Hypothesis

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Retinoic Acid Action in Cumulus Cells: Implications to Oocyte Development and In Vitro Fertilization

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Abstract: **In** the field of human in vitro fertilization (IVF), selecting the best oocyte for freezing or embryo for transfer remains an important focus of clinical practice. Although several techniques are and have been used for this goal, results have generally not been favorable and/or are invasive such that damage to some embryos occurs, resulting in a reduced number of healthy births. Therefore, the search continues for non-invasive oocyte and embryo quality markers that signal the development of high-quality embryos. Multiple studies indicate the important positive effects of retinoic acid (RA) on oocyte maturation and function. Here, we present evidence that part of these effects are via the ability of RA to regulate the activity of connexin 43 (Cx43), the main subunit of gap junction channels in human cumulus granulosa cells (CGC). These channels play an important role in regulating the micronutrient environment of the oocyte by allowing the transfer of ions, metabolites, and small molecules. Multiple studies have demonstrated the requirement for Cx43 in CGC for the normal progression of folliculogenesis, and that increased expression of this connexin is linked to improved developmental competence of the oocyte. The data has shown that RA can up-regulate gap junction intercellular communication (GJIC) in the cumulus-oocyte complex by a non-genomic mechanism that results in the dephosphorylation of Cx43 and enhanced GJIC. Recognizing the positive role played by gap junctions in CGC on oocyte development and the regulation of Cx43 by RA, the findings have highlighted the possibility that CGC RA levels may serve as a non-invasive indicator for selecting high-quality oocytes for IVF procedures. In addition, the data suggests that manipulation of Cx43 with retinoid compounds could provide new pharmacological approaches to improve IVF outcomes in cases of failed implantation, recurrent miscarriage, or in certain diseases that are characterized by reduced fecundity, such as endometriosis.

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1. The Search for High Quality Oocytes

In the field of human in vitro fertilization (IVF), selecting the best oocyte for freezing or embryo for transfer remains an important focus of clinical practice because it improves outcome. In the earliest days of IVF, enhanced selection was achieved by extending the time embryos were cultured from day 2, when embryos have about four cells, to day 3, when they have about eight cells, to the current practice of transferring blastocysts on day 5 or 6 of culture. This extended culture substantially increased the chance for successful implantation. However, with this approach the implantation rate still rarely exceeds 50%, even in the youngest patients. So other approaches to improve implantation rates by finding the best embryos continue to be pursued. Of these more recent attempts, preimplantation genetic testing has gained a foothold in clinical practice [1, 2]. In this process, a small number of trophectoderm cells are removed from blastocysts which are then assessed for ploidy status. When initially investigated, biopsies were done on day 3 (removing 1 or 2 cells of the 8) and evaluated by "fluorescence in situ hybridization". The results were not favorable. In more recent years, the biopsy procedure has been delayed to day 5 or 6 of culture when embryos have more cells (so the biopsy can be larger and have less impact), and the testing platform is typically next generation sequencing, which is both more accurate and comprehensive. In this iteration, randomized trials have shown a shorter time to pregnancy and fewer miscarriages [2]. However, this approach, now called "preimplantation genetic testing for aneuploidy" (PGT-A), is not without risk [3]. Risks include damage to some embryos, misdiagnosis of the true ploidy status (testing errors), false positives and negatives (due to mosaicism, the ploidy status of the inner cell

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may not be the same as the cells biopsied), and loss due to an extra cryopreservation step. It is therefore likely that PGT-A reduces the number of successful pregnancies from a set of embryos that would occur had this invasive testing not been done. Therefore, the search continues for non-invasive oocyte and embryo quality markers that signal the best embryo [4, 5].

