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Role of RhoA/ROCK pathway in angiogenesis and their potential values in prostate cancer treatment

Jury

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ABSTRACT

Prostate cancer remains a major cause of mortality among males in western countries. Treatment options for metastatic castration-resistant disease remain limited. There is a continuing unmet need for new systemic interventions in patients with progressive prostate cancer. RhoA/Rho-associated protein kinases (ROCK) are key regulators of the cytoskeleton and have been implicated in PCa angiogenesis and tumour invasion. In the first study (Part I), we investigated the anti-angiogenic effects of fasudil, a ROCK inhibitor, on PCa-induced angiogenesis in vitro. Proliferation of PCa-conditioned human umbilical vein endothelial cells (HUVECs) was assessed using a bromodeoxyuridine (BrdU) assay, and migration was assessed with a wound healing assay. In vitro angiogenesis of PCa-conditioned HUVECs was evaluated by tube formation and a spheroid sprouting assay. Fasudil inhibited PCa-induced endothelial cell proliferation, and also decreased PCa-induced endothelial cell migration. In the *in vitro* angiogenesis assay, tube formation and spheroid sprouts were significantly inhibited by fasudil in a dose dependent manner. Western blotting results showed that expression of phosphorylated myosin phosphatase target subunit 1 (MYPT-1) was significantly lower after fasudil treatment, confirming that fasudil inhibited ROCK activity in these model systems. In the second study (Part II & III), we evaluated RhoA/ROCK expression and RhoA activity in a total of 34 paraffin embedded and 20 frozen prostate specimens, respectively, obtained from 45 patients treated with radical prostatectomy for clinically localized cancer. The expression patterns of RhoA/ROCK were tested by immunohistochemical staining and Western blotting, and further compared between the tumour centre, tumour front and distant peritumoral tissue. RhoA activity was assessed by G-LISA. Our results showed an increasing gradient of expression from the centre to the periphery of index tumour foci. RhoA expression was indeed significantly higher at the tumour front compared to tumour centre, using immunohistochemistry ($p=0.001$). Also, Gleason scores were significantly higher in patients with higher RhoA expression in both tumour front and tumour centre ($p=0.044$ and 0.039, respectively). After a median follow-up of 52 months, the rate of PSA relapse was higher in patients with a higher RhoA expression at the tumour front (62.5% vs 35%), although the difference was not significant ($p=0.089$). There was no association between RhoA expression and PSA, pathological stage. We also found ROCK2 expression, but not ROCK1 expression, was significantly higher in the prostate cancer tumor front. In conclusion, we found fasudil significantly inhibits the key steps of endothelial cell angiogenesis, including proliferation, migration, capillary tube formation and spheroid sprouting, in a dose-dependent manner. These effects may due to inhibition of ROCK activity induced by PCa cell secretions. We also identified higher RhoA and ROCK2 expression in human prostate tumour front. The correlation of higher RhoA expression with higher Gleason score and higher rate of cancer relapse. This indicated the association of RhoA/ROCK2 pathway with aggressiveness of prostate cancer. The insights described here may provide the foundation for novel therapeutic approaches targeting RhoA/ROCK pathway to inhibit angiogenesis and clinically aggressiveness of PCa. Fasudil may be a useful anti-angiogenic agent and should be investigated further for its potential role in PCa treatment.

Key words: prostate cancer, RhoA, ROCK, angiogenesis, invasion

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ABREVIATIONS

AA	arachidonic acid
ADT	androgen deprivation therapy
bFGF	basic fibroblast growth factor
BPH	benign prostate hyperplasia
CALGB	Cancer and Leukemia Group B
Cdc42	cell division control protein 42
CRD	cystein-rich domain
CRIK	citron kinase
CRPC	castration-resistant PCa
DMPK	myotonic dystrophy kinase
DRFs	diaphanous-related formins
EC	endothelial cell
EMT	epithelial-mesenchymal transition
ERM	ezrin/radixin/moesin
FERM	N-terminal 4.1/ezrin/radixin/moesin
GAP	GTPase-activating proteins
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine exchange factor
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase
HGF	hepatocyte growth factor
LIMK	LIM-Kinase
MAP	mitogen-activated protein
MBS	myosin binding subunit
MCP-1	monocyte chemoattractant protein 1
MLC	myosin light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
	DMPK-related cell division control protein 42
MRCK	binding kinase
MVD	microvessel density
MYPT-1	myosin phosphatase target subunit 1
PC-3	human prostate cancer cell line
PC3CM	PCa cell line PC3-conditioned medium
PCa	prostate cancer

