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Mirage or miracle—Intraovarian autologous platelet cytokines for infertility and menopause

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Running title: Platelet factors and ovarian rejuvenation

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Abstract

On a bleak therapeutic landscape unchanged since the 1980's, IVF with egg donation still stand as the lone medical answer to diminished reserve and premature ovarian insufficiency. Intraovarian platelet-rich plasma (PRP) crossed the horizon in 2016 as hopeful answer to these intertwined problems. The once remote mirage of platelet cytokine effects on gene regulation or telomere stabilization is now in sharper focus, and current work is clarifying how PRP corrects oxidative stress, rectifies tissue hypoxia, downregulates apoptosis, and enhances cellular metabolism. Not yet ready for routine use, this investigational treatment does offer one point of general agreement: How intraovarian PRP results should be classified—Patients are either responders or non-responders. From this, it is intriguing that no published PRP protocol has reported a supranormal ovarian rebound or hyperstimulation effect. This could be explained by baseline age-related ovarian conditions prevalent among poor responders, but since dysregulated or malignant transformations are also absent in other tissue contexts following autologous PRP treatment, the contribution of some platelet product which intrinsically delimits regenerative action cannot be discounted. Here we summarize results with recent experimental and clinical platelet research, framing those most likely to help advance reproductive endocrinology practice.

Key words: ovary; PRP; senescence; rejuvenation; hypoxia; telomere length

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Now I am ready to tell
how bodies are changed
into different bodies.

Descend again, be pleased
to reanimate this revival
of those marvels.

Show now exactly
how they were performed
from the beginning,
up to this moment.

The Metamorphoses, Book I
Ovid (43 B.C. - 17)

Background

Nilsson *et al* (2006) were among the first to describe how cross-signaling by multiple growth factors can modulate both rate of transition and developmental pace of ovarian follicles. Their work enlarged the understanding on how ovarian tissue cultured with platelet (PLT) derived growth factor (PDGF) elicits increased Kit ligand expression (Parrott & Skinner, 1999). Another important product of PLT activation is basic fibroblast growth factor (Qian *et al*, 2017), which prompts primordial follicles to transition via Kit ligand (Nilsson *et al*, 2001; Nilsson & Skinner, 2004). Classifying autologous PLT releaseate as a recuperative construct for inactive or senescent ovaries (Pantos *et al*, 2019) invites a new way to boost precursor cell populations while dampening apoptosis (Moussa *et al*, 2017). Numerous cytokines of PLT source with relevance to ovarian tissue have now been catalogued (Wood & Sills, 2020). For connective tissue, PRP secures this this by dose-dependent declines in MMP3, MMP13, and ADAMTS-5, IL-6 and COX-2 while increasing TGF-β, type-2 collagen, and intracellular IL-4, IL-10, IL-13 (Moussa *et al*, 2017). But how might such mediators perform in adult ovarian tissue?

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Signaling renewal

Influenced by experience in blood banking, growth differentiation factor-9 (GDF-9), transforming growth factor-beta1 (TGFβ9), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1) (Bieback *et al*, 2019) are receiving attention in functional ovarian studies. PDGF and the BMPs are consistently central to cell migration, vascular support, and general ovarian function. Remarkably, when these moieties are secreted by tissue grafted adjacent to undifferentiated oocyte stem precursors, the latency phase transitions to delineate follicle development and restored reproductive potential. Direct intraovarian injection of these derivative platelet cytokines can similarly restart menses, ovulation, and enable term livebirth (Sills & Wood, 2022). PRP action was recently observed in rat chondrocytes (Yang *et al*, 2021) by expressing hypoxia-inducible factor 2α (HIF-2α) using small-interfering RNA (siRNA) specific for HIF-2α and adenovirus-mediated HIF-2α overexpression, measured with matrix metalloproteinase (MMP)-1, 3, -9 and -13, and extracellular matrix-related genes. Using this design, 10% PRP offered chondrocyte protection against IL-1β-induced apoptosis and matrix loss, while also suppressing HIF-2α activation, illustrating how PRP-mediated chondroprotection works via HIF-2α targeting (Yang *et al*, 2021). Specific tissue targets could respond to varied PRP dilutions (Sills, 2022) so this too invites notice in reproductive biology.

Using a defined dose protocol in a controlled animal research setting, PRP alleviated ovarian damage and preserved ovarian reserve after tissue hypoxia (Bostancı *et al*, 2022). This rescue via platelet-derived cytokines to improve perfusion and ovarian follicular oxygen (Wood & Sills, 2020) awaits to be directly observed, but the conclusions of Bostancı *et al* (2022) are likely appliable for human ovarian tissue.

