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A novel role of Shc adaptor proteins in steroid hormone-regulated cancers

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Abstract

Tyrosine phosphorylation plays a critical role in growth regulation, and its aberrant regulation can be involved in carcinogenesis. The association of Shc (Src homolog and collagen homolog) adaptor protein family members in tyrosine phosphorylation signaling pathway is well recognized. Shc adaptor proteins transmit activated tyrosine phosphorylation signaling that suggest their plausible role in growth regulation including carcinogenesis and metastasis. In parallel, by sharing a similar mechanism of carcinogenesis, the steroids are involved in the early stage of carcinogenesis as well as the regulation of cancer progression and metastatic processes. Recent evidence indicates a cross-talk between tyrosine phosphorylation signaling and steroid hormone action in epithelial cells, including prostate and breast cancer cells. Therefore, the members of Shc proteins may function as mediators between tyrosine phosphorylation and steroid signaling in steroid-regulated cell proliferation and carcinogenesis. In this communication, we discuss the novel roles of Shc proteins, specifically $p52^{Shc}$ and $p66^{Shc}$, in steroid hormone-regulated cancers and a novel molecular mechanism by which redox signaling induced by $p66^{Shc}$ mediates steroid action via a non-genomic pathway. The $p66^{Shc}$ protein may serve as an effective biomarker for predicting cancer prognosis as well as a useful target for treatment.

Introduction

Cancers in steroid hormone-responsive tissues presently account for more than 35% in men and more than 40% in women of all newly diagnosed cancers in the United States (Henderson & Feigelson 2000). Extensive research has clearly demonstrated that the abnormal changes in the levels, frequencies, and types of steroid hormones are important contributors to the development of major cancer types such as the cancers of the prostate (androgen, estrogen), testes (*in utero* estrogen), breast (estrogen, progesterone), ovary (FSH, estrogen, androgen), uterine endometrium (estrogen), and thyroid (TSH, estrogen; Henderson & Feigelson 2000). Thus, many studies have been focused on the involvement of steroids in the regulation of tumor development, cancer cell proliferation, progression, and metastatic processes. It is now evident that either in the early stage of carcinogenesis or in the advanced metastatic phenotype, steroid hormone action goes far beyond the classical receptor-mediated gene regulation. These steroid

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Declaration of interest
All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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hormone-related cancers may share common mechanisms of carcinogenesis, such as DNA damage/mutation as well as the elevated levels of various growth factors induced by the excess of steroids, leading to aberrant growth regulation (Dickson & Lippman 1987, Dabrosin *et al.* 1997, Devanesan *et al.* 2001). Recent advances further indicate that these hormones could also induce rapid, non-genomic responses and a convoluted network of interactions with different intracellular signaling pathways.

Non-genomic actions by steroid hormones

Numerous studies have demonstrated the non-genomic action of steroid hormones, including androgens and estrogens, in cellular processes such as cell proliferation and motility (Berridge *et al.* 1998). The most intriguing facts on the non-genomic nature of steroids are that the effects depend on their rapid response and insisting no direct binding of nuclear receptors to gene expression, i.e., seconds to minutes and their insensitivity to the inhibitors of transcription and translation and/or the antagonists of the classical intracellular steroid hormone receptors. The non-genomic effects of steroids could be mediated by multiple pathways, and are discussed briefly as follows.

Direct and acute action of steroids

Steroids may have direct action on target molecules independent of steroid receptors. One such effect, for example, is the direct binding and activation of protein kinase C (PKC) isoforms such as PKC_{α} and PKC_{δ} by aldosterone and 17β-estradiol (E_2) respectively via binding directly to their regulatory domains C_2 that mediates calcium (Ca^{2+}) binding and results in the autophosphorylation of these kinases (Alzamora & Harvey 2008). Furthermore, aldosterone and E_2 rapidly and directly stimulate phospholipase A2 (PLA_2) and cyclooxygenase (COX), which also result in the rapid increase in intracellular $[Ca^{2+}]_i$ (Harvey *et al.* 2002). The other direct and acute effects of steroids are their rapid action on voltage-gated and calcium-activated ion channels. E_2 activates the calcium-activated potassium channels via binding directly to its regulatory β-subunit (Valverde *et al.* 1999). Testosterone rapidly activates ATP-sensitive K^+ channels (K_{ATP}) via opening K_{ATP} channels (Er *et al.* 2004) and inhibits the L-type and T-type calcium channels ($I_{Ca,L}$; Michels *et al.* 2006, Er *et al.* 2007).

Rapid action of steroids involving classical intracellular steroid receptors

Steroids may bind to the classical intracellular steroid receptors and activate the second messenger pathways such as c-Src kinase that rapidly stimulate the MAPK/ERK and PI3K/AKT kinase pathways (Migliaccio *et al.* 2000). Interestingly, an androgen receptor (AR)/Src/modulator of non-genomic action of estrogen receptor (MNAR) complex and the cooperative association of c-Src, estrogen receptors (ERs), and AR activates MAPK and c-Src kinase pathways respectively (Kousteni *et al.* 2001, Unni *et al.* 2004). Estrogens on binding to ERα may also serve as a transcriptional co-activator activating several transcriptional factors, such as activator protein 1 (AP-1), nuclear factor kappa B (NF-κB), and SP-1 in a non-genomic manner (Ray *et al.* 1997, Jakacka *et al.* 2001, Safe 2001). Steroids can also activate cAMP-dependent protein kinase A (PKA) via the transmembrane sex hormone-binding globulin (SHBG) receptor in association with transmembrane G-protein-coupled receptor (GPCR; Fortunati 1999, Rosner *et al.* 1999). The activation of PKA via the induction of cAMP by SHBG is observed in both prostate and breast cancer cells (Fortunati *et al.* 1996, Nakhla *et al.* 1997).

Rapid action of steroids involving non-classical membrane-bound steroid receptors

Steroids may undergo non-genomic action by binding to distinct non-classical membrane-bound steroid receptors. Several reports have presumed the presence of androgen- and estrogen-binding sites in a number of cells (Benten *et al.* 1999a,b, Armen & Gay 2000, Kampa

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et al. 2002). Interestingly, both the membrane androgen receptor (mAR) and the membrane ER (mER) are found to be associated with an integral membrane protein caveolin that facilitates the assembling of several signaling molecules, including phosphatidylinositol 3-kinase (PI3K), Ras, and Src kinase in their scaffold domain (Okamoto *et al.* 1998, Kim *et al.* 1999, Lu *et al.* 2001). Furthermore, mER perhaps exists as in a cytoplasmic pool and the rapid action requires their interaction with caveolin in association with MNAR, Shc and growth factor receptors, and striatin that translocated ER to the plasma membrane (Wong *et al.* 2002, Lu *et al.* 2004, Song *et al.* 2005).

Rapid action of membrane steroid receptors involving GPCR—The most preserved non-genomic action of steroid hormones is the rapid increase in intracellular calcium concentration [Ca^{2+}] mediated via GPCR that constitutes α-, β-, and γ-subunits (Lieberherr & Grosse 1994, Benten *et al.* 1998), which ultimately results in the rapid activation of MAPK/ERK and PI3K/AKT pathways, leading to the activation of PKC and PKA (Kelly *et al.* 1999, Estrada *et al.* 2003). The interaction of mAR with GPCR results in the dissociation of Gα-subunit and the signal is transmitted from Gβγ through the activation of effector molecules including c-Src, Raf, and phospholipase C (PLC; Pierce *et al.* 2002). GPCR itself may also serve as the membrane receptor, i.e., binding of E_2 to an orphan GPCR, termed GPR30, plays a critical role in the rapid signaling of E_2-mediated stimulation of Ras-dependent MAPK activation through the phosphorylation of Shc (Luttrell *et al.* 1996).