PDGF	platelet-derived endothelial cell growth factor
PDGF-a	platelet-derived growth factor alpha
PH	pleckstrin-homology
PKN	protein kinase N
pMYPT-1	phosphorylated myosin phosphatase target subunit 1
PRK	protein kinase C-related kinase
PSMA	prostate specific membrane antigen
RBD	Rho-binding domain
Rho	Ras homology
RhoA	Ras homolog gene family, member A
ROCK	Rho associated coiled-coil kinase
RP	radical prostatectomy
SPC	sphingosine phosphorylcholine
SRF	serum response factor
TNF-a	tumour necrosis factor alpha
TRAMP	transgenic adenocarcinoma of the mouse prostate
VE	vascular endothelial
VE-Cadherin	vascular endothelial (VE) cadherin
VEGF	vascular endothelial growth factor
ET	endothelin

INTRODUCTION

1 EPIDERMIOLOGY OF PROSTATE CANCER

Prostate cancer (PCa) is the most prevalent malignancy in men in Western countries (Siegel, Naishadham et al. 2013). In France, 56800 PCa are newly diagnosed in the year of 2012, with age standardized incidence about 100 per 100,000 (Invs 2013). While in the other western European countries the incidence rate varies between 73.1 and 104.2 per 100,000 in the year of 2008 (UK 2012). It is the second leading cause of cancer-related death in this area. 8900 cases of PCa related death are recorded in France in 2012. Several autopsy studies have shown that 60-70% of older men have histological PCa(Heidenreich, Bastian et al. 2014). PCa is diagnosed in 15-20% of men during their lifetime (Heidenreich, Bastian et al. 2014). In China, the incidence of PCa increased rapidly during the past decades, partly because of the application of PSA screening in the elder male patients. 994 cases of prostate cancer were diagnosed in Shanghai in 2009, with an incidence of 32.23 per 100,000, fifth cause of tumour in men ("Incidence of malignancy". 2012).

2 CURRENT STATUS OF PROSTATE CANCER TREATMENT

Data from autopsy showed that advanced and metastatic stages of the disease are found in 35% of patients with PCa (Bubendorf, Schöpfer et al. 2000). With the widely application of PSA screening, the diagnosis of PCa becomes earlier. But till now, there are still a rather large portion of patients are diagnosis as advanced stage, as reported 11.7% in USA and 31.2 in Europe in 2013. A multicenter report in China showed at least 36.2% of PCa patients were diagnosed at advanced stage (Peyromaure, Debre et al. 2005).

For PCa patients who are diagnosed with early stage, therapeutic options include radical prostatectomy (RP), radiotherapy, and active surveillance. Active surveillance has become more accepted by the patients and doctors. But it is still strictly limited to some of the low risk patients who don't want to receive the operation immediately and who can comply with regular follow-up under surveillance. Although there is some controversy on the treatment choice between RP and radiotherapy, RP is still considered as the best treatment for localized early stage patients. It has also been reported to have a little benefit on overall survival as compared with radiotherapy in a 15 years' long term cohort study (Sooriakumaran, Nyberg et al. 2014). Thus, RP is the most common treatment choice for these patients. Even for patients who are eligible for radical prostatectomy, approximately 35% will develop recurrence (metastatic disease) within 10 years of surgery (Hull, Rabbani et al. 2002, Roehl, Han et al. 2004).

For most of the patients who present with or progress to advanced or metastatic disease, hormone therapy can effectively palliate the symptoms. This is also referred to androgen deprivation therapy (ADT), including castration with or without anti-androgens. However, there is currently no conclusive evidence to show that it extends life. Furthermore, the median duration of response to ADT is limited to between 8 months and 3 years (Daneshgari and Crawford 1993) and these patients will eventually become castration resistant. When the patients progress to CRPC, there are few effective alternative treatments for them, such as chemotherapy. Chemotherapy regime including docetaxel is effective treatment for castration-resistant PCa. Docetaxel was shown to mildly prolong survival in patients with CRPC. Despite demonstrating an improvement in overall survival, responses are not durable and eventual progression of the disease is inevitable. The median duration of response is only 10.3 months (Eymard, Oudard et al. 2010). As regard to the high incidence rate and large portion of progressive stage

patients, there is clearly an urgent need to develop additional systemic interventions for these patients.