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Recruitment & assembly

After puberty, primordial follicles in the ovary are mainly dormant with only a select few moving to maturation and further selection for mono-ovulation each month (Zhao *et al*, 2018). Cell recruitment, follicle assembly, and oocyte development are orchestrated by local growth factors such as Kit ligand, leukemia inhibitory factor, bone morphogenic proteins, keratinocyte growth factor, and basic fibroblast growth factor (Skinner, 2005; Sills & Wood, 2019). This is contingent on mammalian target of rapamycin complex 1 (mTORC1)-Kit ligand (KITL) signals within pre-granulosa cells, and receptor (KIT)-phosphoinositol 3 kinase (PI3K) oocyte signaling. Zhao *et al* (2018) identified a phosphorylated mitogen-activated protein kinase3/1 (MAPK3/1) protein expressed in all growing follicles and in some primordial follicles (Zhao *et al*, 2018). The presence of MAPKs in PLTs has been known for decades, and much published work has accumulated since then to describe conditions for MAPK-mediated PLT activation—but not without contradictory conclusions (Patel & Naik, 2020). In latent follicles, the MAPK3/1 protein engages the mTORC1 signaling pathway to start maturational change (Zhao *et al*, 2018). Later, Li *et al* (2020) reported on a phosphorylated cAMP response element-binding protein (CREB), activated by the MAPK3/1–mTORC1 signal, bound to the promoter region of the receptor protein tyrosine kinase ligand (KITL) enhancing its expression. Blunted primordial follicle activation with accelerated oocyte apoptosis resulted from adding CREB inhibitor KG-501 and CREB siRNA (Zhang *et al*, 2021). Notably, KG-501 and CREB knockdown significantly dampened phosphorylated Akt, reducing oocyte number. From this, it was seen that CREB is needed for MAPK3/1-mTORC1 signal-promoted KITL expression followed by activation of primordial follicles (Li *et al*, 2020). To explore the MAPK3/1 signal system in primordial follicle activation, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) inhibitor bpV(HOpic) was studied. MAPK3/1 was seen to contribute to primordial follicle activation via mTORC1-KITL signaling (Zhao *et al*, 2018), but upstream regulators of mTORC1 signaling have not been fully characterized.

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Consistent with its previously discussed anti-inflammatory actions (Sills & Wood, 2019), PRP modulates and occasionally squelches completely the TNFα induction of NF-κB-responsive genes (CCL5 or RELA), and interferon signatures with blunted IRF1, MX1, or MX2 expression (Zahir *et al,* 2021). Pathway mapping in PRP-treated fibroblasts (compared to controls) confirmed modulation of functional pathways for cell survival with repressed ATF4, ATF6, and PI3K/AKT signaling vs. untreated controls.

With an emphasis on the PI3K/Akt/GSK3β signal pathway, Ma *et al* (2021) reported on a PRP intervention which promoted cell proliferation, reduced markers of cellular stress, increased antioxidant enzyme activity, and attenuated DNA damage in experimental cells. A technique to activate dormant adult mammalian ovarian follicles was proposed involving the PTEN/PI3K/AktFoxo3 pathway (Li *et al,* 2010). This was successfully used in mice with oocyte-specific gene deletions (Li *et al,* 2010). Working with human oocytes, Danish experts explored transcriptome dynamics in primordial follicles during directed activation (Ernst *et al,* 2017). Theirs was the first work to identify new pathways governing egg dormancy and activation, where differential regulation involving >400 genes influenced the primordial-to-primary ovarian follicle maturation (Ernst *et al,* 2017).

However, culture of human ovarian tissue with PI3K/Akt activators could bring untoward effects on follicle function vs. spontaneous follicular growth, as seen from low E_2 production post-treatment. In contrast, transient incubation with an mTORC1 inhibitor partially blocks spontaneous activation by curtailing follicular growth, granulosa cell proliferation, and Kit ligand expression with unchanged steroidogenesis (Gabardi & Baroletti 2010). Functional connections exist between PI3K/Akt/mTOR and Hippo pathways, but any gain in manipulating this sequence in the ovary must be balanced against the risk of sparking a sudden, diffuse collapse of the entire follicle stock (Grosbois & Demeestere, 2018). Indeed, mice without PTEN (phosphatase & tensin homolog deleted on chromosome 10) in oocytes—a major PI3K suppressor—produce this 'flash activation' where the entire primordial follicle set is at risk. In response, pharmacological inhibition has been applied to the PI3K/Akt/mTOR pathway as a way to temper spontaneous follicular activation, thus permitting a more controlled, physiologic response (Gabardi &