2. Retinoic Acid Levels in Cumulus Cells as a Non-Invasive Predictor of Oocyte Quality

ultiple evidence indicates important effects of vitamin A compounds (retinoids) on oocyte maturation and function [6-9]. To this end, in vivo administration of retinoids in cattle, sheep and pigs has been shown to enhance oocyte fertilization competence [7, 10, 11] while in vitro studies identified all-trans retinoic acid (RA) as the active vitamin A metabolite in this activity [12-14]. Recognizing that cumulus granulosa cells (CGC) have tight connections to oocytes via their gap junctions, and based on prior work indicating a positive role for these connections in oocyte development [15, 16], more recent studies have focused on retinoids which support this intercellular communication through its effect on connexin 43 (Cx43) [17-20]. Concentrations of retinol (ROL, the substrate for RA synthesis) in the follicular fluid (FF) of women and animals have been reported by several laboratories [21-23] and our group directly quantified the FF levels of RA [24]. This work demonstrated that high follicular fluid RA concentrations at the time of oocyte retrieval in IVF protocols were associated with oocytes giving rise to the highest quality embryos [24]. Importantly, for follicles from which a mature egg was retrieved ("mature egg follicle"), an increase in the percentage of grade I embryos was observed across the tertiles of RA distribution; 57% of the embryos generated from mature oocytes in the highest RA tertile were classified as grade I versus only 18% of those derived from mature oocytes in the lowest tertile (**Figure 1**). As such, the follicular fluid RA concentration appeared to be an additional discriminating factor beyond the usual morphologic criteria that could be used for predicting successful oocyte fertilization and generation of high-quality embryos. Subsequently, we quantified RA and other retinoids in primary CGC from women undergoing IVF [17]. The results showed high levels of retinoids in CGC and the active biosynthesis of RA from its ROL substrate. These findings were compared with retinoid levels quantified in human endometrium and mammary glands, tissues previously shown to actively synthesize RA from ROL [25, 26]. In the endometrium, the action of RA is essential for proper decidualization [27] while in the mammary gland, RA signaling plays an essential role in ductal morphogenesis [28]. The data showed higher concentrations of RA in CGC compared with these other ROL metabolizing tissues indicating that CGC are a major target of retinoid uptake and an important source of RA production in the ovary. These studies demonstrated that CGC are a primary source of follicle RA biosynthesis, and that higher mean RA levels in the CGC of IVF patients yielded the highest percentage of successfully fertilized oocytes [17]. These findings were supported by Read and Dyce [18] who showed that bovine cumulus-oocyte complexes exposed to RA during maturation caused a significant increase in their maturation, cleavage, and blastocyst rates.

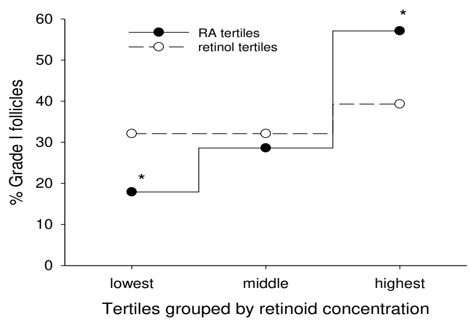

Figure 1. Percentage of grade I embryos derived from "mature egg follicles" (n=84) stratified by tertiles according to RA or ROL concentrations. Percentage of grade I follicles = # of grade I follicles /28 (number of mature egg follicles in each tertile). *Significant difference between values at $P < 0.005$. RA concentrations in pmol/mL for each tertile (mean±s.d.): lowest, 2.3 ±1.2; middle, 4.8 ±0.4; highest, 8.8±3. Reproduced from Figure 3 from Pauli SA et al.,[24].

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3. RA Can Promote Oocyte Competency Via Regulation of Cx43 in CGC

