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MEDICAL HYPOTHESIS

Growth factors, gene activation, and cell recruitment: From intraovarian condensed platelet cytokines to *de novo* oocyte development

E. Scott Sills[1,2*], Samuel H. Wood[2,3]

[1]Reproductive Research Section, Center for Advanced Genetics, San Clemente, California, Unites States, [2]Department of Obstetrics and Gynecology, Palomar Medical Center, Escondido, California, Unites States, [3]Gen 5 Fertility Center, San Diego, California, Unites States

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**Corresponding author:*
E. Scott Sills
Reproductive Research Section, Center for Advanced Genetics; San Clemente, Department of Obstetrics and Gynecology, Palomar Medical Center, Escondido, California, Unites States.
Email: ess@prp.md

ABSTRACT

Background: Interest in decelerating or reversing reproductive aging is unlikely to diminish in the era of molecular genetics. For the adult human ovary, meeting the challenge of menopause without synthetic hormone replacement has now moved beyond proof-of-concept, as shown from treatments validated with standard metabolic markers and ovarian reserve estimates. However, without proper recruitment and differentiation of oocytes, such outcomes would be impossible. The full inventory of factors required for such folliculogenesis is not yet final, but growth differentiation factor-9, transforming growth factor-beta1, vascular endothelial growth factor, and insulin-like growth factor-1 are consistently identified as relevant. Platelet-derived growth factor and, more recently, bone morphogenic proteins are also central to cell migration, vascular support, and general ovarian function. Interestingly, when cells secreting these moieties are surgically grafted near undifferentiated oocyte stem precursors, the latency phase transitions to delineate follicle development and restoration of reproductive capacity. Direct intraovarian injection of condensed platelet-derived cytokines (a platelet-rich plasma/PRP product) likewise enables return of menses, ovulation, and term live birth.
Aim: This report extends our previous work on the proangiogenic effects of intraovarian PRP by connecting clinical responses to specific cytokine-dependent gene activation pathways likely needed to induce oocyte differentiation.
Relevance for Patients: Ovarian rejuvenation is a promising new application for platelet-rich plasma and/or condensed plasma cytokines of platelet origin, which are injected into older ovarian tissue.

1. Introduction

Experience with *in vitro* fertilization has provided repeated confirmation of oocyte primacy in the human fertility equation, as availability and competency of the egg correlate inversely with maternal age [1]. Yearly adult ovarian reserve losses are probably negligible until about age 30-35, with successful pregnancies declining sharply afterward [2]. Oocytes must be recruited and advanced through a precise maturational sequence to acquire the capacity for normal fertilization. Impairments in the precursor pool or interference in its required signaling ensemble will adversely impact reproductive outcome.

In the years since fertility medications first entered clinical practice, millions of babies worldwide have been delivered following their use. Of note, such pharmacologic agents work best when the ovary is sufficiently receptive to recruit additional eggs. On entering the selectable stage during the preceding luteal phase, ovarian follicles — if present — can acquire responsiveness to FSH [3]. With increasing age this cascade fails given the age-contingent gradual loss of early follicle targets capable of such response. In 2016, an innovative technique for "ovarian rejuvenation" was described aiming to reset this

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sensitivity [4]. Pantos *et al*. were the first to confront successfully the established teaching [4] which held that *de novo* oocytes cannot develop after the postnatal period [2]. Research in animal models had already suggested that oogenesis might be possible in adult mammals [5], yet if this were correct, an important follow-up question emerges next: What factors or conditions elicit the signals to favor building new oocytes?

2. Recruitment and Differentiation

How human primordial germ cells become competent oocytes has only been partially characterized, but this sequence is most likely under control of transcriptional regulators operant within a dynamic gene ensemble [6]. Among determinative signaling moieties involved in oocyte differentiation, the cytokine suite discharged during platelet activation seems crucial. In humans, advancing precursor cells into the oocyte pool shares some features with murine egg development. Both include somatic (mesodermal) gene activation early in embryogenesis, followed by preferential suppression of neural components and DNA methylation [7,8]. A common developmental feature in vertebrates is early segregation of the germ line from somatic cells. To detail this process more closely, Chatfield *et al.* [9] examined axolotl embryos as a tetrapod ancestor. This revealed primordial germ cells arising from within mesoderm under stochastic signaling, mediated by fibroblast growth factor and bone morphogenic protein (BMP)-4, showing conditional induction of these precursors [9]. Growth differentiation factor (GDF)-9 appears to be released both by platelets and oocytes, and is a potent regulator of folliculogenesis across several species [10,11]. Similarly, transforming growth factor-beta1 (TGF-β1), vascular endothelial growth factor (VEGF), and insulin-like growth factor one (IGF-1) are well represented in platelet products after activation [10,12]. For BMP-4 specifically, Fujiwara *et al*. [13] established this signal as critical in induction of oocyte precursors and allantois in adjacent epiblast, as homozygous BMP-4 null mutants showed complete absence of both cell types. BMP-6, BMP-15, and others in this protein family help orchestrate folliculogenesis, including regulating granulosa cell sensitivity to gonadotropins, controlling apoptosis, and coordinating follicle support and eventual ovulation [14-17].