3 HALLMARKS IN CANCER DEVELOPMENT

There are ten hallmarks which represent the crucial biological capabilities acquired for the multistep development of human tumours, including sustaining genome instability & mutation, escaping growth suppressors, evading cell death, enabling replicative immortality, proliferative signaling, deregulating cellular energetics, promoting inflammation, inducing angiogenesis, avoiding immune destruction and activating invasion and metastasis. Many new therapies are developing targeting these hallmarks. Many studies have been conducted to interfere with these acquired capabilities in the tumour growth and progress. Researches targeting angiogenesis and tumour metastasis are among those which are actively being pursued.

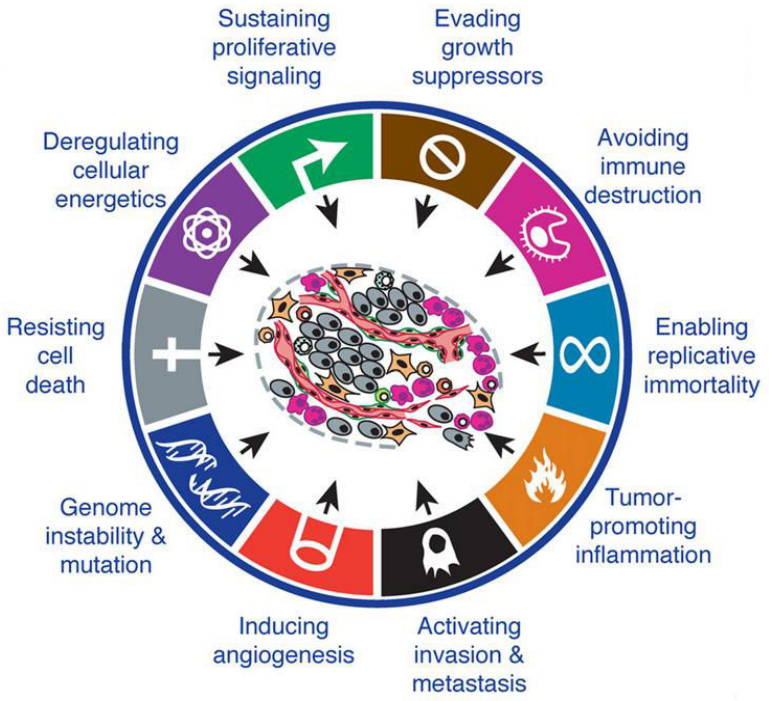

Figure 1 The hallmarks of cancer (Hanahan and Weinberg 2011).

4 ANGIOGENESIS IN CANCER DEVELOPMENT

The tumour-associated neovasculature is a prerequisite for the expansion of solid tumours beyond 1-3 mm^3(Folkman, Cole et al. 1966). During the very early stage of the tumour, these lesions may stay dormant by reaching a steady state between growth and apoptosis. Dormant tumours have been discovered in autopsies of patients who died of causes other than cancer (Black and Welch 1993). The tumour-associated neovasculature mainly generated by the process of angiogenesis. During embryogenesis, neovasculature includes the birth of new endothelial cells and their assembly into new vessels (vasculogenesis) as well as the sprouting (angiogenesis) of new vessels from existing ones. In the adult, the normal vasculature becomes largely quiescent. This balance of angiogenesis is sophistically governed by the balance of counteracting factors: pro-angiogenic and anti-angiogenic factors. Only some of physiologic processes such as female reproductive cycling or wound healing, pro-angiogenetic factors are only transiently elevated, resulting in transiently turn on of angiogenesis. These are strictly controlled by the balance of angiogenic stimulators or inhibitors. In contrast, during solid tumour progression, in which consistently elevated pro-angiogenetic factors are always found, angiogenesis is nearly always activated and remains on, causing continually sprouting of new vessels into the expanding neoplasm (Hanahan and Folkman 1996). This step has been termed as ‘‘angiogenic switch’’ (Fig. 2). Tumours acquire the ability to recruit their own blood supply is one of the important hallmarks in the progress of the tumour (Fig. 1).

Figure 2 The balance of the angiogenesis control.

