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Hedgehog 對於前列腺癌血管及淋巴管生成之調控角色
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Abstract

Metastasis is the major cause of prostate cancer deaths, involving angiogenesis and lymphangiogenesis. Hedgehog (Hh) is a morphogene which plays critical roles in cell fate determination and tissue patterning during embryonic development and has been shown to involve in vasculogenesis during embryogenesis and in angiogenesis within adult mice. Aberrant Hh signaling has been linked with some human malignant tumors, including prostate cancer. Our studies have shown that (1) Hh could stimulate tumor growth via autocrine and paracrine stimulations and promote metastasis, (2) a new mouse prostate cancer model mimicking human status has been established by Hh-overexpression, and (3) lymph node metastasis occurs up to 80% in our prostate cancer model, with indication that Hh can upregulate the expression of VEGF-A, VEGF-C and VEGF-D a major metastasis gene, and thus promotes prostate cancer metastasis into peripheral lymph nodes. Despite previous knowledge, however, it remains unclear whether Hh is involved in or may even direct angiogenesis and lymphangiogenesis in prostate cancer. In this project, we have demonstrated that Hh can stimulate tumor angiogenesis and lymphangiogenesis via direct and indirect pathway during prostate cancer progression. The results of this study will facilitate understanding of Hh involvement in tumor angiogenesis and lymphangiogenesis of prostate cancer and will provide baseline information of anti-angiogenic or anti-lymphangiogenic effects through Hh signaling blockage.

KeyWords: Hedgehog, prostate cancer, mouse model,metastasis, angiogenesis, lymphangiogenesis

中文摘要

癌細胞轉移是前列腺癌致死的最重要原因，而轉移和血管新生及淋巴管新生作用有關。Hedgehog 蛋白是發育生長因子，於胚胎發育過程中，細胞命運的決定及形態生成時扮演重要角色，也參與胚胎期的血管生成和成年期的血管新生。Hedgehog 的訊息傳遞異常和惡性腫瘤發生有關，包括前列腺癌。我們的研究顯示(1) Hedgehog 可藉由自體訊號或周邊訊號刺激前列腺癌細胞生長和轉移，(2) Hedgehog 高度表現下會誘導前列腺癌化，藉此可建立小鼠的前列腺癌研究模式，(3) 分析小鼠的前列腺癌研究模式發現有高達 80%有轉移到淋巴結，並且 Hedgehog 高度表現會誘發 VEGF-A, VEGF-C and VEGF-D 高度表現，為主要的腫瘤轉移相關基因。研究結果可知 Hedgehog 和腫瘤轉移相關，並且參與主導前列腺癌的血管新生及淋巴管新生作用。我們利用人類的前列腺癌檢體和先前已建立的小鼠研究模式，來探討 Hedgehog 於前列腺癌的血管新生及淋巴管新生作用所扮演的角色，並探討可能的機制，並且嘗試以 Hedgehog 的訊息傳遞阻斷劑，探討以抑制 Hedgehog 訊息傳遞來壓制前列腺癌的血管新生及淋巴管新生作用，或是甚至壓制前列腺癌腫瘤轉移的可能性。

關鍵字: Hedgehog 蛋白、前列腺癌、動物模式、腫瘤轉移、血管新生、淋巴管新生

Background

Incidence of prostate cancer and prostate cancer progression

Prostate cancer poses a great risk for men around the world and the incidence of prostate cancer has increased in the past 50 years. Among American men, most particularly in African-American men, prostate cancer is the most prevalent cancer than any other nonskin cancer. In Taiwan, several studies have indicated a gradual increase of prostate cancer incidence over the past decade (Jeng et al., 2002; Pu, 2000; Wu & Huang, 2001). By the year 2000, for example, prostate cancer has become the 7th most common malignancy in Taiwan (Jeng et al., 2002) and it was said that the incidence of prostate cancer was rapidly increasing during 1990 – 2000 (Pu, 2000) . The increase in Taiwan prostate cases may be due to many recently developed diagnostic tests such as digital rectal examination (DRE), transrectal ultrasound (TRUS), prostate specific antigen (PSA), PSA density (PSAD), PSA velocity, age-specific PSA, and free-to-total PSA, instead of true differences in underlying risk. Alternatively, new risk factors, novel environmental contaminants for example, may be responsible and has yet to be identified.

