



## Estrogen in vascular smooth muscle cells: A friend or a foe?

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### ABSTRACT

Cardiovascular disease (CVD) continues to be the leading cause of death worldwide. The effect of estrogen on these diseases has been assessed in *in vitro* and *in vivo* models, as well as in observational studies. Collectively, these studies alluded to a cardiovascular-protective effect of estrogen. However, comprehensive clinical investigation failed to produce concrete proof of a cardiovascular protective effect for hormone replacement therapy (HRT), let alone rule out potential harm. These seemingly paradoxical effects of estrogen were explained by the ‘theory of timing and opportunity’. This theory states that the effect of estrogen, whether cardiovascular-protective or pathological, significantly depends on the age of the individual when estrogen administration takes place. Here, we review the conflicting effects of estrogen on vascular smooth muscle cells, mainly proliferation and migration as two cellular capacities intimately related to physiology and pathophysiology of the cardiovascular system. Furthermore, we critically discuss the major parameters and signaling pathways that may account for the aforementioned paradoxical observations, as well as the key molecular players involved.

### 1. Introduction

Estrogens represent a class of hormones that is mainly composed of 17- $\beta$ -estradiol ( $E_2$ ), in addition to estrone ( $E_1$ ) and estriol ( $E_3$ ), with 17- $\beta$ -estradiol being the most potent [1,2]. They are synthesized by the ovaries, adrenal cortices and liver, as well as by the placenta during pregnancy [1]. Estrogens are responsible for the development of primary and secondary female sex characteristics. Furthermore, they take part in the induction of female growth spurts by stimulating bone growth, increasing body metabolism and enhancing fat and protein deposition [1]. Two nuclear receptors, namely estrogen receptor alpha ( $ER\alpha$ ) and beta ( $ER\beta$ ), in addition to the G protein-coupled estrogen receptor (GPER) mediate the actions of estrogens [3–6].

It has been suggested that estrogens impart a beneficial effect on the cardiovascular system. This was primarily based on multiple cohort studies which showed a negative correlation between the use of estrogen supplements and coronary heart disease (CHD), as well as other fatal cardiovascular diseases among postmenopausal women [7,8]. The Danish Osteoporosis Prevention Study's randomized controlled trial

(RCT) provided some support for this protective effect [9]. This study showed that postmenopausal women who received hormone replacement therapy (HRT) with a cyclical protocol including synthetic 17- $\beta$ -estradiol and a combination of 17- $\beta$ -estradiol and norethisterone (for patients with intact uterus) or 17- $\beta$ -estradiol alone (for patients with a hysterectomy) were at lower risk of myocardial infarction (MI) [9].

Yet, not all studies support a cardio-vasculoprotective effect of estrogen. Indeed, a RCT conducted by Women's Health Initiative revealed that postmenopausal women on HRT experienced higher rates of CHD, stroke, venous thromboembolism (VTE), deep vein thrombosis (DVT) and pulmonary embolism (PE) [10]. However, it is noteworthy that patients in this study received conjugated equine estrogens with extensive first pass metabolism and variable *in vivo* potency [11]. Additionally, the difference in mean subject age between the above mentioned trials could potentially underlie this discrepancy, with an older patient cohort in the Women's health Initiative Study. In fact, some authors suggest that the vast difference in the dosage of estrogenic component between oral and other routes of administration, including transdermal application, that is driven by the first pass metabolism,

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triggers unfavorable cardiovascular effects caused by disproportionate hepatic exposure to estrogens in oral preparations [12]. Detailed discussion of these effects is beyond the scope of this review.