4.1 CELLS INVOLVED IN TUMOUR ANGIOGENESIS

Angiogenesis is a multiple step process involving a complex series of events. Firstly, an increase in the permeability of the basement membrane allows a new capillary to sprout. Next, endothelial cells(ECs) activated by angiogenic factors migrate through the basement membrane into the extracellular matrix, towards the angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of ECs. This is then followed by re-organization of ECs to form tubules with a central lumen, together with the recruitment of peri-endothelial cells (pericytes) and vascular smooth muscle cells for new capillary stabilization(Sakamoto, Ryan et al. 2008). Besides cancer cells, many other cells in the cancer microenvironments including, fibroblast, inflammatory cells, pericytes and immunocytes take part in this process.

Tumours growth results in the increasing of diffusion distances from the existing vascular and decreasing oxygen supply. Tumour hypoxia can also arise as a result to

increased metabolic activity and oxygen consumption by rapidly proliferating tumour cells. As a reaction to hypoxia, the transcription factor hypoxia inducible factor (HIF) -1 is activated. Among which, HIF-1α is the most ubiquitously expressed, and functions as the master regulator of oxygen homeostasis, which is also the key regulator of hypoxia-induced angiogenesis (Krock, Skuli et al. 2011). HIF-1 can directly activate many gene transcription by HRE binding (Manalo, Rowan et al. 2005). Multiple HIF-1 target genes have been demonstrated to modulate angiogenesis. These lead to induction of VEGF RNA stability, transcription and translation. It also reduces the levels of the endogenous anti-angiogenesis factor TSP1. Some other pro-angiogenic factor expression also increase due to HIF-1 activation. Tumour cell gene instability and mutation may be another reason for the elevated other pro-angiogenic factor expression of tumour cells. There are abundant reports on elevated pro-angiogenic factors secretion by tumour cells (Santin, Hermonat et al. 1999).

Stromal fibroblasts in tumour tissue also plays an important role in tumour angiogenesis. During tumour growth, fibroblasts become activated, secreting various ECM components, such as collagens, fibronectin, heparan sulfate proteoglycans, which are very important to provide a support to neovasculature. These cells also secrete soluble angiogenic growth factors such as VEGF (Kellouche, Mourah et al. 2007), transforming growth factor-β(TGF-β) (Paunescu, Bojin et al. 2011), and platelet-derived growth factor (PDGF) (Paunescu, Bojin et al. 2011), Several studies have reported that these fibroblasts, also called carcinoma-associated fibroblasts (CAFs), promote tumour growth partially through promotion of angiogenesis (Newman, Nakatsu et al. 2011).

Since long time, there have been many researches on the relation between inflammation and cancer. The inflammation in some organs may increase the risk of tumour. In the tumour microenvironment. Inflammation also contributes to tumour angiogenesis, as

well as metastasis etc. It is known as cancer-related inflammation (CRI). Characteristics of cancer-related inflammation include the infiltration of white blood cells, prominently tumour-associated macrophages (TAMs), the presence of inflammation factors such as cytokines (TNF, IL-1, IL-6), which can promote angiogenesis in the tumour stroma. Recently, more and more evidences were shown that one subset of monocytes expressing the Tie2 receptor (TEM) has a crucial role in tumour angiogenesis (De Palma, Murdoch et al. 2007) (De Palma, Venneri et al. 2005). These peri-tumoural inflammatory cells, as well as inflammatory factors which they have secreted, help to trigger the angiogenic switch in previously quiescent tumour tissue and to maintain ongoing angiogenesis along with tumour growth.

4.2 OTHER MECHANISM OF THE FORMATION OF TUMOUR-ASSOCIATED VASCULATURE

Besides angiogenesis, there are several other mechanisms also involved in tumour vessel formation, including vascular co-option, mosaicism, vasculogenic mimicry and postnatal vasculogenesis.

4.2.1 VASCULAR CO-OPTION

Some tumours can grow and metastasize without angiogenesis. Studies have found an alternative way for the tumour to gain its blood supply, vessel co-option. It means tumour cells migrate to and along the preexisting blood vessels of the host organ. Vessel co-option occur in many malignancies, and may be more important in some highly vascularized tissues such as lung, brain, and liver. It may also be important as a potential mechanism of anti-angiogenic drug resistance, as it has been reported that anti-angiogenic therapy promoted tumour invasion and metastasis by facilitating the tumour cells migration towards the less hypoxia region.