Zhou *et al*. [18] recently examined BMP-11 (also termed GDF-11) in mice to define its contribution to mammalian ovarian function, reporting that dietary intake of rec-GDF-11 ameliorated cellular aging in the ovary. Such BMP-11 use in reproductive biology was a logical continuation of prior work where this intervention had already corrected age-related myocardial hypertrophy [19], improved brain capillary and muscle function [20,21], successfully deferred production of age-specific biomarkers [22], and even extended lifespan of experimental animals [23]. Reassuringly, murine response to exogenous BMP-11 was confined to functional enhancement of ovarian tissue, with negligible effects on gross body mass, gonadal weight, or overall metabolism [18]. Given known platelet dynamics and recuperative ovary effects reported in clinical fertility practice, it is unsurprising that BMP-11 is among the molecular secretome elements which are locally available on platelet activation [24].

Ovarian stem cells localize to sub-epithelial regions and are the source material for primordial follicle assembly and oocyte development [8]. Over time, human ovarian tissue gradually acquires an altered microenvironment unable to support differentiation which otherwise could lead to oocytes [25]. As aging progresses, cells increasingly feature a senescence-associated secretory phenotype which includes several pro-inflammatory factors [26]. For example, theca-interstitial cell senescence is associated with higher ambient levels of chemokine C-C-motif ligand 5 (CCL5) with further aging [27]. Accumulation of CCL5 in the follicular microenvironment is reproductively significant, as this promotes granulosa cell apoptosis, restricts preantral follicle growth, and dampens estradiol output [27]. Preferential expression of certain TGF-β ligands and receptors has been localized to mural granulosa cells (MGCs), and TGF-β significantly increases gene and protein levels of natriuretic peptide type C (NPPC) in MGCs cultured *in vitro* [28]. More recently, MGCs have been shown to secrete NPPC through guanylyl cyclase-linked natriuretic peptide receptor 2, to maintain eggs in meiotic arrest [28].

Animal research has isolated candidate growth factors which coordinate and direct oocyte maturation through oolemma binding. In particular, C-X-C motif chemokine ligand 12, VEGF-A, and Wingless-type MMTV integration site family member 5A/WNT5A, all have been identified as maternal cytokines needed for oocyte maturation [29]. Another moiety, platelet-derived growth factor, evokes connexin 43 (Cx43) expression through β-catenin, the latter as a recognized nexus for numerous signaling pathways [30]. Augmented Cx43 expression has been confirmed in an enriched human platelet lysate milieu [31]. Other research has helped characterize the constellation of switching elements involved in advancing primordial germ cells towards oocyte commitment. For example, WNT3 induces many transcription factors closely associated with mesoderm in pluripotent epiblast-like cells, likely mediated by β-catenin. Furthermore, relevant is a highly-conserved mesodermal signal "T" (Brachyury), which activates two known germline determinants — Blimp1 and Prdm14 [32].

Interleukin-7 (IL-7) is another component of platelet releasate [33] with a role in oocyte development and maturation confirmed in mice [34]. As part of a complex signaling cascade, IL-7 itself can promote proliferation and secretion of interferon-γ, tumor necrosis factor-α, and IL-10, which appear to regulate genes central to oocyte maturation [33,35]. Interestingly, when the oocyte-specific homeobox gene (NOBOX) is silenced, the murine postnatal oocyte pool is severely curtailed by interrupting the transition from primordial to growing follicles. Echoing features of human menopause and diminished ovarian reserve, normal follicles in mice are overtaken by fibrosis if NOBOX is missing. Rajkovic *et al*. [36] reported that NOBOX-knockout results in steep downregulation of Oct4 and Gdf9, genes preferentially expressed in oocytes [36]. Of note, these pluripotency markers (along with SOX2, SALL4, and NANOG) are amplified following local platelet-rich plasma (PRP) exposure [10,37]. At present,

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two methods are known to initiate this sequence by intraovarian dosing (Figure 1).