Rapid action of membrane steroid receptors via trans-activation of growth factor receptors—The rapid non-genomic actions of membrane steroid receptors may function via trans-activation of the growth factor receptors (Levin 2005), and of all membrane steroid receptors, mER is the paramount and well studied. The phenomenon is further confirmed by the co-existence of endogenous membrane receptors, including AR and ER, G-proteins, GPCR, growth factor receptors (EGFR, IGFR), non-receptor tyrosine kinases (Src, Ras), and linker proteins such as MNAR and striatin in the plasma membrane termed as 'signalosomes' (Hammes & Levin 2007). Alternatively, steroids may activate growth factor receptor kinase activity by inhibiting the regulatory phosphatases (Meng *et al.* 2000).

Rapid non-transcriptional action of membrane steroid receptors—The other non-genomic action of steroids involving membrane receptors is the non-transcriptional effects of these receptors that provoke the posttranslational amendments including phosphorylation. By regulating kinases and phosphatases, steroids influence the cell functions such as cell motility via modifying actin cytoskeleton (Kampa *et al.* 2002, Meyer & Feldman 2002, Levin 2005).

Rapid action of steroids on membrane fluidity

Steroids may mediate the non-genomic fashion through changes in membrane flexibility. Androgens via interacting with phospholipids in the lipid bilayer decrease the membrane fluidity, and subsequently alter the function of Na^+/K^+ and Ca^{2+} ATPase systems and also influence cellular adhesion and cell–cell interaction (Duval *et al.* 1983, Van Bömmel *et al.* 1987).

In summary, we propose that though the non-genomic actions of steroids are mediated through multiple pathways, both genomic and non-genomic effects are interlinked as non-genomic actions of steroids ultimately influencing at least one of the classical genomic-mediated transcriptional activities. Nevertheless, the molecular mechanisms of steroids, especially androgens and estrogens, in both the development and progression of human endocrine-related cancers at the non-genomic levels need further investigations. Along with these observations, in this review, we emphasize a novel non-genomic action of steroids promoting various stages of carcinogenesis via Shc proteins.

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Cross-talk of tyrosine phosphorylation signaling and steroids in carcinogenesis

Every step of carcinogenesis is essentially controlled by various growth factors and their receptors, either it is steroid regulated or not. Growth factors, including nerve growth factor (Engebraaten *et al.* 1993, Sachs *et al.* 1996), fibroblast growth factor (Mignatti *et al.* 1991, Engebraaten *et al.* 1993, Taylor *et al.* 1993), platelet-derived growth factor (Engebraaten *et al.* 1993, Choudhury *et al.* 1997), epidermal growth factor (EGF; Engebraaten *et al.* 1993, Hamada *et al.* 1995), keratin growth factor (Sachs *et al.* 1996), hepatocyte growth factor (Pelicci *et al.* 1995, Sachs *et al.* 1996), interleukin 2 (Ratner *et al.* 1992), insulin, and insulin-like growth factors (Stracke *et al.* 1989) are known to be involved in regulating cell proliferation, motility, invasion, and/or migration of various cell types. The activated receptors triggered by growth factors, cytokines, or adhesion molecules facilitate the docking of Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domain-containing adaptor molecules that transduce signals via downstream intracellular cascades. Each of the receptors for the ligands described above activates the c-Src homology and collagen homolog (Shc) adaptor proteins for signal transduction, which suggests a conceivable role of the Shc proteins in various stages of carcinogenesis.

Recently, several lines of evidence indicate that Shc proteins mediate diverse biological activities; for example, they may mediate steroid actions other than serving as adaptors in tyrosine phosphorylation signaling. Since the role of Shc proteins in mediating tyrosine phosphorylation signaling and in regulating oxidative stress-induced apoptosis has received much attention (Migliaccio *et al.* 1997, 1999, Ravichandran 2001), in this communication, we will first briefly overview Shc proteins and then focus our efforts on discussing the novel roles of Shc proteins, specifically $p52^{Shc}$ and $p66^{Shc}$, in steroid-regulated cancers.

Members of Shc family: structure and function in tyrosine phosphorylation signaling

Molecular structure of Shc isoforms

Shc proteins were first cloned using an SH2-coding sequence as a probe, and the Shc family includes three isoforms with molecular masses of 46, 52 and 66 kDa, which are encoded by the same gene at chromosome 1q21 (Pelicci *et al.* 1992). A promoter in the first intron of Shc locus transcribes the mRNA of $p66^{Shc}$, whereas an alternate promoter and splicing generates the other two Shc isoforms, i.e., $p46^{Shc}$ and $p52^{Shc}$ (Ventura *et al.* 2002). These three isoforms of Shc protein contain overlapping amino acid sequences that contribute to a SH2 domain at the COOH-terminal and a PTB domain at the NH_2-terminal, separated by a central region enriched in proline and glycine residues, i.e., collagen homology (CH1) domain (Ravichandran 2001), as shown in Fig. 1. The SH2 domain (~100 amino acids) is the prototype for protein–protein interaction modules that mediate the formation of multiprotein complexes during signaling (Pawson & Scott 1997, Yoshida *et al.* 2004). Structurally, $p66^{Shc}$ differs from $p52^{Shc}$ and $p46^{Shc}$ by virtue of its unique NH_2-terminal, a 110-amino acid CH_2 region, which is also rich in proline and glycine residues (Migliaccio *et al.* 1997).

Subcellular localization of Shc isoforms

$p66^{Shc}$ is expressed primarily in epithelial cells, while $p52^{Shc}$ and $p46^{Shc}$ are expressed ubiquitously (Migliaccio *et al.* 1997). Most of $p66^{Shc}$ protein is distributed throughout the cytosol and a fraction of $p66^{Shc}$ localizes within the inner membrane and intermembrane spaces of mitochondria (Orsini *et al.* 2004, Ventura *et al.* 2004, Giorgio *et al.* 2005, Nemoto *et al.* 2006). $p46^{Shc}$ is found to be localized in the mitochondrial matrix (Orsini *et al.* 2004, Ventura

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et al. 2004, Nemoto *et al.* 2006). Unlike p46^{Shc} and p66^{Shc}, p52^{Shc} is translocated to the plasma membrane from cytosol upon stimulation by growth factors, e.g., EGF (Migliaccio *et al.* 1997).