The unfavorable effects of estrogens become further evident in yet another vascular disease, namely Raynaud's phenomenon (RP). RP is an exaggerated, cold-induced constriction of peripheral cutaneous arterioles [13]. Although patients with RP exhibited signs of improvement upon the short-term administration of estrogen [14], a 10-year literature review alluded to a positive association between increased risk of RP and estrogen therapy [15]. This is not surprising given that nearly 70% of RP patients are females [16,17], and the prevalence of this disease in premenopausal females is nine times higher than in age-matched men [18–20]. Furthermore, RP is more predominant in premenopausal women compared to postmenopausal ones [13]. Interestingly, postmenopausal women receiving HRT have a significantly higher incidence rate of RP than the control group [21]. In this study 114 women received estrogen replacement therapy, the majority of who received conjugated equine estrogens while only 12 patients reported using the transdermal patch. Clearly, these findings support the notion that estrogen plays a major contributing role to the onset or pathogenesis of RP [22].

The Flavahan group elegantly showed that the entirety of cold-induced vasoconstriction was shown to be mediated by vascular alpha 2C-adrenoceptors ( $\alpha_{2C}$ -ARs) [22,23]. The same group further showed that  $\alpha_{2C}$ -AR, once-dubbed a “vestigial” receptor, assumes a rather unique biology. Indeed, while this receptor remains intracellularly trapped in the endoplasmic reticulum and Golgi apparatus at 37 °C, it is spatially and functionally rescued to the plasma membrane upon moderate cold exposure [24–26]. Once at the membrane, this receptor can readily bind to epinephrine, its natural agonist, and thus elicit vasoconstriction. It was later showed that estrogen leads to heightened cold-induced vasoconstriction by virtue of its ability to increase the expression and function of  $\alpha_{2C}$ -ARs in human arteriolar smooth muscle cells [22].

VSMCs are major structural and functional components of the vessel wall. They carry out several functions, such as regulation of vasotone and blood flow, both of which are critical for tissue perfusion, metabolic demand and homeostasis. In addition to their role in physiology, VSMCs are also important players in the pathophysiology of several diseases including atherosclerosis [27–29]. Atherosclerosis is the most common cause of ischemic heart disease as well as stroke, both of which are major contributors to CVD-associated morbidity and mortality [30,31]. Indeed, VSMCs contribute to the formation of the atheroma within the tunica intima either by proliferating and migrating from tunica media [32,33], or by arising from proliferating multipotent vascular stem cells (MVSCs) that reside in the vessel wall [34]. Additionally, VSMCs are involved in restenosis, the re-narrowing of arterial lumen that occurs post-angioplasty [35]. Restenosis results from excessive migration and proliferation of VSMCs which eventually culminate in the formation of neointimal hyperplasia [35,36].

Due to the controversy that has arisen in regards to the use of estrogens in HRT, our aim in this review is to dissect and critically discuss the effects of estrogens on proliferation, migration and apoptosis of VSMCs. These parameters represent major hallmarks of VSMC phenotype, a crucial player in cardiovascular physiology and pathophysiology.

## 2. Modulation of VSMC proliferation and migration by estrogen

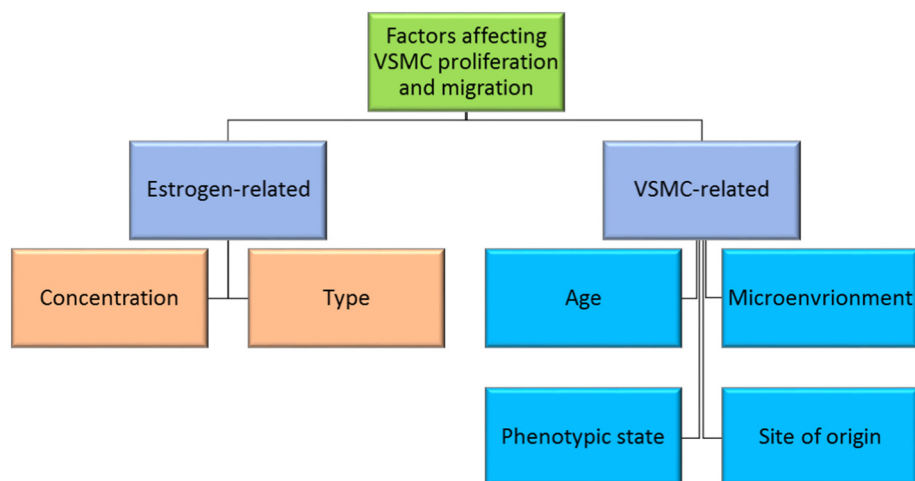
The literature contains contradictory reports on the effect of estrogen on VSMC proliferation and migration. These discrepancies are likely due to several factors related to VSMCs, such as the vascular bed from where cells were isolated, age of the animal, cell population number, cell phenotype in culture and whether cells are stimulated or not (Fig. 1). The chemical type of estrogen used, as well as its concentration, are also among the factors that contribute to the variable effects of estrogen on VSMCs' proliferation and migration (Fig. 1).