3. Platelet Cytokines: Merits and Misgivings

The reprogramming or recruitment actions presented here all depend on signals reaching ovarian stem cells. In practice, varied techniques are already being used to place PRP (or its derivatives) within ovarian tissue, either through laparoscopy or by ultrasound-guided needle injection. While several approaches have been successful, equivalence or superiority has not yet been established. Despite many years of safe PRP experience in other clinical areas, acceptance of intraovarian PRP is understandably muted until a mechanism of action specific to reproductive targets is established and confirmed [38].

The first ovarian PRP clinical trial was a proof-of-concept study measuring ovarian reserve as estimated by serum AMH, and the treatment did produce significant AMH increases in >25% of patients after intraovarian PRP [39]. The similarity in post-treatment AMH response as a function of bilateral versus unilateral ovary injection raised more questions than answers. Because baseline platelet concentration was noted to influence serum AMH response after intraovarian PRP [40], claims that sham injection alone is sufficient to evoke a response are difficult to validate.

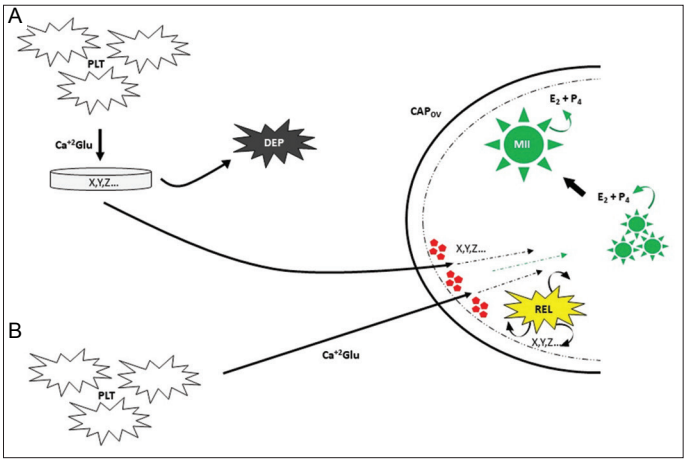

Figure 1. Outline for "ovarian rejuvenation" and *de novo* oocyte recruitment through sub-cortical injection of autologous platelet-rich plasma (PRP) and condensed plasma cytokines. Sample preparation methods include platelet-derived cytokines isolated after *in vitro* platelet (PLT) culture **(A)**, and conventional PRP injection **(B)**, both utilizing calcium gluconate for PLT activation. For method A, depleted platelets (DEP) are subtracted after concentration of releasate consisting of PLT-derived signaling moieties (X, Y, Z…). In B, activated platelets (yellow) arrive intact as PRP within ovarian tissue to secrete a cargo protein complex. For both techniques, surgical placement of specimen is by needle insertion including cortex and subcapsular space (CAP_{OV}). Distribution of ovarian germ cells (red) permits local exposure to PLT-derived growth factors and promoters, which direct noncommitted precursors to develop into early preantral follicles (green). Regained functionality leads to cyclic production of estradiol and progesterone ($E_2 + P_4$), increased anti-mullerian hormone output, and finally, arrival of competent *de novo* metaphase II (MII) oocytes.

One cautionary theme concerns PRP potentially initiating tumorigenic changes if the procedure stimulates or modifies ovarian stem cells. This likely reflects only a theoretical risk, as it has never been observed in any clinical context where pluripotent cells are nearby. Moreover, since platelet cytokines interact with cell membrane receptors — not the nucleus — the physiologic role is unlike trophic hormones [8]. Further reassurance comes from experience with other tissues treated with PRP, where optimized growth of healthy cells was noted with no induced malignancy [40].

4. Conclusions

Human platelet outputs embrace a complex set of biologicals, including lipids, mRNAs, and miRNAs. These constituents make their own contributions to oocyte recruitment from ovarian precursors; transcriptional signature analyses [41] are one way to inform future clinical practice. Grafting of mesenchymal stem cells has been completed with good results in animal research as well as in experimental human settings [42]. Although the roster of cytokines generated by such mesenchymal implants awaits full characterization, placing such cells near ovarian stem cells means any latent oocyte precursors can avail of programming inputs essential for *de novo* oocyte differentiation [8,42]. Considering the overlap with platelet releasate, this likely explains how intraovarian injection of platelet cytokines can restore regular menses [43] as well as achieve term live births [44-46]. Among all elements modulating the genetic controls on ovarian stem cells, the platelet-derived cytokines discussed here appear central. However, we agree with Chang *et al*. [47] regarding the importance of growth factor research to improve accuracy in diagnosis and efficacy in treatment.

Conflict of Interest

ESS has been awarded U.S. Trademark #6009685 for relevant intraovarian technology.

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