4.2.2 VASCULOGENIC MIMICRY

Vasculogenic mimicry (VM) is a newly-defined pattern that tumour gains ts blood supply. Cancer stem cells (CSCs) are found to have the capacity of self-renewal and multipotent differentiation, and may contribute to the new vessels formation in the tumour tissue. It has been known for many years that tumour endothelia has differences in molecular markers and the phenotype with normal endothelium. While some of them can be found in malignant cells (Hida, Hida et al. 2004). A fraction of the tumour vascular endothelial cells has been proven to be of neoplastic origin (Pezzolo, Parodi et al. 2007). CSCs have been proved to be capable of forming functional blood vessels de novo by trans-differentiating into EPCs, ECs, or vascular smooth muscle-like cells.

4.2.3 VASCULOGENESIS

Tumour vasculogenesis means that new vasculatures are constituted via recruitment of endothelial progenitor cells (EPCs) from the bone marrow. Peripheral tissue hypoxia results in increased secretion of EPC-mobilizing factors (for example granulocyte colony–stimulating factor (G-CSF), vascular endothelial growth factor(VEGF), basic fibroblast growth factor (bFGF), placental growth factor, erythropoietin or SDF-1) which can mobilize EPC in the BM, leading to an increase of EPC in blood (De Falco, Porcelli et al. 2004). Once in circulation, EPCs response to chemokine signaling in the tumour tissue and home to the tumour site. They cross the endothelial monolayer, migrate through the blood vessel basement membrane and through the interstitial extracellular matrix (ECM) alonging the gradient of chemokines to exert their functions.

5 ANGIOGENESIS IN PROSTATE CANCER

Angiogenesis plays a critical role in cancer progression and metastasis, and its significance in human PCa has been firmly established. The microvessel density (MVD)

was more prominent in carcinomas than in benign prostatic hyperplasia (BPH) and normal tissue (Bigler, Deering et al. 1993, Stefanou, Batistatou et al. 2004). It has been reported that MVD increases with increased Gleason's score, especially in poorly differentiated PCa (Weidner, Carroll et al. 1993). Increased MVD was significantly associated with high-grade carcinomas (Stefanou, Batistatou et al. 2004). Weidner showed that MVD was significantly increased in prostate cancer tissues of those patients with metastatic disease as compared with those without metastasis (Weidner, Carroll et al. 1993). Borre et al reported that MVD of PCa samples was significantly correlated with stage, grade and disease specific survival in 221 prostate cancer patients with a median followup of 15 years (Borre, Offersen et al. 1998).

5.1 REACTIVE STROMA

Till now, mechanism of angiogenesis in PCa has not yet been well elucidated. Although there are several mechanisms of tumour neovascularization, only angiogenesis was well explored in PCa. Microenvironment of PCa comprises a reactive stroma, characterized by an increasing number and abnormal function in myofibroblasts and fibroblasts, a corresponding augmentation in extracellular matrix components production, and an increase in microvessel density (Tuxhorn, Ayala et al. 2002). Prostate cancer cells, together with cells in their reactive stroma, secreted increasing level of angiogenic stimulator and decreasing level of angiogenic inhibitor, resulting in the angiogenic switch in local foci. Many stimulators were found to be significantly higher in the PCa tissue, including HIF-1, VEGF, bFGF, PDGF, IL-8 etc.