Shc isoforms in tyrosine phosphorylation signaling

p46^{Shc}—Shc proteins are expressed in distinct patterns and exhibit diverse biological functions. Ventura *et al.* (2004) have reported specifically p46^{Shc} as the first example to be localized in the mitochondrial matrix by means of a mitochondrion-targeting signal, which is inactive in p52^{Shc} and p66^{Shc}. Thus, p46^{Shc} may play a role in the signal transduction pathways regulating mitochondrial physiology (Ventura *et al.* 2004). In addition to mediating tyrosine phosphorylation signaling (McGlade *et al.* 1992, Migliaccio *et al.* 1997), p46^{Shc} may also modulate steroid action since steroids can regulate mitochondrial enzymatic activities (Ripple *et al.* 1997, 1999). Nevertheless, due to the limited studies on p46^{Shc} in steroid action in carcinogenesis, it will not be discussed further in this communication.

p52^{Shc}—p52^{Shc} is responsible for transducing anchorage-dependent growth signaling (Pelicci *et al.* 1992). In general, when cells are stimulated by growth factors, p52^{Shc} is recruited and binds to tyrosine kinase receptors through its PTB or SH2 domain, leading to its phosphorylation at tyrosine residues 239, 240, and 317 within the CH1 domain (Fig. 1; Rozakis-Adcock *et al.* 1992, van der Geer *et al.* 1996, Gotoh *et al.* 1996). Upon tyrosine phosphorylation, p52^{Shc} recruits Grb2/SOS through a binding event between the SH2 domain of Grb2 and Shc phosphotyrosine residues (Pelicci *et al.* 1992, Rozakis-Adcock *et al.* 1992), which ultimately results in the activation of Ras and the MAPK cascade for mitogenesis (Fig. 2; Bonfini *et al.* 1996). The PTB domain of p52^{Shc} binds to the phosphorylated tyrosine residues of receptor protein tyrosine kinases and functions similar to the SH2 domain. The CH1 and CH2 domains are putative SH3-binding regions (Lotti *et al.* 1996). Studies have also suggested that p52^{Shc} mediates steroid action on cell proliferation as well as cell survival via tyrosine phosphorylation signal pathway (Kousteni *et al.* 2001, Lee *et al.* 2004a). Its aberrant expression and activation may lead to the dysregulation of one of the multi-pathways in carcinogenesis by steroids, a topic of focus in this review.

p66^{Shc}—p66^{Shc} is also phosphorylated at its tyrosine residues as p52^{Shc} and p46^{Shc} upon growth factor stimulation, e.g., EGF treatment, and forms complexes with Grb2. However, there are certain functional differences between p66^{Shc} and the other two Shc members. Unlike p52^{Shc}, p66^{Shc} is unable to transform NIH3T3 mouse fibroblasts in culture (Migliaccio *et al.* 1999) and it could not augment EGF-induced extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK) activation in cell cultures such as HeLa, CHO, and COS-1 cells. One possible explanation is that increased expression of p66^{Shc} has resulted in an elevated level of the basal activity of ERK/MAPK in the absence of stimulus, which thus minimizes the extent of further activation by growth factors (Migliaccio *et al.* 1997, Okada *et al.* 1997, Veeramani *et al.* 2005b). Recently, the task of p66^{Shc} in mediating stress-induced apoptosis has received much attention (Migliaccio *et al.* 1999, Orsini *et al.* 2004, Giorgio *et al.* 2005). Once p66^{Shc} is phosphorylated at Ser-36 in its CH2 domain in response to various stress factors, such as H_2O_2, UV radiation, and chemicals, e.g., Taxol, a fraction of cytosolic p66^{Shc} associates with heat-shock proteins to mediate apoptotic response (Fig. 3; Orsini *et al.* 2004) and serves as an apoptotic sensitizer to those signals (Migliaccio *et al.* 1999). p66^{Shc} also acts as a negative regulator of human and mouse T-cell survival and proliferation (Pacini *et al.* 2004). In parallel, p66^{Shc} knockout mice exhibit a prolonged life span by 30% and those mouse embryo fibroblast (MEF) cells have increased resistance to oxidative and hypoxic stress (Migliaccio *et al.* 1999, Trinei *et al.* 2002, Zaccagnini *et al.* 2004). Thus, p66^{Shc} may function as a longevity gene in mammals. Interestingly, p66^{Shc} expression level in human dermal fibroblasts increases with aging, opposite to the knockout mouse model

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(Pandolfi *et al.* 2005). Collectively, these data indicate that p66^{Shc} could function as a sensor and transduce signals in response to cellular stress while more studies are required for its role in human longevity.

Several lines of evidence suggest that aberrant expression of p66^{Shc} could be involved in various stages of carcinogenesis (Jackson *et al.* 2000, Luzi *et al.* 2000, Ravichandran 2001, Davol *et al.* 2003, Lee *et al.* 2004b, De *et al.* 2005, Grossman *et al.* 2007). However, the role and the molecular mechanisms of p66^{Shc} in this mode of regulation remain to be elucidated. In this review, we focus on discussing a novel functional role of p66^{Shc} adaptor protein involved in steroid-related carcinogenesis, leading to its metastasis. This member of Shc protein family may serve as a new target for preventing tumor progression and metastasis.

Role of Shc proteins in steroid-regulated tumor progression and metastasis

Tumor progression and metastasis are the features of cancer. Cell proliferation, migration, and adhesion to the target tissues are the critical steps that allow tumor cells to obtain the metastatic phenotype. The process of metastasis requires the interaction of malignant cells with at least three distinct microenvironments, including the primary organ, the circulation or lymphatic channels, and the target organ where a metastatic lesion will develop (Radinsky & Fidler 1992, Mundy 1997, Cooper *et al.* 2003). Within these microenvironments, several factors are involved in the metastatic cascade (Gopalkrishnan *et al.* 2001). Tumor cells, after reaching to target organs and tissues, establish as successful foci in the conducible environments. Subsequently, tumor cells proliferate in the new, supportive microenvironment as micrometastasis where they induce angiogenesis for maintaining the growth of new lesion. Induction of angiogenesis is necessary for successful metastasis to meet the nutrient requirements when the tumor size becomes more than 2 mm in size (Ellis & Fidler 1996, Gopalkrishnan *et al.* 2001). Cell proliferation and migration depend on intracellular signals transmitted by the growth factors and adhesion proteins within the extracellular matrix (Pages *et al.* 1993). Both these processes employ many common intracellular signaling molecules, e.g., Rho family proteins and ERK cascades (Pages *et al.* 1993, Olson *et al.* 1995, Anand-Apte *et al.* 1997, Klemke *et al.* 1997). It is evident now that Shc isoforms, specifically p52^{Shc} and p66^{Shc}, play a crucial role in cell migration and adhesion, in addition to their roles in mediating cell proliferation induced by growth factor receptor signaling (Fig. 4; Klemke *et al.* 1994, Huttenlocher *et al.* 1995). These processes involve the rearrangement of actin cytoskeleton, the formation of new integrin substratum contacts, cell contraction, and the release of pre-existing cell-matrix contacts (Lauffenburger & Horwitz 1996). We will thus discuss the role of p52^{Shc} and p66^{Shc} in these processes and emphasize on steroid regulation.