Under histopathological examinations, prostate cancer shares a number of features with benign prostatic hyperplasia (BPH) and the putative precursor of cancer, prostatic intraepithelial neoplasia (PIN) (Abate-Shen & Shen, 2000). All of the features increase in prevalence with host age, require androgens for growth and development, and respond to androgen-deprivation treatment (Abate-Shen & Shen, 2000). The continuum that culminates in high-grade PIN and early invasive cancer is characterized by progressive basal cell layer disruption, abnormalities in markers of secretory differentiation, increasing nuclear and nucleolar alterations, increasing cell proliferation, variation in DNA content, and increasing genetic instability (Abate-Shen & Shen, 2002). Androgen-deprivation therapy will almost fail in most cases as the prostate malignancy develops to later stages (Isaacs et al., 2002; Nelson et al., 2003). Some biomarkers, like cytokeratins, show up-regulation or gain in the progression from benign prostatic epithelium to high-grade PIN and cancer, whereas others are down-regulated or lost (Isaacs et al., 2002; Nelson et al., 2003). Existing data indicate that more biomarkers are up-regulated, but the relative importance of each is unknown. There is a significant increase in microvessel density in PIN and carcinoma compared with normal prostatic tissue (De Marzo et al., 2004; De Marzo et al., 2003).

Shh is originally identified one of the three vertebrate homologues, Shh, Ihh and Dhh, of the segment-polarity gene hedgehog of the *Drosophila* (Ingham & McMahon, 2001; McMahon et al., 2003). It has been reported to be expressed at numerous sites of epithelial and mesenchymal cell lineages during development (Marti et al., 1995; Roberts et al., 1995), and generally regarded as a morphogen to exert its biological functions, including dorsoventral patterning of body axis, specification of neuronal and oligodendrocytes cell fates, cell proliferation, cell differentiation, axonal outgrowth, and cell survival (Marti & Bovolenta, 2002). Both *Drosophila* and mouse genetics show that the seven transmembrane protein, Smo (*Smoothened*) is required for Shh signaling (Alcedo et al., 1996; van den Heuvel & Ingham, 1996; Van Den Heuvel & Ingham, 1996; Wang et al., 2003). Ptc (*Patched*) appears to negatively regulate Smo in the absence of Shh

(Ingham et al., 2000; Ingham et al., 1991; Taylor et al., 1993). Although it is widely accepted that Shh binds to Ptc (Ingham & McMahon, 2001; Ingham et al., 2000; Strutt et al., 2001) with high affinity, it is unclear how Shh binding results in the activation of its downstream target Smo, as well as the subsequent signaling pathway. The original model suggested that Ptc binds directly to Smo and represses its activity in the absence of Shh. Upon Shh binding the normal inhibition by Ptc is released, and Smo initiates signaling. However, recent studies in *Drosophila* have suggested that Ptc may not repress Smo activity through a direct interaction but rather that Ptc inhibits Smo activity from a distance (Bailey et al., 2002; Chen & Struhl, 1996; Chen & Struhl, 1998; Johnson et al., 2000), possibly through the regulation of vesicular trafficking. The main target of Shh activity is a family of zinc finger transcription factors known as Gli or Ci in the fly. In vertebrates there are three Ci orthologues: Gli1, Gli2, and Gli3 (Ingham & McMahon, 2001; Ingham et al., 1991).

Like many other genes involved in development, disruption or mutation of the Shh pathway results in developmental disorders (Ramalho-Santos et al., 2000; Villavicencio et al., 2000; Walterhouse et al., 2003) and are highly associated with several human diseases including basal cell carcinoma (Basset-Seguin & Soufir, 2004; Oro et al., 1997) and medulloblastomas (Rao et al., 2004; Romer et al., 2004). Plant alkaloid cyclopamine, an antagonist on Shh signaling by blocking the activity of Smo (Cooper et al., 1998; Incardona et al., 2000) has shown promises in pre-clinical models of medulloblastomas (Beachy et al., 2004a; Beachy et al., 2004b; Berman et al., 2002) and could be proven useful in the treatment of Shh-associated tumors.