In terms of its action in the follicle, it has been suggested that RA may promote cytoplasmic maturation of oocytes via its modulatory effects on the gene expression of gonadotropin receptors, midkine (neurite outgrowth-promoting factor 2), cyclooxygenase-2, and/or nitric oxide synthase in CGC [7]. Oocyte competency is known to positively correlate with efficient gap junction intercellular communication (GJIC) among granulosa cells in the cumulus-oocyte complex. Cx43 is the main subunit of gap junction channels in human CGC and plays an important role in regulating the micronutrient environment of the oocyte by allowing transfer of ions, metabolites, and small molecules up to 1 kDa [15, 16]. In the ovary, Cx43 appears to be the only connexin contributing to gap junctions between the CGC of growing follicles [29, 30] while Cx37 is the main connexin expressed in oocytes that link them with the CGC. Although, the identity of the connexin in the granulosa cells that form the oocyte-granulosa channels is not known in humans, in mice those gap junctions were found to be composed of Cx37 in the oocytes linked with Cx37 exclusively expressed at the trans-zonal projections of the CGC [31, 32]. Multiple studies have demonstrated the requirement for Cx43 in CGC in order for the normal progression of folliculogenesis and that increased expression of this connexin, but not other connexins, is linked to improved developmental competence of the oocyte [16, 33]. In addition, patients with higher Cx43-expressing CGC cells were found to have more successful embryo transfer and implantation rates and were more likely to have a successful pregnancy outcome [16]. Ovarian Cx43 has been shown to be regulated by luteinizing hormone (LH); exposure of pre-ovulatory follicles to LH deactivates gap junctions through induced phosphorylation of Cx43 [34, 35]. This activity prevents the intercellular transport of cAMP, a meiosis inhibitor, leading to resumption of meiosis [36]. The importance of this activity was highlighted by showing that Cx43 knockout mice have impaired folliculogenesis as the follicles are unable to proceed beyond the pre-antral follicular stage [37]. In addition to its expression level, the phosphorylation status of Cx43 plays an integral part in processes that influence GJIC, including gap junction assembly and channel gating [38]. The overall effects of Cx43 phosphorylation on GJIC are dependent both on the cell type and the modified amino acid residues. In human cardiac cells, most studies have shown that phosphorylation of Cx43 by PKC correlates with reduced GJIC [38-41]. However, in guinea pig cardiomyocytes and transfected HeLa cells, increases in GJIC following PKC activation have been reported [42]. Cx43 phosphorylation within the same cell type can show opposite effects depending on the residues involved. For example, in human cardiac cells, Cx43 serine phosphorylation at serine (S) residues S364, S365, and S369 increased GJIC, while that at residues S262, S368, and S372, reduced GJIC [43]. In contrast, phosphorylation of Cx43 at S368 in folliculostellate cells increased GJIC [44]. Studies from our laboratory showed that RA can upregulate Cx43 activity and GJIC in human endometrial stromal cells (ESC) and CGC through a non-genomic mechanism that involves rapid dephosphorylation of Cx43 (**Figure 2**) [17, 20]. In ESC, this action was shown to be mediated through increased interaction of Cx43 with its primary phosphatase, protein phosphatase 2A (PP2A) [20] (**Figure 3**). A similar mechanism of action involving reduced phosphorylation of the ERK- and Akt-mediated pathways via actions of PP2A has been demonstrated for RA reduction of interferon-gamma and nitric oxide production in certain animal cells [45, 46]. The RA-induced dephosphorylation of Cx43 in ESC was shown to be at S262 [20], a residue of Cx43 whose phosphorylation is implicated in the GJIC-inhibitory action of certain growth factors (e.g. epidermal growth factor) and other PKC activators [47]. This action appears to be distinctive to ESC and CGC since RA-induced dephosphorylation of Cx43 has heretofore not been detected in other cell types. The determination that RA rapidly increases GJIC in CGC through this action on Cx43 provides a mechanism by which follicles and CGC containing higher levels of RA can increase the probability of generating high-quality grade 1 embryos. This hypothesis has been supported by studies showing that cumulus-oocyte complexes exposed to RA during maturation had significantly higher Cx43 expression correlating with increased gap junction coupling, and an increase in maturation, cleavage, and blastocyst rates [18].

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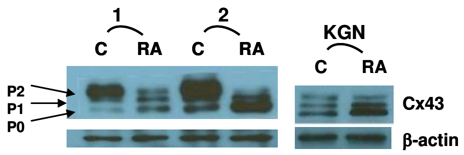

Figure 2. Effects of all-trans retinoic acid (RA) on Cx43 phosphorylation in cumulus granulosa cells. Total cellular protein was isolated from CGC and the human granulosa cell line KGN and assayed for Cx43 expression by western blotting. Representative experiments of primary CGC from two patients and KGN cells treated with 10 µM RA or vehicle control (C) as indicated for 48 h show changes in the band intensity and distribution of the non-phosphorylated (P0) and phosphorylated (P1 and P2) species of Cx43. The non-phosphorylated P0 band is the most biologically active form of Cx43 in these cells. Reproduced from. Figure 3A inset from Best MW et al.,[17].