Role of p52^{Shc} in steroid-regulated cell proliferation and migration

In addition to the classical role in mediating tyrosine kinase-activated pathways, p52^{Shc} functions as a primary adaptor protein for mediating the mitogenic signals of steroids at the non-genomic level in human breast and prostate cancer cells (Stevenson *et al.* 1999, Lee *et al.* 2004a). In prostate cancer, the data clearly showed that p52^{Shc} is responsible for transducing androgen-activated ErbB-2 signaling, which leads to prostate cancer cell proliferation (Lee *et al.* 2004a). This androgen-stimulated cell proliferation requires Y317 phosphorylation since p52^{Shc} Y317F mutant effectively abolishes androgen stimulation, and that inhibition occurs at certain phases of the cell cycle (Lee *et al.* 2004a). This observation on the role of p52^{Shc} in androgen action on prostate cancer cells is in parallel to that of p52^{Shc} in estrogen action on breast cancer cells, correlating with ErbB-2 activity (Stevenson & Frackelton 1998). In addition, vitamin D treatment caused a significant decrease in LNCaP cell growth, which is closely associated with the reduction in ErbB-2 activity and its downstream signaling mediator p52^{Shc} via dephosphorylating Y317, thereby emphasizing the involvement of tyrosine phosphorylation of p52^{Shc} in human prostate cancer cell proliferation (Stewart *et al.* 2005).

Furthermore, the $p52^{Shc}$ Y317F mutant blocks estrogen-induced cell cycle progression at both the G_0–G_1 and G_2–M junctions (Stevenson *et al.* 1999). Additionally, in the presence of steroids, ER α-transfected HeLa cells exhibit chemoresistance, where $p52^{Shc}$ mediates the anti-apoptotic activity of steroids and thus prevents those cells from etoposide-induced apoptosis (Kousteni *et al.* 2001). Clearly, $p52^{Shc}$ plays a critical role in mediating steroid action, at least in part, via tyrosine phosphorylation non-genomic signaling.

$p52^{Shc}$ may also be involved in the adhesion process of cancer cells. In rapidly adhering prostate cancer cells, e.g., LNCaP C-81 and PC-3 cells, the phosphorylation level of $p52^{Shc}$ protein at Y317 correlates with ErbB-2 activation, higher than in slow-adhering LNCaP C-33 cells (Lee *et al.* 2004a, Yuan *et al.* 2007). Yet, both the adhesion rate and the Y317 phosphorylation of $p52^{Shc}$ as well as ErbB-2 tyrosine phosphorylation level in slow-adhering LNCaP C-33 cells can be up-regulated by steroids. Thus, by correlating tyrosine phosphorylation and adhesion, $p52^{Shc}$ may play a crucial role in the adhesion of cancer cells during steroid hormone-induced metastasis. Furthermore, a direct involvement of $p52^{Shc}$ in breast cancer metastasis in transgenic mice that express polyomavirus middle T antigen with a mutated Shc-binding site has been demonstrated (Webster *et al.* 1998). Polyomavirus middle T antigen couples with and activates signaling molecules, such as Src, Shc, and phosphatidylinositol 3′-kinase (PI3K) for its oncogenic capacity. Importantly, in transgenic mice, which have metastatic tumors, the mutated $p52^{Shc}$-binding site on middle T antigen had reverted to the wild type and regained its function, thus emphasizing the potential importance of the functional $p52^{Shc}$ in the process of metastasis *in vivo* (Webster *et al.* 1998). In addition, it has been revealed that in integrin signaling, Shc recruitment to the actin-associated cytoskeleton is important (McGlade *et al.* 1992, Schlaepfer *et al.* 1998, Wary *et al.* 1998). $p52^{Shc}$ potentiates integrin signaling, and integrin ligation results in the activation of non-receptor tyrosine kinases, such as Src, Fyn, and focal adhesion kinase (FAK), which phosphorylates $p52^{Shc}$, leading to Ras activation and entering into the cell cycle (McGlade *et al.* 1992, Mainiero *et al.* 1995, Wary *et al.* 1996, 1998). Besides, the SH3 domain of Fyn interacts with the proline-rich region in the CH1 domain of $p52^{Shc}$ (Thomas & Bradshaw 1997) and the amino-terminal domain of $p52^{Shc}$ is shown to mediate the association of this adaptor protein to an actin-rich cellular fraction (Thomas & Bradshaw 1997). Additionally, a mutation of the PTB domain (S154P-$p52^{Shc}$) abolishes integrin-induced $p52^{Shc}$ tyrosine phosphorylation where the SH2 domain of $p52^{Shc}$ is dispensable (Collins *et al.* 1999). $p52^{Shc}$ phosphorylation by c-Src can be augmented when the PTB domain binds to phospholipids (Zhou *et al.* 1995, Sato *et al.* 1997). These observations explain how the PTB domain localizes $p52^{Shc}$ to the membrane where it becomes phosphorylated by cytoskeleton-associated tyrosine kinases, which finally results in cell migration. It should be noted that these non-receptor tyrosine kinases, e.g., Src, closely interact with steroid hormone signaling pathway (Migliaccio *et al.* 2000, Guo *et al.* 2006). The molecular mechanism by which steroids induce cell adhesion and/or migration via $p52^{Shc}$ requires further investigation.

Role of $p66^{Shc}$ in steroid-regulated cell proliferation

In tyrosine phosphorylation signal transduction pathway, $p66^{Shc}$ conventionally known as an adaptor protein. Interestingly, $p66^{Shc}$ expression closely correlates with the growth rate of prostate cancer cells. For example, $p66^{Shc}$ protein levels in the rapidly growing cells, e.g., PC-3 and DU145, are approximately 4- to 13-fold higher than that in the slow-growing LNCaP C-33 cells and are over tenfold higher than that in even slower MDA PCa2b cells (Veeramani *et al.* 2005b). Recent data indicate that $p66^{Shc}$ may play a critical role in mediating steroid-stimulated cell proliferation. In the presence of steroid hormones (androgen and estrogen), $p66^{Shc}$ protein level as well as cell proliferation rate is increased in hormone-sensitive human prostate (LNCaP C-33 and MDA PCa2b), testicular (Tera-1 and Tera-2), and breast (MCF-7)

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cancer cells, higher than those cells cultured in the absence of steroids (Lee *et al.* 2004b). Thus, steroids increase $p66^{Shc}$ protein level and concurrently cell growth.

To elucidate directly the functional role of $p66^{Shc}$ protein in steroid-regulated cells, both cDNA and siRNA approaches were employed (Veeramani *et al.* 2005b). In both $p66^{Shc}$ cDNA, transiently transfected cell population and stable subclones of slow-growing LNCaP C-33 cells, elevated expression of $p66^{Shc}$ correlates with increased cell proliferation. On the contrary, a decreased cell growth rate is observed when $p66^{Shc}$ protein is knocked down by its siRNA in the rapidly growing LNCaP C-81 and PC-3 cells. The data clearly establish the causal relationship of $p66^{Shc}$ protein and cell growth. Furthermore, $p66^{Shc}$ mediates growth stimulation by androgens (Veeramani *et al.* 2008). The clinical relevance of these data is supported by the observations that in prostate cancer archival specimens, $p66^{Shc}$ protein level is significantly higher in prostate adenocarcinomatous cells than in adjacent benign glandular cells (Lee *et al.* 2004b). Similarly, the expression level of $p66^{Shc}$ is elevated in metastatic breast, ovarian, and thyroid tumors and may serve as a useful prognostic marker for stage-IIA colon cancer (Jackson *et al.* 2000, Abdollahi *et al.* 2003, Park *et al.* 2005, Grossman *et al.* 2007). Nevertheless, some studies showed that $p66^{Shc}$ protein is down-regulated in the primary tumors of breast cancers (Davol *et al.* 2003). Due to the potential importance of $p66^{Shc}$ in carcinogenesis, further studies are needed to clarify the correlation of $p66^{Shc}$ expression with breast cancer. In summary, cross-talks between tyrosine phosphorylation signaling and steroid hormones have been well established (Weigel 1996, Meng *et al.* 2000, Grossmann *et al.* 2001, Guo *et al.* 2006, Kraus *et al.* 2006, Migliaccio *et al.* 2006, Weigel & Moore 2007); it is thus reasonable to propose that $p66^{Shc}$ mediates steroid action in steroid-responsive epithelial cells. We therefore hypothesize that the elevated level of $p66^{Shc}$ protein in steroid-related cancer cells plays a critical role in up-regulating those cancer cell proliferation and thus contributes to the tumorigenicity of those cancers (Fig. 2). The role of $p66^{Shc}$ in this mode of regulation requires further investigation.