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Baroletti, 2010). Primordial follicle activation thus embraces many ovary research themes, including phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and Hippo signaling (Ford *et al*, 2020). The mammalian oocyte itself guides follicle activation, and this oocyte PTEN/PI3K path governs follicle activation by controlling oocyte growth (Reddy *et al*, 2008). This growth requires proper calibration, as oocytes with uncontrolled PI3K action will irreversibly transform granulosa cells into a tumorigenic phenotype (Kim *et al*, 2016). Across all stages of follicular development, c-Myc expression during local ovarian tissue remodeling accompanies luteolysis and atresia in an apparently pro-apoptotic function (Nandedkar & Dharma, 2001; Seyyed Anvari *et al*, 2019). This signal is included in the PRP cytokine suite which was observed to ameliorate hormone imbalances in a murine polycystic ovary syndrome model (Seyyed Anvari *et al*, 2019).

A synergy between Hippo and PI3K/Akt/mTOR signaling appears to evoke early primordial follicle recruitment in thawed human ovarian tissue (Grosbois & Demeestere, 2018), and pregnancies have been achieved after grafting ovarian fragments cultured with PI3K/Akt activators into patients diagnosed with premature ovarian insufficiency (Kawamura *et al*, 2013). This activation is a tightly controlled, closely regulated process entailing bilateral communication between oocytes and granulosa cells. The latter group maintains proper oocyte metabolism and directs development (Sills & Wood, 2019). Likewise, oocytes send signals to regulate differentiation and function of their granulosa cells. This prevailing ovarian microclimate includes the theca compartment, vascular elements, and extracellular matrix which collectively influence follicular dormancy and activation (Wood & Sills, 2020; Zhang *et al*, 2021). A paracrine restorative effect and active Hippo signaling appears to be possible without major surgery (Zhang *et al*, 2019). When stem cells derived from menstrual blood, PRP, and both together were studied, expression of human-derived secretory protein IGF-1, SDF-1, and TSP-1 was confirmed. All three therapy methods improved fertility with gene expressions for cell proliferation, development, and Hippo signaling recorded. While CTGF, Wnt5a, and Gdf5 were significantly upregulated, the observed response was synergistically enhanced by PRP (Zhang *et al*, 2019).

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Further study of Hippo disruption understandably prefigured theoretical use in clinical fertility practice (Reig *et al*, 2021). After encouraging murine data, human clinical trials (Kawamura *et al*, 2013) blended stimulation of ovarian Akt signaling in excised cortex fragments with disruption of Hippo-signaling (Hsueh *et al*, 2015). Cortical ovarian fragments taken from POI patients were frozen, warmed, diced into 1-2mm^2 cubes and then incubated with PTEN-inhibition + PI3K-Akt-stimulation x2 days, followed by surgical re-grafting to tubal serosa (Griesinger & Fauser, 2020). Targeted stimulation of *in vitro* follicles by Hippo-signal disruption persists as an emerging research theme, although this technique has other objectionable aspects (Griesinger & Fauser, 2020) not seen with PRP. For example, the lack of requirements for laparoscopy favors intraovarian PRP, since activated PRP may be injected to ovarian tissue by direct ultrasound guidance without need for sedation or anesthesia. Second, directed inhibition or promotion of protein kinases is unnecessary when the injected sample is autologous, as with PRP or its derivatives. Concerns about cancer risk from pharmacologic *in vitro* activation and Hippo signal disruption are valid, but experience with PRP when used in multiple tissues has never caused malignant transformation (Sills, 2021). Finally, *in vitro* ovarian tissue culture is not a feature of any known clinical fertility PRP protocol (Sills & Wood, 2022).

Oocyte quantity vs. quality

An equally important challenge in reproductive endocrinology practice is the inverse relation between oocyte quality and maternal age, where incidence of embryo aneuploidy rises sharply over time. In human polar bodies, reduced telomere length is associated with genetic imbalance (Treff *et al*, 2011) and telomerase activity is substantially lowered when ovarian function is impaired (Xu *et al*, 2017; Polonio *et al*, 2020). Although data from an ovarian PRP context is pending, authors working in cosmetic dental surgery have reported on telomere measurements after treatment with enriched platelet factors. Not only were correlations noted between PLT-based therapy and increased telomere length, but the extent of this effect was also related to number of treatment events (Nacopoulos *et al*, 2019). If reversal of telomere loss

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were likewise attained by locally administered intraovarian PLT cytokines, this would offer a plausible mechanism for embryo 'ploidy rescue' with IVF (Sills *et al*, 2019).