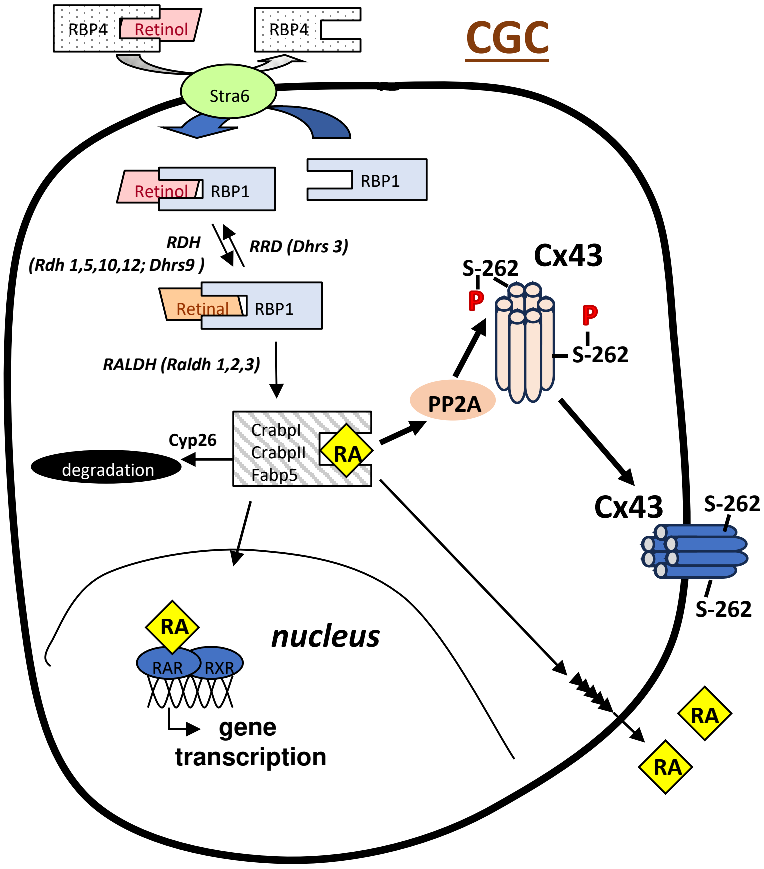

Figure 3. Schematic of retinoid metabolism and RA regulation of Cx43. Uptake of retinol (ROL) from circulating RBP4-bound retinol is controlled by the membrane receptor for vitamin A, Stra6. Intracellular chaperone cellular retinol-binding protein, type 1 (RBP1) physically interacts with Stra6 to pick up ROL and then delivers it to retinol dehydrogenase enzymes (RDH) which reversibly catalyze conversion of ROL to retinal. RBP1 then chaperones retinal to retinal dehydrogenases (RALDH) which irreversibly convert retinal to RA. For the canonical/genomic regulation of certain target genes, RA is then chaperoned into the cell nucleus by a distinct set of RA-binding proteins (CrabpI, CrabpII, Fabp5) to activate gene transcription. To regulate CGC gap junction activity, RA acts through a non-genomic mechanism to dephosphorylate Cx43 at the serine 262 (S-262) residue. This action is mediated through increased interaction of Cx43 with its primary phosphatase, protein phosphatase 2A (PP2A). Once formed, RA can be: (1) utilized in the RA-producing cell; (2) transported to neighboring cells (e.g., oocyte) to initiate RA-mediated signaling; and/or (3) degraded. RBP1 has been shown to be reduced in endometrial stromal cells from endometriosis patients leading to reduced RA production and Cx43 expression. .

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4. A Role for RA in Endometriosis-Associated Infertility Via Action on Cx43