A role of Shh in prostate tumorigenesis is implicated by several lines of evidences. Abundant Gli-1 expression was found in 9 of 11 prostate cancer tissues examined, suggesting that Hedgehog signaling could play a role in prostate tumorigenesis (Dahmane et al., 1997). Yet, there is so far no Hedgehog pathway gene mutation reported in prostate cancer. More recently, Fan et al. established a xenograft model to elucidate paracrine interactions between Shh-expressing human LNCaP tumor cells and host mouse stromal cells (Fan et al., 2004). The genetically engineered Shh-overexpressing LNCaP cells, when subcutaneously co-injected with Matrigel, was shown to increase stromal Gli-1 expression and dramatically accelerate tumor growth (Fan et al., 2004). Subsequent studies have demonstrated important roles of Shh-signaling pathway in the progressing of prostate cancer with important therapeutic implications, both in the mouse models and in human, as well as in prostate cancer cell lines (Olsen et al., 2004; Sanchez et al., 2004; Sheng et al., 2004). Despite these data, there is so far no mouse prostate cancer model caused initially by Hedgehog dysregulation and a potential role of Hedgehog in the initiation and progression of prostate cancer remains to be elucidated.

Hedgehog pathway in cancer angiogenesis

A parallel line of evidence that may depict Shh as an important factor during prostate tumorigenesis lies in its roles in angiogenesis. Many observations already point to the involvement of Shh in vascularization certain embryonic as well as adult tissues. First, hypervascularization of neuroectoderm is seen following transgenic overexpression of Shh in the dorsal neural tube (Rowitch et al., 1999). Second, Shh-deficient zebrafish exhibits disorganization of endothelial precursors and an inability to form the dorsal aorta or axial vein (Brown et al., 2000). Third, Shh-deficient mice lack proper vascularization of the developing

lung (Bergers et al., 2000; Vu et al., 1998). Fourth, Ihh, expressed by prehypertrophic chondrocytes, regulates the rate of chondrocyte maturation, a process closely correlated to the induction of angiogenesis in bone (Zhou et al., 2000). Fifth, the induction of anagen in the hair follicle requires both Shh and angiogenesis (Mecklenburg et al., 2000; Wang et al., 2000). Sixth, Shh signaling pathway is found present in adult cardiovascular tissues and can be activated in vivo, where vascular endothelial growth factor-1 (VEGF-1) and angiopoietin-1 and -2 (Ang-1 and Ang-2) (Pola et al., 2001; Trelles et al., 2002). Seventh, the human GLI-1, the major downstream effector gene of Sonic hedgehog, was found to cooperate with TWIST in many rhabdomyosarcomas, suggesting that one of Twist's primary role is the regulation of Gli-1 (Villavicencio et al., 2002). Twist is a master regulator of morphogenesis, playing an essential role in epithelial-mesenchymal interactions that lead to angiogenesis and tumor metastasis (Kang & Massague, 2004; Yang et al., 2004). Eighth, Shh and VEGF have been reported to act upstream of the Notch pathway during arterial endothelial differentiation (Lawson et al., 2002; Weinstein & Lawson, 2002). Ninth, postnatal recapitulation of embryonic Hedgehog pathway happens in response to skeletal muscle ischemia (Pola et al., 2003). Tenth, Shh was found to induce capillary morphogenesis by endothelial cells through phosphoinositide 3-kinase (Kanda et al., 2003). More recently, Hedgehog signaling was demonstrated to be downstream of retinoic acid, acting act independently for vascular remodeling and endothelial cell proliferation (Bohnsack et al., 2004), and being essential for endothelial tube formation during vasculogenesis (Vokes et al., 2004). Moreover, The Hedgehog-interacting protein (HIP) is highly expressed in endothelial cells and down-regulated during angiogenesis in several human tumors (Olsen et al., 2004). These previous studies have substantiated Shh as involved in capillary and artery morphogenesis, be it during embryonic development or under adult physiological or pathophysiological situations. Noticeably, however, a potential role of Sonic hedgehog during lymphangiogenesis remains not yet explored. As mentioned, these previous studies have substantiated Shh as involved in capillary and artery morphogenesis, be it during embryonic development or under adult physiological or pathophysiological situations (Bohnsack et al., 2004; Olsen et al., 2004; Vokes et al., 2004). We were surprised to find that none of previous studies have provided information regarding a potential role of Sonic hedgehog during lymphangiogenesis.