One mechanism not yet associated with intraovarian PRP effects is DNA methylation (*i.e.*, epigenetic alterations regulated by DNA methyltransferases). For mammals, this involves covalent transfer of methyl groups from S-adenosyl-1-methionine (SAM) to cytosine within CpG islands (Fouad *et al*, 2021). In this process, gene expression is suppressed by steric interference with transcriptive access to chromatin or by recruitment of methyl binding proteins (Romero-Garcia *et al*, 2020). Methylation is a dynamic process much more likely to be influenced by diet, environment, or exercise patterns than by PLT cytokines, but this remains an intriguing issue for future study. What seems more plausible, as discussed here, is that instead of PLT releasate inducing specific morphological changes, it affects expression patterns of genes directing tissue regeneration (Fukuda *et al*, 2017).

Le Rolle *et al* (2021) described crosstalk between nuclear β-catenin and a germ cell pluripotency factor, POU5F1. Specifically, passage of this complex outside the nucleus correlated with germ cell differentiation, promoted by up-regulation of Znrf3 which itself inhibits WNT/β-catenin signaling. The Valrose Institute team characterized this molecular pathway to shepherd the primordial germ cell transition forward to oogonia (LeRolle *et al*, 2021), revealing how β-catenin is a key conductor of *de novo* oocyte construction. This mediator is retained in PRP, being among the discharge products following granule exocytosis accompanying PLT activation (Heijnen *et al*, 1999; Liu *et al*, 2019).

Open topics

Adenosine can either prompt proliferation or induce apoptosis, depending on the target cell (Merhigi *et al*, 2002). A2a-adenosine receptor (A2bAR) mediates PLT response in mouse megakaryocytes and PLTs expressing A2bAR transcripts. The gene displays an *in vivo* uptick after injury or inflammation, when A2bAR-mediated inhibition of PLT aggregation increases (Sirotkin, 2011). Distribution of adenosine

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receptor in human ovaries is not well mapped, but earlier work confirmed its expression in rat oocytes (Li et al, 2001). Another driver in recruitment of functional PLTs from megakaryocytes is thrombopoietin. Given the inducible nature of A2bAR, Folman *et al* (2000) reported on potential upregulation of this gene by thrombopoietin and found this also applied to megakaryocytes. PLTs themselves are thrombopoietin reservoirs, most likely stored in PLT granules (Folman *et al,* 2000). Activated PLTs release thrombopoietin where mammalian research suggests a 'physiological filter' can sort cells by inducing proliferation of viable cells or scavenging non-viable cells (Sarkar *et al,* 2011).

The degree to which PLT cytokines may modify gated ion channels or alter cellular messaging in ovary/oocyte is not well studied, although membrane transport of 5-hydroxytryptamine (5-HT) after PLT activation in non-reproductive tissue reveals potential both to absorb or release this signal under conditions of reduced perfusion via 5-HT(3) receptor (Fu *et al,* 2002). Given numerous serotonin-specific receptors already localized to ovarian tissue and oocytes (Dube *et al,* 2007), PLT cytokines may exert a comparable effect here as well. Gated membrane channel function is largely driven by ambient oxygen conditions (Lopez-Barneo *et al,* 1988), and since the senescent or non-responsive ovary is more likely to suffer from reduced tissue perfusion (Wood & Sills, 2020) the role of such ion channels in cellular bio-conductivity is not trivial. Animal PRP/collagen and PRP/thrombin studies found multiple sympathetic afferents responding via 5-HT(3) receptors (Fu *et al,* 2002). The intracellular chloride channel works in response to mitochondrial stress (*i.e.,* increased reactive oxygen species), which next promotes NLRP3 in both mouse and human cell lines (Tang *et al,* 2017). Because NLRP3 is partially under PLT control this suggests electrophysiologic parameters might be changed by autologous PRP (Cornelius *et al,* 2019) independent of any effects by $CaCl_2$ or calcium gluconate, often used for PLT activation.