Endometriosis affects more than 8 million women in North America alone and gives rise to a variety of symptoms, the most common being chronic pelvic pain and infertility [48, 49]. In IVF procedures, women with endometriosis ovulate fewer oocytes than healthy women while the oocytes ovulated by these women are often compromised [50-52]. Thus, it has been reported that fertilization and/or embryo cleavage rates after IVF, both in stimulated and unstimulated cycles, are significantly lower in women with endometriosis compared with healthy controls. Fertilization and embryo cleavage rates of endometriosis oocytes remain impaired after sperm from their partners are substituted with sperm from donors [53]. Additionally, implantation rates of oocytes from donors with endometriosis are reduced in recipients who do not have endometriosis [54]. We have determined that FF from women with endometriosis undergoing IVF have a significantly lower mean concentration of RA than control participants[24]. In contrast, ROL concentrations did not differ between the 2 groups, indicating that impairment of RA biosynthesis in the follicle, rather than uptake of the ROL precursor, is responsible for the reduced RA levels. This finding is consistent with our earlier data showing that in endometrial cells from endometriosis patients, dysregulation of the retinoid metabolic pathway results in reduced production of RA and plays a fundamental early role in the ability of the cellsto implant and grow at ectopic sites [25]. In those studies, we determined that a very early event in the ability of endometrial cells to form ectopic lesions is the reduced expression of cellular retinol-binding protein type 1 (RBP1; also known as Crbp1), a retinol chaperone protein that serves as the preferred substrate for retinol dehydrogenase enzymes as the rate-limiting step in RA biosynthesis [55] (**Figure 3**). Studies from our group have shown that expression of RBP1 is important for maintaining a normal flux through the retinoid biosynthetic pathway and is reduced in ESC from endometriosis patients [25]. Consistent with the role of RA in regulating Cx43 expression, we have demonstrated a direct correlation between reduced Cx43 expression in ESC from endometriosis patients and the diminished ability of the cells to synthesize RA from retinol (**Figure 4**). If the same relationship between RA production and Cx43 expression occurs in the CGC from these patients, this correlation would provide a mechanistic pathway by which reduced RA levels contribute to the decreased fecundity that is associated with this disease.

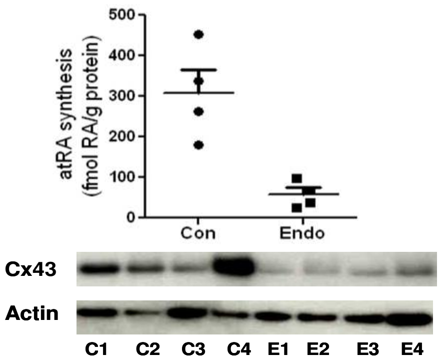

Figure 4. Synthesis of RA (top) and western blotting of Cx43 (bottom) in endometrial stromal cells from the eutopic endometrium (proliferative phase) of four control (C) and four endometriosis patients (E). RA levels were quantified in cells by liquid chromatography-tandem mass spectrometry following treatment with 2 μM retinol for 18 hr [17, 24]. In the absence of retinol, RA was not detected in all cases (unpublished data). .

5. Concluding Remarks

Our understanding of the role played by RA in the acquisition of oocyte competence may provide critical information for developing novel non-invasive approaches for selecting high quality oocytes for vitrification and/or use in single embryo transfers. As such, we propose studying CGC RA production as a predictor of oocyte competence. There are two reasons that make this goal especially appealing. First, we know RA plays a critical role in the acquisition of oocyte competence [7, 10-14, 56], and thus may mark that competence. Recent improvements in assessing RA activity now make it possible to evaluate the activity in individual follicles. This allows for the performance of individual oocytes to be evaluated and correlated with CGC RA levels and relevant clinical outcomes. Second, RA is on the causal chain influencing oocyte competence and thus may also be considered in therapeutic applications. Studying a biomarker that is also on the causal chain for producing healthy oocytes is an appealing approach. Through its action on Cx43, RA levels affect the formation of tight gap junctions within the cumulus-oocyte complex that promote the transfer of essential materials into the oocyte and improve their potential to produce a healthy pregnancy. Thus, RA is more than just a biomarker, it may also be a driver of the acquisition of that competence, offering the possibility that it could also become a treatment as well. In such a case, co-treatment with RA during folliculogenesis could improve patient outcomes. Although RA and its derivatives (e.g. isotretinoin, acitretin) have been shown to be efficacious and safe in a variety of clinical applications [57-59], they are highly teratogenic and they should only be used under strict monitoring of pregnancy prevention. However, the natural RA compound (all-trans retinoic acid) has a short half-life of only 1 - 2 hours [60] and could therefore be used during ovarian stimulation, withheld upon the hormonal trigger, and be gone by oocyte retrieval, eliminating any concern about teratogenesis. Obviously, the safety profile of this potential therapeutic application to improve IVF patient outcomes will need to be assiduously vetted in animal studies before clinical trials in human pregnancy could be considered. We look forward to these future studies with enthusiasm.

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