldn d, g, h, 1, 12, ocln* and *ocln b*) are hormone sensitive in this tissue and that these cell-cell adhesion factors may contribute significantly in the process of ovarian follicle development in zebrafish.

To further understand the role of the TJ complex in zebrafish ovarian follicles, the effects of exogenously applied growth factors would be of interest on the basis that several have been reported to play key roles in zebrafish folliculogenesis [7,11,21,25,45] and emerging evidence suggests that growth factors contribute to the regulation of TJ dynamics in diverse tissues [29,34,44,48]. More specifically, Activin stimulates meiotic maturation in mid to late vitellogenic zebrafish follicles [11,45], while Transforming Growth Factor β1 (TGFβ) and Bone Morphogenetic Protein 15 (BMP15) inhibit hCG and MIH induced zebrafish follicle maturation [6,7,21,20]. Furthermore, GDF9 is present in zebrafish ovarian follicles where it exhibits highest mRNA expression in primary follicles and declines as folliculogenesis progresses [25]. If the above knowledge is coupled with recent reports that GDF9 is involved in the regulation of TJ protein expression in Sertoli cells of mammalian testis [29,34], TGFβ regulates TJ proteins in the retina [44] and ovarian surface epithelial cells [48] and Activin regulates occludin expression in avian granulosa cells [39], a rationale for examining whether the application of exogenous Activin A, BMP15, TGFβ or GDF9 would alter TJ protein transcript abundance in zebrafish ovarian follicles emerges. Therefore, these studies offer the first insight into a potential role for growth factors in regulating the TJ complex during follicle development in fishes.

2. Materials and methods

2.1. Animals

Zebrafish were purchased from a local supplier and held in a 200 l tank at 26 °C under a 14 h light, 10 h dark photoperiod. Fish were allowed to acclimate for a minimum of 2 weeks prior to experimental use. At sampling, fish were anaesthetized in 0.5 g/l Tricaine Methanesulfonate (Aqua Life TMS, Syndal Laboratories, Vancouver, British Columbia) then decapitated. Ovaries were then excised into Cortland's media (Cortland saline supplemented with 1 g/l BSA and 100 IU/ml penicillin–streptomycin) and teased into separate follicles by repeated aspiration into a small bore transfer pipette. Follicles were staged according to Clelland et al. [7]; stage four follicles as being those which exceeded 0.69 mm diameter but which did not exhibit significant ooplasmic clearing or GVBD. Following *in vitro* incubation (described below), follicles were flash frozen in liquid nitrogen and stored at −80 °C until processing. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care guide to the care and use of experimental animals and an experimental protocol approved by York University Animal Care Committee.

2.2. PCR primer design

Primers for zebrafish glyceraldehyde phosphate dehydrogenase (GAPDH), occludins and claudins were designed as previously described [4]. Primers for zebrafish Steroidogenic Acute Regulatory Peptide (StAR) (For 5′-ctgagaatggacccacctgt-3′ and Rev-5′-gcaataaacgtcagcaagca-3′) were designed utilizing Primer 3 (http://www.frodo.wt.mit.edu/primer3/) and ClustalX (http://www.igbmc.u-strasbg.fr/BioInfo/) [41]. These primers produced a 222 bp amplicon of zebrafish *StAR* (Accession # NM131663), which was confirmed by sequence analysis (York University Core Molecular Facility). Endogenous StAR mRNA is highly expressed in pre-vitellogenic (stage 1) and vitellogenic (stage 3) ovarian follicles, but declines in stage 2 (cortical alveolar) and stage 4 (maturing) follicles [15].

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from follicles using TRIzol Reagent (Invitrogen Canada, Burlington, Ontario) and treated with DNase-1 (amplification grade, Invitrogen), following the manufacturer's protocols. Single strand cDNA was synthesized using 2 μg of DNase-1 treated template RNA and Superscript III (Invitrogen) reverse transcriptase. All cDNA was stored at -20 °C until required.

2.4. Reverse transcriptase-PCR and quantitative real-time PCR (qRT-PCR)

Routine RT-PCR was performed under the following conditions: 95 °C/4 min; 95 °C/30 s–59 °C/45 s–72 °C/30 s (30–35 cycles); 72 °C/5 min, using a MyCycler (Bio-Rad Canada, Mississauga, Ontario) thermal cycler. Amplicons were loaded onto 1.5% agarose gels, electrophoresed at 150 V for 50 min, stained with ethidium bromide and visualized using a Gel Doc XR system and Quantity Software (Bio-Rad). qRT-PCR was conducted according to [4] using the relative Ct (ΔΔCt) method (outlined in the ABI Prism 7700 Technical Bulletin # 2). Briefly, iQ SYBR Green Supermix (Bio-Rad) was used with a Chromo 4 (Bio-Rad) quantitative thermocycler. Reactions were performed under the following conditions: 95 °C/4 min; 95 °C/30 s–59 °C/45 s–72 °C/30 s (40 cycles), followed by melting curve analysis from 58 °C to 95 °C. GAPDH was used to normalize mRNA abundance according to an established protocol [6]. StAR primers were optimized and validated using serially diluted mixed stage follicle cDNA as previous described [4]. In the current study, transcript abundance for *cldn d, g, h, 1* and *12*, *ocln* and *ocln b* were assessed for all incubation studies; *StAR* was assessed for GDF9 follicle incubations only.

2.5. Incubation of zebrafish follicles with growth factors

For *in vitro* assays, ovaries from 8 females were excised and follicles isolated and staged as described (see Section 2.1 Animals). The follicles were incubated in 24-well culture plates (20 follicles per well, 4 wells per treatment) in Cortland's media (control) or media supplemented with growth factors for 24 h at 28 °C. Recombinant human Activin A (Cedarlane Laboratories Limited, Burlington, Ontario) was utilized at 100 ng/ml [45]. Recombinant human BMP15 (R&D Systems, Inc., Minneapolis, Minnesota) was used at a dose of 200 ng/ml [7] and recombinant human TGFβ1 (R&D Systems, Inc., Minneapolis, Minnesota) at 10 ng/ml [21]. Recombinant mouse GDF9 (R&D Systems, Inc., Minneapolis, Minnesota) was utilized at 100 ng/ml following a dose response analysis of follicles incubated with 25 ng/ml, 50 ng/ml or 100 ng/ml GDF9.

Initial studies were conducted using mid-stage 3 (mid-vitellogenic) ovarian follicles. Following incubation, follicles were scored for oocyte maturation [20], flash frozen in liquid nitrogen, and