Role of $p66^{Shc}$ in metastasis

Several studies have shown the involvement of $p66^{Shc}$ in cellular invasion, motility, migration, and/or metastasis. Jackson *et al.* (2000) have shown that xenograft bone metastasis of breast cancer cell line, MDA-MB-231, expresses $p66^{Shc}$ and its metastatic variant F-11 cells have a threefold higher $p66^{Shc}$ expression level. Furthermore, increased expression of $p66^{Shc}$ in lymph node-positive breast cancers correlates with an increased number of positive lymph nodes (Jackson *et al.* 2000). As higher levels of $p66^{Shc}$ protein are observed in breast cancer specimens with higher metastatic potential, it suggests the possibility that $p66^{Shc}$ influences cell motility and invasion other than the MAPK pathway (Jackson *et al.* 2000). Furthermore, Northey *et al.* (2008) have demonstrated that decrease in the ShcA levels or the expression of a dominant-negative ShcA mutant blocked TGF-β-induced motility and the invasion of Neu/ErbB-2-expressing breast cancer cells, thus exploiting the crucial role of $p66^{Shc}$ in the migration and invasion of cancer cells. In addition, elevated expression of $p66^{Shc}$ by cDNA transfection in LNCaP C-33 cells is associated with increased motility and invasion (Yuan TC, Lin FF & Lin MF, unpublished data). In parallel, LNCaP C-81 and PC-3 prostate cancer cells express higher levels of $p66^{Shc}$ and exhibit higher metastatic potential than LNCaP C-33 cells in xenograft animals (Veeramani *et al.* 2005b, Sebeger J & Lin MF, unpublished observation). The observations on the increased expression of $p66^{Shc}$, but not $p52^{Shc}$ or $p46^{Shc}$, in cell lines with higher metastatic ability and in the node-positive primary breast cancers also imply that $p66^{Shc}$ functions in metastatic pathway.

Integrins play a vital role in cancer progression because of their ability to regulate various intracellular signaling molecules that are essential for cell motility, cell survival, and proliferation (Ruoslahti & Reed 1994, Hynes *et al.* 1999). It has been suggested that $\alpha_v\beta_3$

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integrin plays a critical role in the metastasis of cancer cells to bone marrow (Cooper *et al.* 2002). $\alpha_v\beta_3$ integrin is expressed in breast and lung cancer cells that were originally derived from the bone marrow aspirates. $\alpha_v\beta_3$ is also expressed in highly tumorigenic, bone foci-derived human PC-3 prostate cancer cells, but not in low tumorigenic, lymph node foci-derived LNCaP cells (Zheng *et al.* 1999). The results of recent studies suggest that activated $\alpha_v\beta_3$ integrin regulates tumor growth *in vivo* by influencing VEGF expression. The up-regulation of VEGF expression depends on $\alpha_v\beta_3$ clustering where it promotes the recruitment of p66^{Shc} and subsequently the phosphorylation of β_3-associated p66^{Shc}. Phosphorylation of p66^{Shc} is a critical step for $\alpha_v\beta_3$-mediated potentiation of VEGF expression and tumor vascularization *in vivo* (De *et al.* 2005). These findings provide insights into the role of $\alpha_v\beta_3$ and p66^{Shc} interaction as a regulator of tumor metastasis and angiogenesis. Thus, down-regulation of p66^{Shc} inhibits VEGF expression as well as the tumor growth and angiogenesis *in vivo* (De *et al.* 2005). Although the molecular mechanism of p66^{Shc} involvement in metastasis requires further investigations, it is hypothesized that p66^{Shc} is involved in an early step of invasion or during cell motility (Jackson *et al.* 2000) and plays a role in steroid-regulated metastatic process.

Molecular mechanisms of p66^{Shc}-mediated steroid action

In determining the prostate origin of metastatic cancers, cellular prostatic acid phosphatase (cPAcP) has been used as a biomarker, due to its cell-specific expression (Sakai *et al.* 1992, Chu & Lin 1998). The results of several studies collectively indicate that cPAcP exhibit the growth inhibitory activity by functioning as a PTPase (Lin & Meng 1996, Lin *et al.* 2001, Veeramani *et al.* 2005a). In parallel, the expression level of cPAcP negatively correlates with prostatic carcinogenesis, i.e., the level of cPAcP decreases in prostate cancer cells, lower than that in the adjacent non-cancerous cells (Reif *et al.* 1973, Foti *et al.* 1977, Loor *et al.* 1981, Chu & Lin 1998, Lin *et al.* 2001). Interestingly, in prostate cancer cells, the level of p66^{Shc} protein shows an inverse correlation with cPAcP expression and has a positive correlation with Erb2 as well as ERK/MAPK activation (Veeramani *et al.* 2005b). In cPAcP cDNA-transfected stable subclone cells, p66^{Shc} protein level is decreased and ErbB-2 as well as ERK/MAPK activity is diminished, correlating with decreased cell proliferation (Veeramani *et al.* 2005b). Conversely, elevated p66^{Shc} protein level as well as ErbB-2 and ERK/MAPK activation is observed in cPAcP-inhibited LNCaP C-33 cells by inhibitors (Veeramani *et al.* 2005b). Similarly, in breast cancer cells, elevated expression of p66^{Shc} protein correlates with ErbB-2 and/or MAPK1/MAPK activation (Stevenson & Frackelton 1998, Lee *et al.* 2004b). In addition, the up-regulation of p66^{Shc} is shown in human ovarian, oral, and lung cancer cells expressing increased levels of ErbB-2 (Xie & Hung 1996). While the molecular mechanism of this inverse relationship between cPAcP and p66^{Shc} protein remains to investigated further, it should be noted that the inverse correlation of cPAcP with p66^{Shc} as well as ERK/MAPK activation is clinically relevant (Loor *et al.* 1981, Pontes *et al.* 1981, Solin *et al.* 1990, Sakai *et al.* 1993, Gioeli *et al.* 1999, Price *et al.* 1999, Lin *et al.* 2001, Lee *et al.* 2004b). Despite the fact that cPAcP may serve as a good prognostic marker for metastatic prostate cancers (Sakai *et al.* 1993), because of decreased cPAcP level upon tumor progression as well as increased p66^{Shc} protein level in PCa cells, we contemplate that the p66^{Shc}/cPAcP ratio may serve as a competitive surrogate biomarker for predicting the prognosis of advanced prostate carcinoma.