Overwhelming evidence clearly shows that the effect of estrogen on VSMCs is largely affected by the site from where they were isolated. For instance, 17 $\beta$ -estradiol inhibited neointimal hyperplasia, characterized by increased proliferation and migration of VSMCs, in the femoral artery of rats and mice [37,38], the aorta and iliac artery of rabbits [39], as well as the carotid artery of mice, rats and pigs [40–44]. Using estrogen receptor knockout mice, Pare et al. attributed these effects to ER $\alpha$  receptor [44]. This was reflected by findings showing that the protective effect of *in vivo* 17 $\beta$ -estradiol treatment against neointimal hyperplasia were abrogated with conditional ER $\alpha$  knockout [38]. On the other hand, 17 $\beta$ -estradiol promoted the proliferation of pulmonary VSMCs obtained from rats and canines in a concentration-dependent manner in the same concentration range reported to inhibit VSMC proliferation in the previous studies [46]. This apparent paradox could potentially be explained by differential regulation of ER $\alpha$  transcriptional activity in VSMCs from different sources. Huang et al. showed that while 17 $\beta$ -estradiol inhibited the proliferation and migration of VSMCs extracted from human saphenous and umbilical veins, the same concentration seemed to increase proliferation of VSMCs from varicose veins of the same patients. This potentiation appears to be due increased expression of IQ-domain GTPase-activating protein 1 (IQGAP1), which is a scaffold protein that aids in the activation of the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) pathway, and thus promotes ER $\alpha$  transcriptional activity as discussed in Section 3 [45]. Whether this is a universal mechanism or other factors contribute to the difference in response to 17 $\beta$ -estradiol observed in VSMCs from different vascular beds remains to be determined. Nevertheless, these results clearly show that VSMCs' response to estrogen is affected by the vascular bed from which they are collected, as well as the initial state of VSMCs prior to estrogen treatment. Accordingly, one may argue that HRT can either be beneficial or harmful depending on the site of vascular injury.

The microenvironment of VSMCs is another factor that determines their response to estrogen. Indeed, 17 $\beta$ -estradiol inhibited the proliferation and migration of VSMCs that were cultured in a medium containing mitogenic factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and serum [47–50]. Ueda et al. showed that this effect is mediated by non-nuclear ER $\alpha$  signaling leading to Akt and Erk dephosphorylation [49]. The same effect of 17 $\beta$ -estradiol was observed for VSMCs grown in conditions that mimic physiologic or pathophysiologic stress such as hyperlipidemia [51], hypoxia [52], or oxidative stress [53]. Interestingly, 17 $\beta$ -estradiol had no effect on unstimulated VSMCs [47]. Therefore, it can be proposed that the microenvironment of the VSMCs alters their phenotypic state, and this affects their response to estrogen.

The phenotypic state of VSMCs can either be contractile under physiologic conditions or synthetic under inflammatory and stimulatory conditions [54]. Synthetic VSMCs are highly proliferative and migratory, and produce a high amount of extracellular matrix (ECM) proteins [55]. Contractile VSMCs, on the other hand, exhibit low proliferative and synthetic states, and they express contractile proteins such as myosin heavy chain (MHC) and elastin [55]. Under the influence of 17 $\beta$ -estradiol, contractile VSMCs had a longer G<sub>0</sub> phase before entering the cell cycle and proliferating extensively [54]. Synthetic VSMCs, however, responded to estrogen by increasing their cell division rate [54]. This finding suggests that the inhibitory effect of estrogen on proliferation and migration of VSMC can be a retardation of the switch from contractile to synthetic phenotype.