Results

(A) Shh signaling in mouse prostate tumorigenesis

We have addressed the potential roles of Shh-signaling in prostate tumorigenesis by overexpressing Hedgehog protein in normal adult mouse prostates. To do this, we conducted intra-prostate injections of a Shh-expressing vector tagged with GFP (pCX-shh-IG), in parallel with vehicle injections containing only GFP tag without Shh insert (pCX-IG) and 0.9% NaCl saline injections as controls (Chen et al. see the Figure 1 in the appendix). To standardize the preparations, all injections were performed postnatally at the 8^{th} week, followed by electroporation to introduce the vector. With the procedure, we managed to introduce Shh-GFP overexpression in the mouse prostates and trace the expression for as long as 90 days after injection.

We first examined the efficiency of the procedure by immunofluorescence microscopy and immunohistochemical detection against GFP at 7, 20, 30, and 90 days following injection.

GFP expression was detected in at least 13 out of 15 prostates injected with pCX-shh-IG (Chen et al.), in parallel with 5/7 of the pCX-GFP injections and in contrast to 0/6 of the normal saline injections. We regarded the efficiency very satisfactory and then checked the effect of Shh overexpression. We were surprised to find that 100% (15/15) of the prostates injected with pCX-shh-IG exhibited PIN, irrespective of injection into either anterior (AP) or dorsolateral prostate (DLP) (Chen et al. submitted; see appendix). This was in contrast to the single PIN-like case of the 7 pCX-IG injections and none of the 6 normal saline injections exhibited PIN. The pCX-shh-IG group also exhibited BPH along with PIN, and even three cases of CaP (prostate carcinoma) at day 30 after injection. We also found extensive stromal growth in most of the pCX-shh-IG injections (Chen et al.; unpublished data), but not in the vehicle or normal saline injections. Noticeably, we demonstrated that pCX-shh-IG injections caused PIN formation at as early as day 7 after the procedure, faster than any other mouse model that had been reported to transform normal prostate epithelium into neoplasia under in vivo conditions. Moreover, we did not find comparable PIN formation in the pCX-IG nor the normal saline injections, indicating that the PIN formation in the pCX-shh-IG group was less likely due to acute inflammatory response to the injection or the electroporation procedures.

To confirm such fast effect caused by Shh overexpression, we examined the presence of GFP, presumably the marker of functional Hedgehog protein, in the three injection groups. Western analyses with anti-GFP antibody showed the presence of Hedgehog protein tagged with GFP in the pCX-shh-IG group, but not in the pCX-IG and 0.9% NaCl saline injections (Chen et al.). We then checked carefully the GFP distribution and correlated it with the sites where Hedgehog protein could be detected by 5E1 anti-Shh antibody. Furthermore, we confirmed the prostatic tumorigenesis by immunohistochemical detection of markers, including E-cadherin, CK14, and p63 (Chen et al.). Within the area of CaP, E-cadherin signals were diminished. Similarly, the basal cell marker CK14 was intensely expressed in a manner of displacement and derangement in the BPH and PIN and was diminished within the area of CaP. Whereas, another basal cell marker p63 was highly expressed within PIN and CaP, as compared to that within BPH. Since both CK14 and p63 are basal cell markers and were only sparsely detected in the normal saline-injected prostates, we concluded that Hedgehog protein overexpression had induced basal cell hyperplasia and transformation. If Hedgehog protein overexpression was responsible for the prostate tumorigenesis, members of its signaling pathway had to be expressed to constitute a functional activation. We performed RT-PCR and immunohistochemistry to examine the expression of Ptc-1, Ptc-2, Gli-1, Gli-2, Gli-3, Smo, and Hip. These are members of Hedgehog signaling pathway and activation of these genes can solidify the observed effects of Hedgehog overexpression. RT-PCR analyses using total prostate RNA preparations showed elevated Ptc-1, Ptc-2, Gli-1, Gli-2, and Gli-3 expression in the pCX-shh-IG injections, whereas Smo and Hip expression appeared not affected (Chen et al.; submitted). Immunohistochemical detection showed Ptc-1 expression in the CaP at a relatively higher level than that in the normal saline-injected luminal epithelium. Ptc-1 expression was also detected in the BPH/PIN epithelial cells and the stromal cells where the signals appeared to be the same or even more intense than those in the CaP. Gli-1 was highly expressed in the CaP, the BPH/PIN epithelial cells, and the stromal cells, in contrast to the absence of signal in the epithelium from normal saline injections. Gli-2 expression was found in the CaP, but not in the epithelium from normal saline injections. Different from Gli-1 expression,