Furthermore, p66^{Shc} may mediate steroid-stimulated cell proliferation via a non-genomic signaling pathway. In the rapidly growing cells, including steroid-stimulated cells, increased oxidative stress by the generation of reactive oxygen species (ROS) might contribute to the elevated p66^{Shc} protein level. p66^{Shc} protein can function as a stress sensor and is involved in regulating the intracellular level of ROS (Trinei *et al.* 2002). Activated metabolic reactions in the rapidly growing cells lead to increased production of ROS (Klaunig & Kamendulis 2004), which may in turn increase p66^{Shc} protein levels to mediate oxidative stress signals (Fig. 3). Evidently, upon H_2O_2 treatment, in MEF cells or DLD-1 colorectal cancer cells, the

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expression level of p66^{Shc} protein is increased (Trinei *et al.* 2002, Pacini *et al.* 2004). Additionally, ROS-induced phosphorylation of p66^{Shc} protein at Ser-36 residue further promotes the generation of ROS (Nemoto & Finkel 2002, Orsini *et al.* 2004). Notably, recent studies reveal that p66^{Shc} protein exhibits endogenous oxidase activity and its amino terminus contains a redox center, which is involved in electron transfer from cytochrome *c* to molecular oxygen and produces H_2O_2. This H_2O_2 mediates the opening of transition pores resulting in increased mitochondrial permeability and thus an abnormal high level of H_2O_2 can lead to apoptosis (Giorgio *et al.* 2005). Nevertheless, physiological levels of H_2O_2 function as growth stimuli. In addition to the above findings, p66^{Shc} may also increase H_2O_2 production through Rac1-SOS-specific pathway (Khanday *et al.* 2006), possibly leading to androgen-independent cell proliferation (Knight-Krajewski *et al.* 2004). ROS therefore mediates diverse biological functions, including cell proliferation, cell adhesion, migration, and apoptosis (Fig. 3).

In prostate cancer archival specimens, the ROS level is higher in cancerous cells than in non-cancerous cells, correlating with the proliferation index (Lim *et al.* 2005). The functional role of ROS as a positive regulator of cell growth, including prostate cancer cells, is apparent in part by inhibiting the PTPase activity, and thus the corresponding RPTK can be activated (Finkel & Holbrook 2000, Liu *et al.* 2002, Lou *et al.* 2008, Veeramani *et al.* 2008). Furthermore, steroid hormones, e.g., androgens and estrogens, and growth factors, such as EGF, can up-regulate ROS production in cells, as such; cell proliferation is promoted (Sundaresan *et al.* 1996, Liu *et al.* 2002). This is consistent with our findings that androgenic treatment of LNCaP C-33 cells promotes cell proliferation via decreasing cellular PAcP and increasing p66^{Shc} protein level and ROS production as well as ErbB-2 tyrosine phosphorylation (Meng *et al.* 2000, Veeramani *et al.* 2008). Therefore, an inverse correlation of p66^{Shc} and cPAcP in prostate cancer cell is observed. Additionally, it has been demonstrated that ROS may play a critical role in the initiation and/or early progression of prostate cancer; as such, antioxidants are used in clinical trials for this cancer prevention (Veeramani & Lin 2007). Collectively, the data indicate that p66^{Shc} can mediate non-genomic steroid action on cell proliferation and carcinogenesis including metastasis, while the molecular mechanisms require further investigations.

Conclusion and perspective

To discover the novel therapeutic targets for the prevention of tumor progression and metastasis, identifying the functional molecules that are involved in enhancing or suppressing these processes is one of the major challenges. The metastatic process in carcinogenesis is regulated dynamically by numerous factors, including growth factors, hormones, and extracellular matrices. To establish a metastatic lesion, tumor cells must complete all the steps in metastatic processes. Several studies have already shown that Shc proteins, including p52^{Shc} and p66^{Shc}, have marked effects on cell proliferation, invasion, and migration. In this review, we discuss a novel role of p52^{Shc} and p66^{Shc} in mediating steroid action on tumor proliferation and metastasis. We further present a novel non-genomic mechanism by which steroid signaling via p52^{Shc} and p66^{Shc} induces cancer cell proliferation, survival, migration and ultimately metastasis (Fig. 5). Nevertheless, the role of other key players engaged with p52^{Shc} and/or p66^{Shc} signaling pathway has yet to be identified. Thus, the molecular mechanisms by which p52^{Shc} and p66^{Shc} mediate steroid hormone-induced carcinogenesis require further investigations. Understanding the role of Shc adaptor proteins in cancer biology including the determination of the upstream regulators and downstream effectors of Shc functional pathways may lead to the development of novel anti-tumor strategies for targeting against steroid-induced epithelial cancers.

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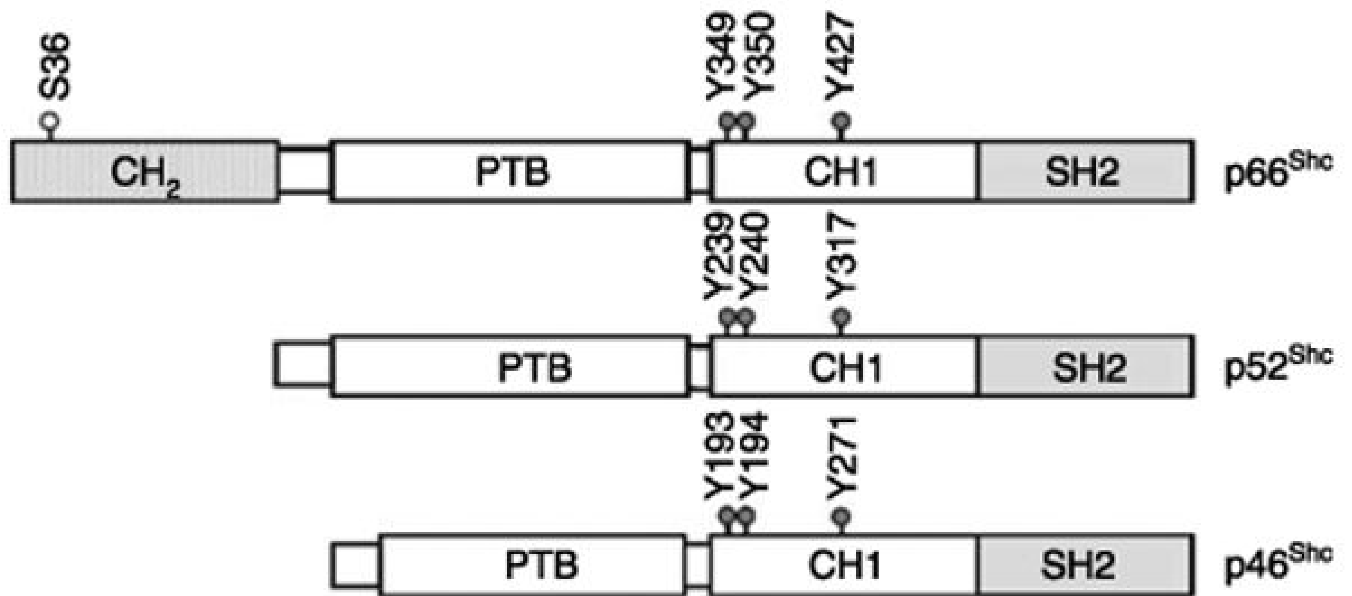

Figure 1.