The age of the animal from which VSMCs are isolated may greatly dictate estrogen's effect on cellular proliferation and migration. In an experiment conducted on old and young mice, estrogen inhibited neointimal hyperplasia in the carotid arteries of young mice, but instigated neointimal hyperplasia in the carotids of old mice [56]. Interestingly, this study implicated both ER $\beta$  and the novel G protein-coupled estrogen receptor (GPR30) in the protective effect observed in



**Fig. 1.** Various factors that may dictate estrogen's effect on proliferation and migration of VSMCs. The concentration/dose as well as the type of estrogen used are two critical factors inherent to estrogen's effects. The age of the subject/animal and the vascular bed from where VSMCs are isolated contribute to phenotypic behavior of these cells in the presence of estrogen. Microenvironmental cues, such as the nature of the extracellular matrix proteins available, are also contributing factors to VSMC phenotype.

young rats. Hence, it can be speculated that with time, VSMCs change their expression of proteins that respond to estrogen from anti-inflammatory molecules to pro-inflammatory ones. Interestingly, barring the differences in route of administration and estrogen/progestin component used, women who benefited from HRT in the Danish Osteoporosis Prevention Study were younger than those who had increased cardiovascular events in the WHI's RCT [9,10]. Indeed, a subgroup analysis of younger patients in the Women's Health Initiative study indicated a potential protective effect [57]. Consequently, all these findings support the “windows of opportunity and timing” hypothesis, which states that an early onset of HRT in postmenopausal women helps reduce the risk of cardiovascular diseases, while a later initiation has no beneficial effect and may actually be harmful [58].

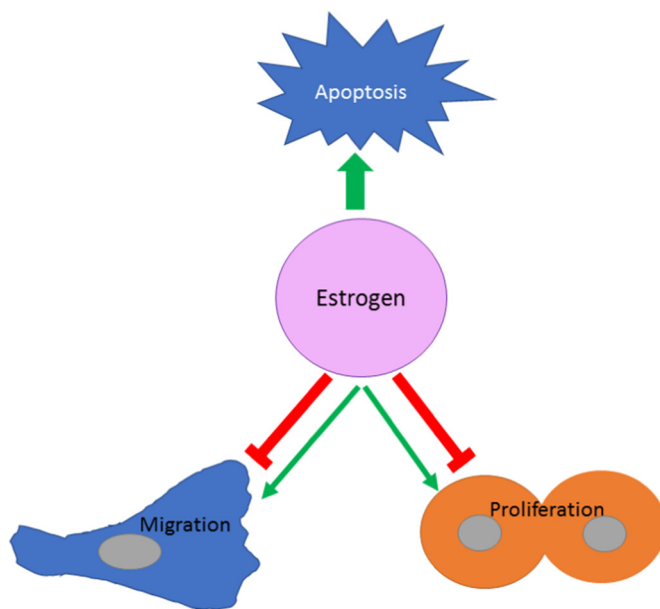
With regards to the age-dependent estrogen effects, it is important to consider the variability in serum estrogen levels in different ages, since opposite effects of estrogen on VSMCs can be also attributed to the concentration/dose used. Serum 17 $\beta$ -estradiol levels were reported to be around 0.2 nM, up to 1.6 nM, and up to 0.15 nM in pre-pubertal, adult, and post-menopausal females, respectively [59]. At concentration levels matching those found in prepubertal and postmenopausal women (0.3 nM), 17 $\beta$ -estradiol promotes the proliferation of human umbilical VSMCs [59]. However, at high concentrations that match those found during the ovulatory phase of premenopausal women (3 nM), 17 $\beta$ -estradiol inhibits VSMC proliferation [59]. Similar results were observed in rat VSMCs, where physiological concentrations of 17 $\beta$ -estradiol (1 nM) stimulated proliferation, but higher concentrations (> 10 nM) inhibited it [60]. Interestingly, women of WHI's RCT received 0.625 mg/day of conjugated equine estrogen and suffered increased risk of cardiovascular diseases [10], while those from the Danish Osteoporosis Prevention Study received 2 mg/day and were at lesser risk of cardiovascular diseases [9]. All these results support the bimodal dose dependency hypothesis, which states that estrogen increases or decreases VSMC proliferation at low or high concentrations respectively [59]. One way to partly explain the bimodal dose dependency hypothesis is by observing 2- and 4-hydroxyestradiols, both of which are estrogen metabolites [60]. As the concentration of estrogen increased, these metabolites accumulated and caused an intracellular increase in reactive oxygen species (ROS), which damaged DNA and resulted in VSMC senescence [60].