Referencing ‘ovarian rejuvenation’ protocols now available, small volume fresh samples are generally preferred over frozen PRP. While freezing can trigger PLT activation after thaw (Fukuda *et al,* 2017) it is possible this technique produces a different growth factor discharge profile compared to fresh releasate. Any hesitancy could change if prior PLT lysate research where specimens using PLTs at 21d

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stimulated fibroblast proliferation the same as if prepared from 2-day old PLTs (Chan *et al*, 2005) is verified by parallel work in the ovary. It may be that while expired PLTs cannot sustain hemostasis, they are still able to emit growth factors for use in other tissue applications (Chan *et al,* 2005).

To study gene regulation patterns in menopause and define reproductive fitness, Shin *et al* (2022) recently cataloged proteins by chronological age, menopausal age, and post-menopause status. Their team found growth differentiation factor-15, insulin-like growth factor binding protein-2, and tumor necrosis factor-α to be positively correlated with chronological age. In addition, they developed parsimonious multiprotein models to clarify the proteomic signature for aging, with key associations measured among GDF15, IFN-γ, IGFBP-2, IGFBP-7, IL-15, IL-1β, IL-17A, IL-8, MCP-1, TIMP-2, TNF-α, VEGF-A, and IP-10 (Shin *et al*, 2022). These observations are especially notable given the experience with a PRP protocol with PLT activation via 10% $CaCl_2$ on human dermal papilla (hair) cells, where differentially expressed and cross-interactive genes were measured during cell proliferation (Shen *et al*, 2017). As noted previously (Sills & Wood, 2022) many of these signaling factors appear in the cytokine suite of fresh PLT releasate upon activation (see Figure 1). Culture media supplemented with PRP is generally recognized to block apoptosis by TGF-β via downstream expression of DAPK1 and BIM (Jang *et al,* 2002). Subsequent gene expression analysis extended PRP regulation to TNF signaling operant via transmembrane receptors TNFRSF10A, TNFRSF25, TNFSF10, TNFRSF9, and TRAF2, and of the Bcl protein superfamily with its related proteins including BAG4, BNIP1, and HRK (Fukaya *et al*, 2012). Evidence now suggests that germ cells fated for apoptosis still make contribution to developing eggs, supporting oocytes through cytoplasmic streaming (Chartier *et al,* 2021). Perhaps the best experimental data that germ cell apoptosis has relevance to oocyte quality during reproductive aging comes from blocked apoptosis (via ced-3 or ced-4 mutation). This interruption yields smaller oocytes from older mothers and increased embryonic death of their offspring (Andux & Ellis, 2008). A protective role has been proposed as similar to what happens in cellular senescence. In other words, the shared effects of aging and response to environmental stress may be explained by a tendency to maintain best-competency gametes, even under non-ideal conditions (Fausett *et*

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al, 2021). Thus germ cell apoptosis seems to help regulate oocyte quality under physiologic stress even if that means absolute egg number declines, a process akin to that observed in maternal aging (Fausett *et al*, 2021).