A schematic organization of Shc isoforms. The Shc proteins include three isoforms that are encoded by the same gene. They contain overlapping sequences. Three major tyrosine phosphorylation sites have been identified within the CH1 domain in all Shc isoforms. The unique CH2 domain of $p66^{Shc}$ isoform consists of 110 amino acids and contains a serine phosphorylation site (Ser-36). The PTB domain of $p46^{Shc}$ is deficient of the first 46 amino acids.

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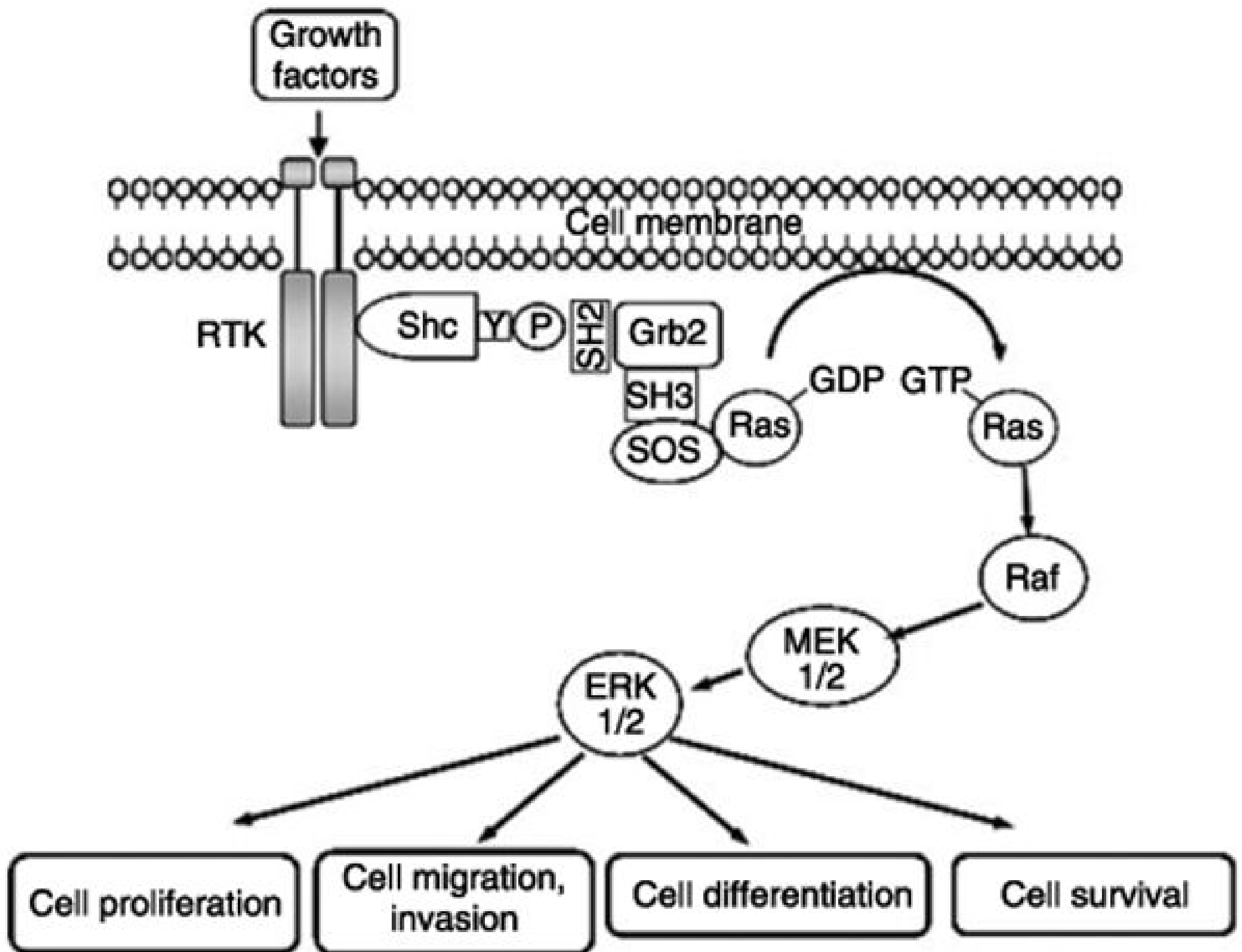

Figure 2.
A schematic representation of Shc proteins activating the Ras and MAP kinase cascade. Upon stimulation by growth factors, RTK undergoes dimerization and tyrosine phosphorylation. Subsequently, Shc proteins are recruited and phosphorylated on tyrosine residue via forming a complex with RTK through the SH2 and/or PTB domain of Shc. The phosphorylated Shc proteins then associate with Grb2 adaptor protein through its tyrosine phosphorylation site to the SH2 domain of Grb2; the latter is constitutively complexed with SOS through its SH3 domains. These events result in the translocation of SOS to the plasma membrane and subsequently activate membrane-bound Ras in the exchange of GDP for GTP and trigger the activation of MAP kinase cascade, resulting in cell proliferation, differentiation, migration, invasion, and survival.

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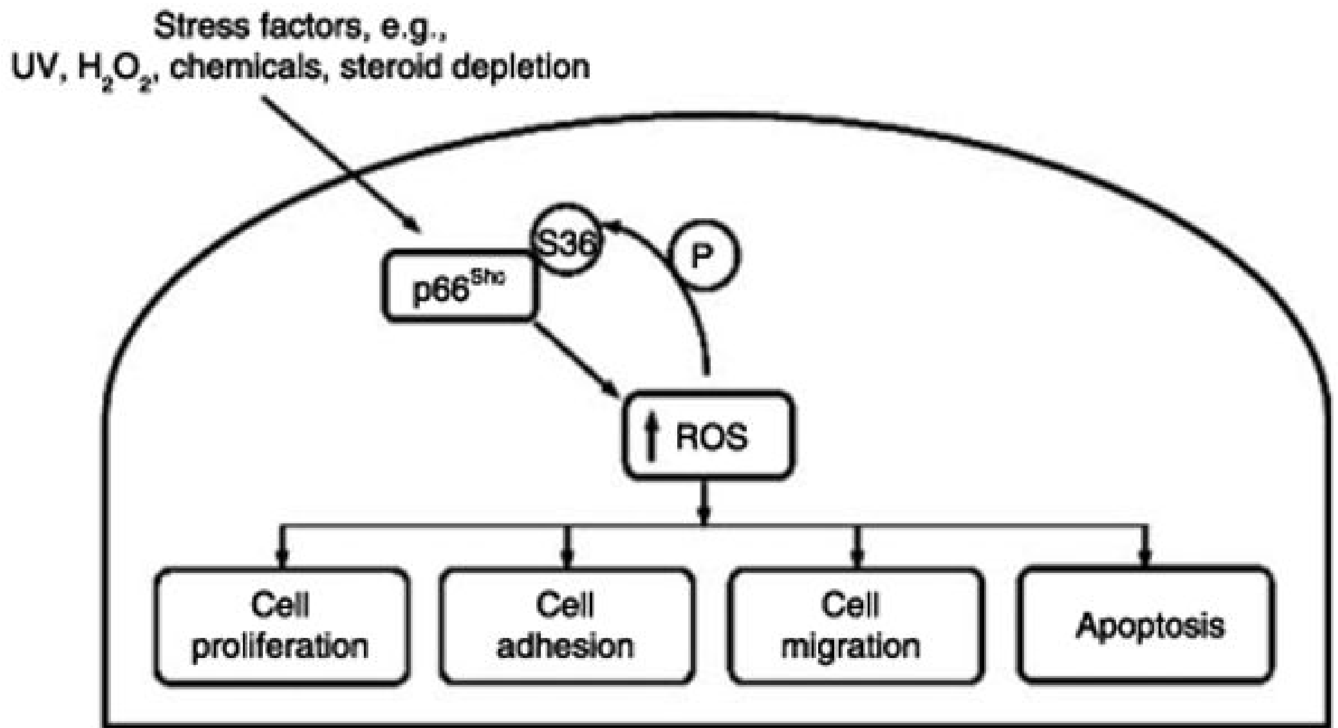