however, Gli-2 seemed not expressed in the BPH/PIN epithelial cells of the anterior lobe, but was intensely expressed in the dorsolateral lobe (Chen et al.; submitted). Similar to Gli-1 and Gli-2, Gli-3 was expressed in the CaP, in contrast to the absence of signal in the epithelium from normal saline injections. Like Gli-1, Gli-3 was also highly expressed in the stromal cells, but no evident Gli-3 signal was detected in the BPH/PIN epithelium from the pCX-shh-IG injections. Hip was detected in the CaP at a level much less than that in the normal saline-injected luminal epithelium (Chen et al. submitted).

(B) Hedgehog signaling promotes tumor vascular and lymphatic angiogenesis in mouse prostate cancer model

We successfully induced prostate BPH/PIN/CaP formation by Hedgehog protein overexpression and observed hypervascularization peripheral to the sites where PIN or even CaP were found. With the knowledge of Shh-induced angiogenesis peripheral to PIN/CaP in our mouse prostate cancer model, we followed observation of vascular and lymphatic vessels. The presence of vascular endothelial cells was confirmed by IHC staining with expression of CD31 and CD34 marker in correlation with pathological stages of prostate cancer; PIN to advance differentiated prostate cancer **(see fig-1)**. The presence of lymphatic endothelial cells, the LYVE-1 positive lymphatic vessels also increased in PIN and advance differentiated prostate cancer **(see fig-1)**. Furthermore, activation of Hedgehog signaling during prostate tumorigenesis was confirmed by the alterations of its downstream signaling members and angiogenesis related protein, such as VEGF-A, VEGF-C, VEGF-D **(see fig-2)** and VEGFR-1, VEGFR-2, VEGFR-3 **(see fig-3)**, FGF-2, FGFR1. Additionally, we demonstrated the angiogenesis and lymphangiogenesis related gene were hedgehog downstream target gene in 3T3L-1 and NIH3T3 cells. Blocked hedgehog signal pathway by Cyclopamine could reduce the expression of these genes **(see fig-4)**. More importantly, we have found the present of vascular and lymphatic vessels was correlated with hedgehog expression in the human prostate cancer specimens. Compare to 7 specimens with not Hedgehog expression; we found higher density of CD31 or LYVE-1 vessels in 9 specimens with high expression of Hedgehog protein.

Discussion

A quick review of current literature has revealed several points that are worthy of considering. Compared to angiogenesis, the lymphangiogenesis during prostate tumor formation has been under-studied. We have shown in this studies that (1) hedgehog overexpression could initiate mouse prostate cancer, starting from the normal status to the stage of malignant CaP formation; (2) Hypervascularization was found peripheral to the hedgehog induced CaP sites; (3) hedgehog overexpression could induce tumor vascular and lymphatic vessels activation in the mouse prostate; (4) hedgehog induced prostate cancer could undergo metastasis, probably through increased angiogenesis and lymphangiogenesis.

We examined the expression of VEGF-A, VEGF-C, VEGF-D and FGF2 (also known as bFGF) in the Shh-induced mouse prostate cancers and found were upregulated in the

pCX-shh-IG-injected prostate CaP. And then was confirm both in 3T3L-1 and NIH3T3 cells. These data showed that Shh overexpression could reactivate not only downstream signal pathway transduction, but also its downstream angiogenetic factors, VEGF-A, VEGF-C, VEGF-D (FGF2). Since VEGF-C and VEGF-D haven been demonstrated and therefore regarded as a master gene responsible for metastasis in the prostate cancer, we also set off to examine whether our mouse prostate cancer model exhibited metastasis through activation of VEGFR-related signaling in the future. Our data showed that Shh-induced prostate cancer cells could undergo metastasis in correlation with Hedgehog pathway activation, as shown by the following paper demonstrating a kidney and lymph node metastasis at 30 days after with pCX-shh-IG injection.