Another determinant of estrogen's effect on VSMC is the type of estrogen used in experiments. In an attempt to differentiate among the clinically used estrogens, it was found out that an inhibitory effect on VSMCs is noted upon the use of estradiol valerate, estradiol cypionate, and estradiol benzoate [61]. Estrone, estrone sulfate, estriol, and 17 $\alpha$ -estradiol, on the other hand, failed to display any inhibitory effect [61]. This result may provide another explanation for the opposing

conclusions met in the aforementioned RCTs, since the Danish Osteoporosis Prevention Study used synthetic 17 $\beta$ -estradiol [9], while WHI used conjugated equine estrogens [10].

The role of individual estrogen receptors, especially ER $\alpha$  and ER $\beta$ , in mediating estrogen's role in vascular injury is also important. Estrogen inhibited vascular injury in both ER $\alpha$ - and ER $\beta$  knock out mice ([62,63]). Interestingly, in double gene knock out mice, 17 $\beta$ -estradiol failed to suppress intimal thickening after vascular injury yet still inhibited proliferation of VSMC [44,64]. These studies alluded to a potential contribution from an ER $\alpha$  splice variant, although the possibility of a yet unidentified estrogen-responsive receptor cannot be eliminated.

Despite the aforementioned varying effects on proliferation and migration of VSMCs, a consistent finding was that estrogen induced apoptosis of VSMCs in all the reviewed studies (Fig. 2). This finding is consistently observed in VSMCs obtained from human or murine aortas [65–67]. This suggests that the anti-proliferative effect of E<sub>2</sub> on VSMCs can be due to E<sub>2</sub>'s induction of apoptosis, which occurred at the G<sub>2</sub>-to-M



**Fig. 2.** Modulation of VSMC proliferation, migration and apoptosis by estrogen. While estrogen-induced apoptosis is a consistent finding, modulation of proliferation and migration by estrogen appears to be affected by several other factors. While estrogen may promote proliferation and migration, it can also inhibit them if the physiological or pathophysiological cues change.

phase, despite the fact that VSMCs had already entered the cell cycle [67].

### 3. Signaling pathways implicated in estrogen's effects on VSMC proliferation and migration

The contentious observations regarding the effect of estrogen on VSMCs are reflected in the experiments that studied the signaling pathways implicated in VSMC proliferation, migration and apoptosis. One of the most extensively studied pathways is the MAPK/ERK pathway. ERK1/2 translocate to the nucleus and phosphorylate transcription factors required for proliferation [68]. Their mode of action is biphasic: the initial step is a transient increase in ERK1/2 for up to 10 min, followed by a lower, but sustained peak for the duration of G<sub>1</sub> phase [69]. These two steps are necessary to drive the G<sub>1</sub>-to-S phase transition into completion [68]. This may appear to suggest that both rapid non-genomic and longer-lasting genomic effects are implicated. Importantly, the notion that both these pathways are based on phosphorylation makes it more likely that the effects are independent on gene expression. However, this remains to be investigated.