Figure 3.

p66^{Shc} acting as a stress sensor and increasing intracellular ROS level. Increased ROS induces the phosphorylation of serine 36 at p66^{Shc} protein that promotes the generation of more ROS, leading to cell proliferation, adhesion, migration, or apoptosis.

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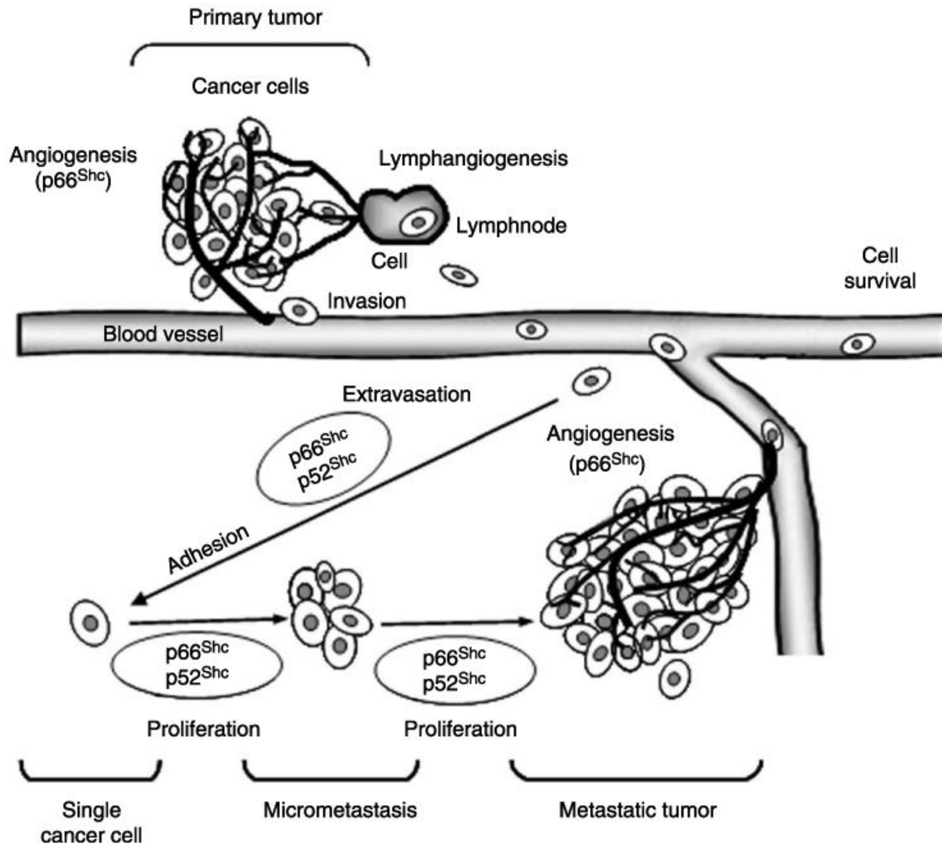

Figure 4.

Involvement of $p66^{Shc}$ and $p52^{Shc}$ in tumor metastasis. Growing primary tumors attract new blood vessels, i.e., angiogenesis, and lymphatic vessels, i.e., lymphangiogenesis, to promote local tumor growth, involvement of regional lymph nodes, and finally distant metastasis. In the process of metastasis after degrading or remodeling the basement membrane, metastatic cells detach from the primary tumor mass, intravasate, survive the stress of vascular transportation, and then evade host defense mechanism, which are in part regulated by the adaptor proteins $p52^{Shc}$ and $p66^{Shc}$. Furthermore, targeting via microvessels and cell adhesion molecules, the increased phosphorylation of $p52^{Shc}$ and $p66^{Shc}$ proteins mediate the proliferation of tumor cells that metastasize to their preferred sites after extravasation into the target organ parenchyma, and are permitted to reside in the target tissue in which cancer cells respond to transendothelial growth factors from that specific organ. (The figure is adapted from La Porta 2000).

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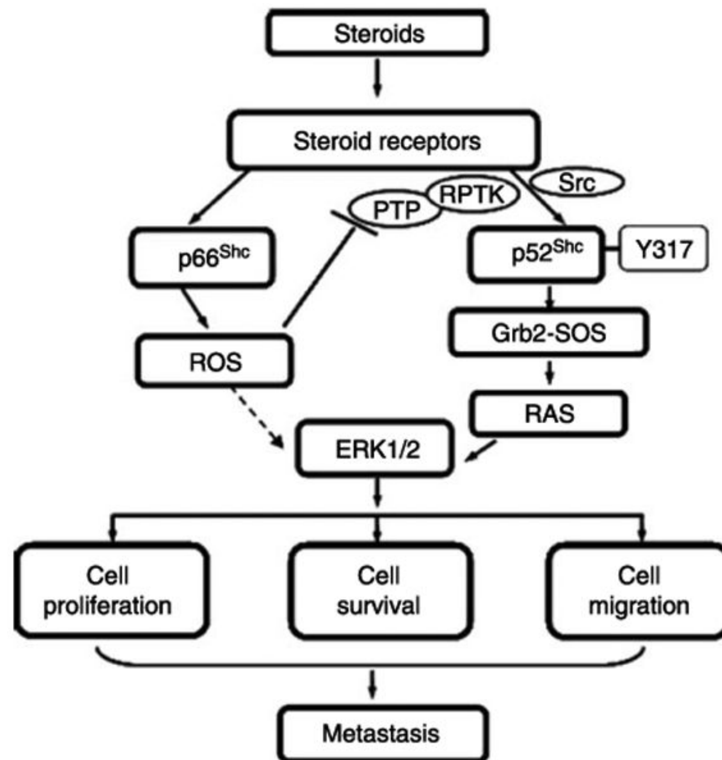

Figure 5.
A proposed scheme of steroid-regulated cancer progression via Shc proteins. Upon steroid activation, p52^{Shc} undergoes phosphorylation at Y317 and promotes tumor growth at least in part via transducing signals through the Grb2–Ras–MAPK pathway. Like p52^{Shc}, p66^{Shc} protein also promotes tumor progression, nevertheless, via mediating oxidative stress signals through the generation of ROS. Upon stimulation by steroids, p66^{Shc} is translocated to mitochondria via Ser-36 phosphorylation-independent manner resulting in the generation of ROS. ROS may then inhibit PTP, resulting in RPTK activation, ERK/MAPK activation, and promote cell proliferation, survival, and migration, which collectively lead to metastasis.