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Fig-1

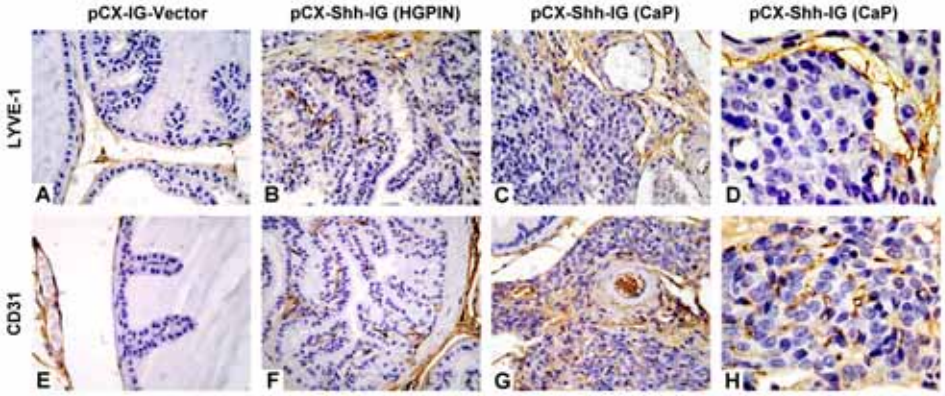
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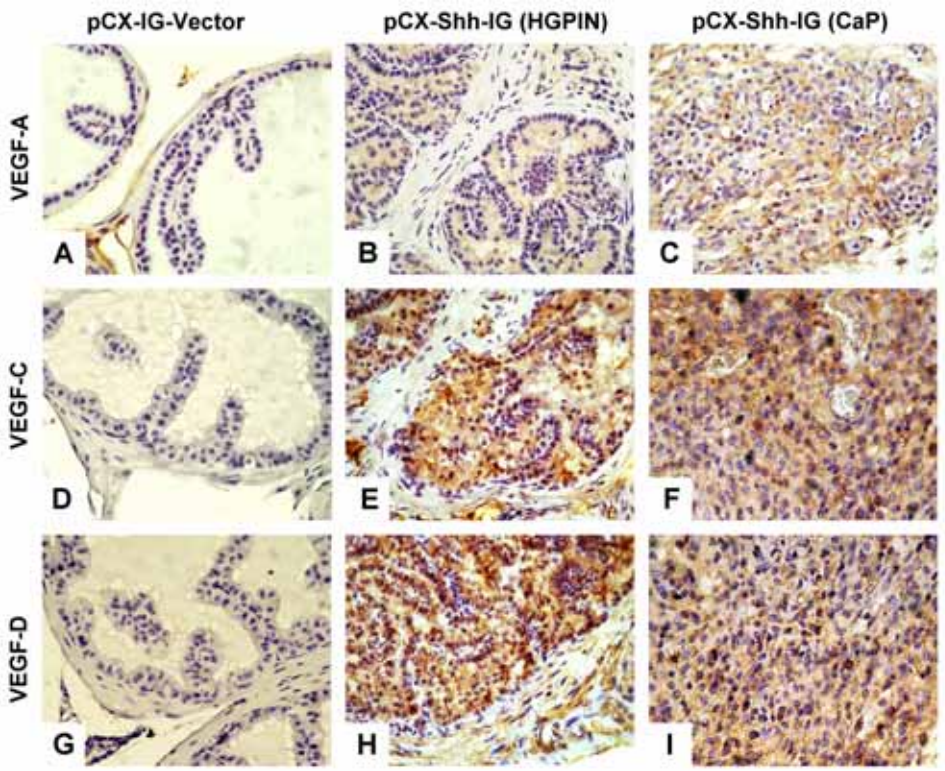
Fig-2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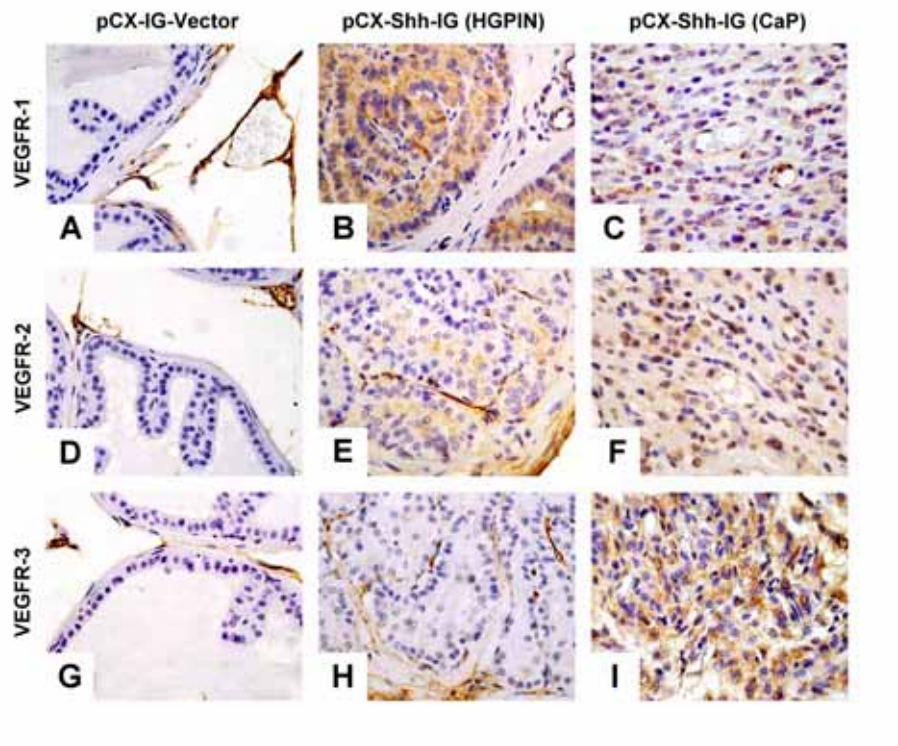