Using rat VSMCs, 17 $\beta$ -estradiol induced proliferation by promoting ERK1/2 phosphorylation [66]. However, it inhibited ERK1/2 phosphorylation and the consequent proliferation and migration in murine, human and porcine VSMCs [70–72], inducing differentiation instead particularly when GPER agonist, G-1, was used [71]. Interestingly, other reports suggest that 17 $\beta$ -estradiol-induced phosphorylation of ERK1/2 may cause inhibition of murine aortic VSMC proliferation and migration [49,73]. This effect appears to be due to estrogen-induced upregulation of striatin [49,73], a calmodulin-dependent scaffolding protein [74].

While these results appear conflicting, a closer look at the underlying molecular mechanisms may help resolve this paradox. Indeed, in human aortic VSMCs, activation of ER $\alpha$  inhibited proliferation by suppressing prolonged ERK phosphorylation via the upregulation of manganese superoxide dismutase (MnSOD) [75,76]. Henceforth, it can be concluded that estrogen induced ERK1/2 phosphorylation momentarily, causing VSMCs to enter the cell cycle and striatin to be expressed, before inhibiting the sustained ERK1/2 phosphorylation of the biphasic model. A limitation to this experiment, however, is that the cells were treated with high glucose (HG) [75], which is known to induce expression of inflammatory genes in VSMCs [77]. Thus, these findings need to be replicated under other conditions before our argument can be cemented.

Another signaling molecule that plays an integral role in cellular proliferation is the retinoblastoma protein (pRb). pRb is a tumor suppressor protein, which binds to and inhibits E2F transcription factors [78]. When phosphorylated, pRb is inactivated, allowing E2F transcription factors to translocate to the nucleus and drive the G<sub>1</sub>-to-S phase transition [78]. In an experiment conducted on rat aortic VSMCs, 17 $\beta$ -estradiol inhibited pRb phosphorylation, resulting in inhibition of proliferation at the G<sub>1</sub> phase [79] (Fig. 3). This study showed that this effect was elicited through ER $\alpha$ , since stimulating A10, a rat aortic smooth muscle cell line that expresses ER $\beta$  but not ER $\alpha$ , with PDGF was not inhibited by 17 $\beta$ -estradiol or raloxifene [79]. However, when these cells were transfected with ER $\alpha$ , estrogen potently inhibited PDGF-induced cyclin D1 expression [79]. This is in marked contrast to findings showing that inhibition of a different related receptor named estrogen related receptor- $\alpha$ , ERR $\alpha$ , induced hyperphosphorylation of pRb, inhibited proliferation and migration of rat aortic smooth muscle cells, as well as attenuated neointima in rat artery subjected to balloon injury [80], with both studies reporting the involvement of the cyclin-dependent kinase inhibitor (CDK), p27<sup>Kip1</sup>. Importantly, by suppressing the Ras-pRb pathway, 17 $\beta$ -estradiol inhibits premature senescence of VSMCs isolated from young (2 months) female rats [81]. Contrarily, this is reversed to a senescence-promoting effect in cells isolated from old (18 months) female rats. Non-selective ER blockade abolished the

protective effect but promoted the senescent effect that appeared likely to be due to estrogen metabolites since it was blocked upon treatment with a cytochrome P450 inhibitor [81]. Together, these findings provide insight into the “time window theory” as well as provides insight into how HRT could be vasculoprotective in younger *versus* being a risk factor in older postmenopausal women.