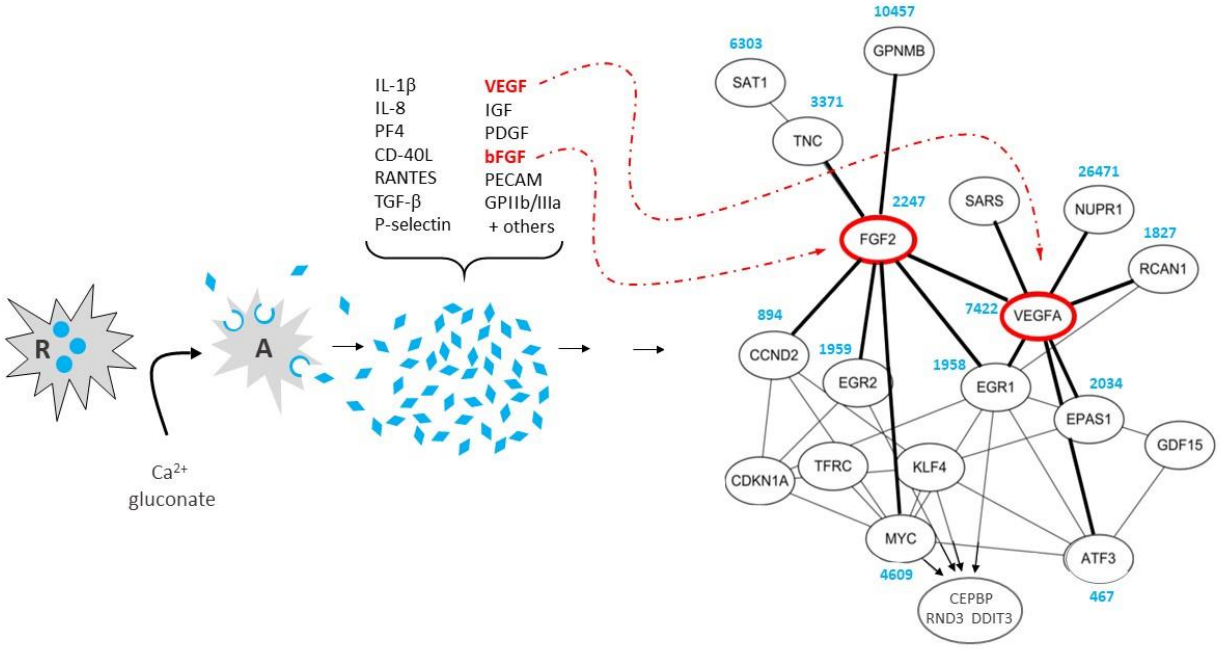

Figure 1. Provisional partial signal multiplier effects with intraovarian PRP. Fresh platelets (PLTs) at rest (R) react with calcium gluconate for activation (A), followed by α-granule exocytosis (blue circles) and cargo protein/cytokine efflux (blue dots). Common PLT releasate components are bracketed with links shown for 2 representative PLT-derived growth factors: VEGF and bFGF (red dashed lines). Gene targets are depicted within a human protein-protein interaction network as reported by Shen *et al* (2017) after 5% PRP. VEGFA/vascular endothelial growth factor A is a member of the PDGF/VEGF growth factor family encoding a heparin-binding protein to induce proliferation and migration of vascular endothelial cells. FGF2/fibroblast growth factor 2 encodes a protein in the fibroblast growth factor family with mitogenic and angiogenic action involved in tissue growth and wound healing. Corresponding NCBI numbers for select regulatory genes influenced by VEGFA and FGF2 are also mapped (blue).

As with other research arenas, reproductive biology is beginning to recognize the contribution of intron variants to regenerative processes. The champion regenerator axolotl (*Ambystoma mexicanum*) has a 14-chromosome diploid genome with massive highly repetitive sequences and exceptionally long introns; this frustrates full genome assembly given the difficulty in acquiring sufficient read-length (Sibai *et al*, 2019). Could critical switch signals not just to direct safe regrowth, but also to disconnect it when

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appropriate, keep an intron address? Interestingly, genes specific to early ovary development have been recently inventoried via expression of enhancer-derived transcripts (eRNAs), and several highly-conserved sequences and rare single intron variants were discovered (Nakagawa *et al,* 2022).

Conclusions

It is not disputed that PLT activation involves a busy discharge of cargo proteins pushed from a common origin (alpha granules) to meet a shared destination (ovarian tissue). With innumerable specified movers, a random scramble describes a forward wave yet individual members would rarely trace the exact same path twice. The growth factor ensemble produced from specified PLT activation has striking similarity with "Yamanaka factors", which impact cell differentiation, recruitment, migration and function as well as inhibition of apoptosis in the mammalian ovary (Zhang *et al,* 2020). How should these research results by interpreted in clinical fertility practice?

The advice of Farquhar *et al* (2019) is by no means a voice in the wilderness for how patients may be better served to seek additional IVF cycles for now, rather than follow-up with poorly defined accessories like intraovarian PRP (Urman & Boza, 2020). Other popular 'add-ons' include autologous mitochondrial transfer to oocytes (Labarta *et al,* 2019) or androgens to increase follicular sensitivity to gonadotropins (Nagels *et al,* 2015). Perhaps the largest series on intraovarian PRP described >500 patients (age 30-45yrs) with a pregnancy rate of 20.5% after treatment, placing this among worthwhile considerations (Cakiroglu *et al,* 2022). When intraovarian PRP before planned IVF achieves an unassisted pregnancy during the wait to start the cycle, any 'ovarian rejuvenation' expense is offset by avoiding the cost and inconvenience of IVF (Sills & Tan, 2022). But a comprehensive audit of intraovarian PRP, dehydroepiandrosterone, hormone replacement therapy, and autologous adipose-derived stromal cell treatment concluded that none of these do anything to recover ovarian function (Kuang *et al,* 2022) and are either provisional or of uncertain clinical value (Greisinger & Fauser, 2020). Close scrutiny in techniques for intraovarian injection should

help normalize the present variance in PRP production and delivery. As a new technique in fertility medicine, intraovarian PRP awaits placebo-controlled studies before its wider use should be expected.

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