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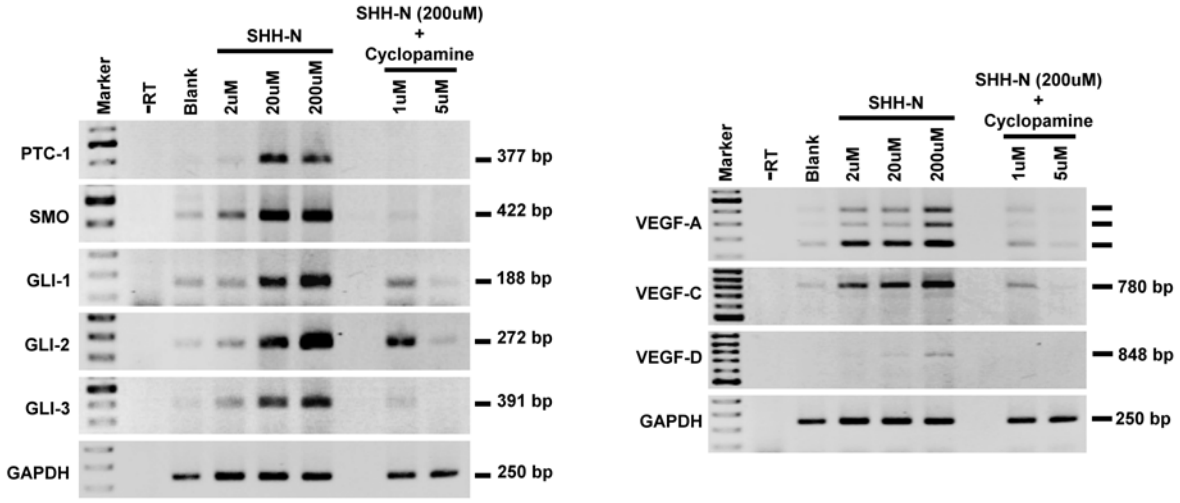
Fig-4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SMO
GLI-1
GLI-2
GLI-3
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