The role of Akt in proliferation is extensively established in various cell types including VSMCs. In particular, Akt is also an important mediator of estrogen-induced inhibition of VSMC proliferation. Indeed, in VSMCs isolated from the carotid artery of human and porcine origin, stimulation of G-protein coupled estrogen receptor 1 (GPER) inhibited proliferation by decreasing Akt phosphorylation and inducing p21 expression [71]. p21 is a CDK inhibitor that induces G<sub>1</sub>/S cell cycle block. Recently, it was also shown that 17 $\beta$ -estradiol, acting *via* Akt, reduced proliferation and migration of rat aortic smooth muscle cells [82]. These effects occur mainly by virtue of estrogen's ability to decrease sirutin 1 (SIRT1) [82]. Importantly, because SIRT1 levels are decreased with aging [83], estrogen may fail to impart its potent anti-proliferative effect on VSMCs. Together, these findings may partly explain the dichotomy of estrogen's effects depending on the age of the cells stimulated with estrogen.

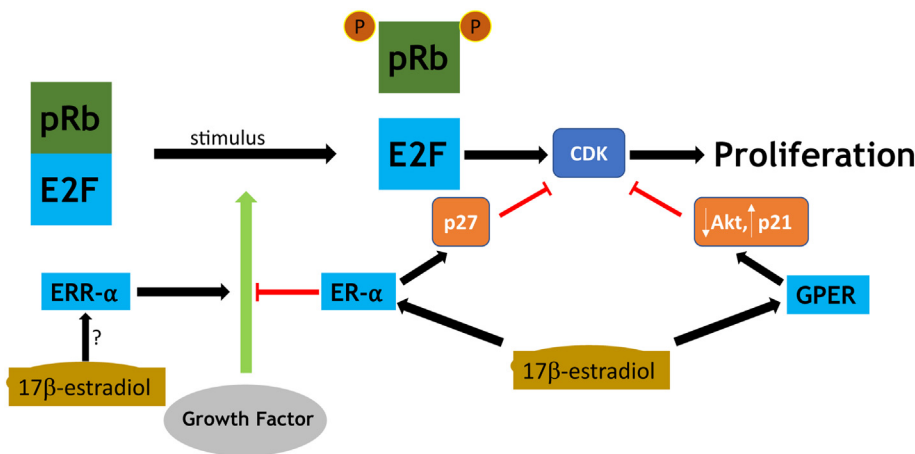
### 4. Signaling pathways implicated in estrogen's effects on VSMC apoptosis

The notion that estrogen induces apoptosis of VSMCs is well-accepted. Indeed, when treated with 17 $\beta$ -estradiol, rat synthetic VSMCs exhibited a dramatic increase in the number of apoptotic cells, marked by an increase in Bax/Bcl-2 ratio at the G<sub>2</sub>-to-M phase [67]. Interestingly, this pro-apoptotic effect of estrogen occurs together with increased expression of cyclin D<sub>1</sub> and CDK4 concomitant with increased G<sub>1</sub> to S transition [67]. Therefore, estrogen established its anti-proliferative effect by inducing apoptosis in the very cells that it accelerated their entry into cell cycle. In human aortic VSMCs, 17 $\beta$ -estradiol also increased apoptosis by promoting rapid and temporary phosphorylation of p38 [65], a MAPK that is usually activated by stress stimuli [84]. Interestingly, ERK1/2 pathway, which caused proliferation, and the p38 pathway, which resulted in apoptosis, were both activated in VSMCs treated with 17 $\beta$ -estradiol [66]. Hence, the ultimate effect of estrogen may result from the balance between these two antagonistic pathways, especially that the inhibition of one of them stimulated the other [66].

One of the key signaling molecules that has been shown to be involved in estrogen's induction of apoptosis is Protein kinase A (PKA). While 17 $\beta$ -estradiol activated the MAPK/ERK pathway, it concomitantly inhibited PKA, resulting in apoptosis [85]. This effect was observed in freshly isolated rat aortic VSMCs [85]. In cultured VSMCs, however, ERK was dephosphorylated and PKA was activated; this resulted in the loss of estrogen-induced apoptosis [85]. A possible explanation to this discrepancy is the expression difference of GPR30, a G-protein coupled receptor of estrogen. Indeed, while GPR30 expression is high in freshly isolated VSMCs, its expression in cultured cells is barely detectable [85]. Importantly, estrogen-GPR30 complex was shown to be responsible for the inhibition of PKA and the induction of apoptosis [85]. It appears that a balance between ER-activated PKA and GRP30-inhibited PKA is critical for defining estrogen's ultimate effect on apoptosis of VSMCs. Consequentially, this indicates that *in vitro* experiments that study estrogen's effect on VSMCs but undermine the role of GPR30 in estrogen's pro-apoptotic effect may need to be revisited.

### 5. The effect of estrogen on VSMC differentiation

Under normal physiological conditions, differentiated VSMCs assume a contractile phenotype [86]. They are characterized by the expression of smooth muscle cell (SMC) differentiation markers such as calponin and smooth muscle  $\alpha$ -actin [86]. The expression of these



**Fig. 3.** Estrogen modulation of growth factor-stimulated, pRb-E2F-mediated proliferation of VSMCs. 17 $\beta$ -estradiol modulates proliferation via different pathways. Its inhibitory actions are elicited by virtue of its ability to increase levels of p21 and p27, which then inhibits CDK and reduces proliferation. It can also act through ER $\alpha$  to inhibit growth-factor-induced stimulation of pRb phosphorylation. However, when it activates ERR $\alpha$ , it may induce the opposite effect by promoting dissociation of pRb from E2F, thereby upregulating CDK levels and promoting proliferation.

markers is downregulated in response to vascular insults that ultimately drive VSMCs to switch to a synthetic phenotype [86]. The effect of estrogen on differentiation of VSMCs and consequently the expression of differentiation markers is not well defined. It may be postulated that estrogen would increase the differentiation markers by virtue of inhibiting VSMC proliferation and migration. However, pathways that increase the expression of contractile molecules and increase the migratory phenotype could occur simultaneously [87–89]. Furthermore, Motague et al. showed that the activation of ER $\alpha$  reduces SMC differentiation markers in aortic SMCs [90]. On the other hand, Huang et al. suggested that the inhibition of ER $\alpha$ , ER $\beta$ , and/or GPR30 reduced the SMC marker, calponin, expression [91]. Furthermore, Myocardin, cardiac and smooth muscle cell specific protein, activates many VSMC differentiation markers [92]. Interestingly, it was shown that this protein interacts with ER co-activator, SRC3, to up-regulate the SMC differentiation markers [92].

## 6. Epigenetic regulation of ERs in VSMCs

Since estrogen elicits its effects through binding to its receptors, the epigenetics of these receptors may affect the cellular response to estrogen. Indeed, ER- $\alpha$  promoter does not appear to be methylated in normal aorta (*in situ*), but exhibits higher methylation status in proliferating VSMCs isolated from human aortae [93]. This hypermethylation is an important contributor to VSMC phenotype [93]. Other studies have shown that hypermethylation of ER- $\alpha$  is correlated with atherosclerosis and ischemic stroke, in a manner proportional to the disease severity [94,95]. This relation is further confirmed in a study showing that insulin potentiated the expression of DNA methyltransferases, leading to increased ER methylation [96]. This in turn caused a loss of ER- $\alpha$ -inhibited proliferation of VSMCs and thus resulted in atherosclerosis [96]. ER- $\beta$  hyper-methylation was also observed in atherosclerotic tissues [97]. Interestingly, unlike ER- $\alpha$ , the correlation of ER- $\beta$  expression with atherosclerosis is independent of age [98].

## 7. Conclusion

It is now evident that the effect of estrogen on cardiovascular diseases depends on age as well as many other factors. Physiological/pathological states, VSMC microenvironment, the particular estrogen derivative used, the predominant estrogen receptor expressed, and the epigenetic status of the estrogen receptor promoter all dictate the outcome of VSMC exposure to estrogen. These factors must be taken into consideration when studying the cardiovascular role of estrogen. Concerted and coordinated efforts among basic science researchers and clinicians are needed to optimize a suitable model to assess the role of estrogen in the vasculature.

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