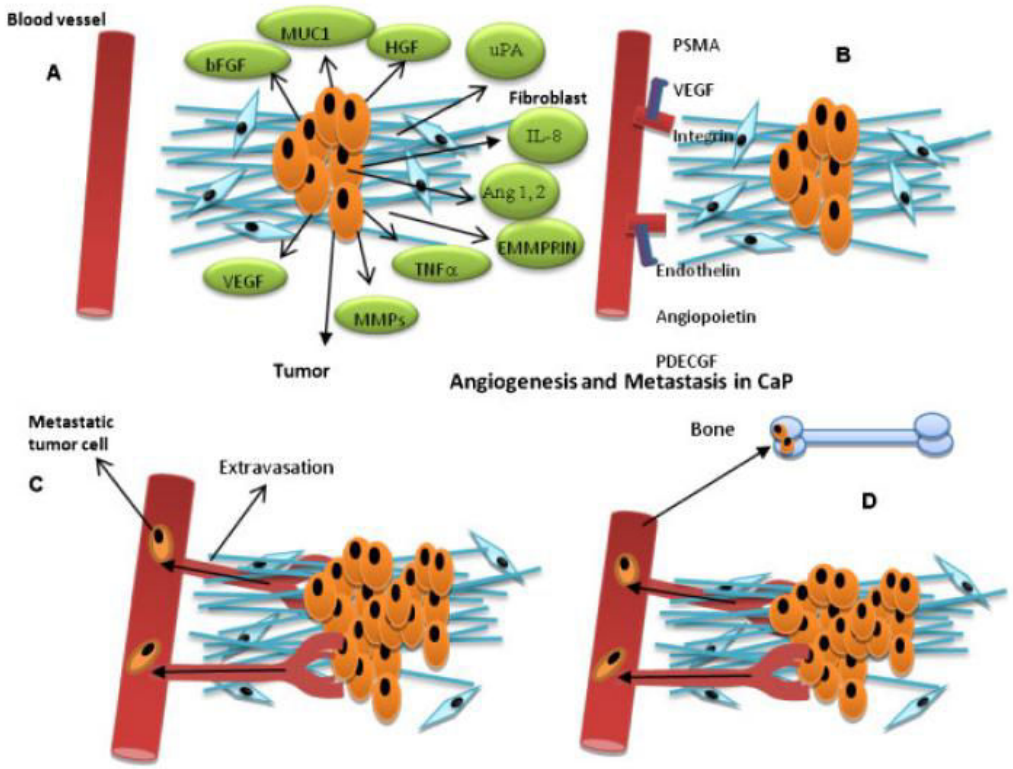

Figure 3 Mechanisms regulating angiogenesis in PCa metastasis(Li and Cozzi 2010).

5.2 ANGIOGENIC STIMULATORS IN PROSTATE CANCER

5.2.1 HIF

Huss et al. demonstrated that the increased expression of HIF-1α and VEGFR-1 in the early stage of angiogenic switch in transgenic adenocarcinoma of the mouse prostate (TRAMP) model, resulting in the increase in neovasculature in high grade prostatic intraepithelial neoplasm and prostate cancer lesions (Huss, Hanrahan et al. 2001) . In PCa patients, the accumulation of HIF-1a protein has also been reported to be an early event in PCa as well as in HGPIN (Hao, Chen et al. 2004) (Zhong, Semenza et al. 2004). Increase expression of HIF-1a directly lead to increasing transcription of the angiogenic factor VEGF. The over expression of VEGF in PCa is associated with the extent of

tumour hypoxia, with according overexpressed HIF-1a in PCa tissues in comparison with benign prostatic tissues (Cvetkovic, Movsas et al. 2001).

5.2.2 IGF-1

IGF-1 is a stimulator of cellular proliferation and cell survival as well as a stimulator of angiogenesis. It has been reported that local levels of IGF-1 and its receptor (IGF-1R) are both increasing during progression of PCa in TRAMP model (Kaplan, Mohan et al. 1999). In this model, serum IGF-I levels correlate with the increase in MVD associated with the development of PCa lesions, indicating the role of IGF-I in the induction of prostatic neovascularization. Clinical study also found increased levels of IGF-I in men with PCa compared with controls (Chokkalingam, Pollak et al. 2001, Woodson, Tangrea et al. 2003). Higher levels of serum IGF-1 have been proven to be a strong risk factor of developing PCa in the aged population (Chan, Stampfer et al. 1998). IGF-1 can upregulate the expression of modulators of endothelial cell function such as VEGF, which has been postulated to be the main downstream events (Akagi, Liu et al. 1998) (Fukuda, Hirota et al. 2002).

5.2.3 ANDROGENS

Androgens have been proven to regulate the vasculature both in PCa animal model and PCa patients. Androgens have been shown to be involved in the up-regulation of HIF-1α expression in PCa (Mabjeesh, Willard et al. 2003). Androgens have also been reported to regulate VEGF expression thus influenced angiogenesis (Cheng, Zhang et al. 2004). Both in the glandular epithelial cells and in smooth muscle cells of the prostate, the secretion of VEGF is regulated by androgens. Richard et al demonstrated an androgen-mediated mechanism of VEGF modulation in a PCa mouse model (Richard, Kim et al. 2002). Androgen deprivation resulted in decrease angiogenesis in PCa in animals models

(Cheng, Zhang et al. 2004). These results indicated that androgen has an important role in the formation of PCa neovsculature and this takes effects mainly through VEGF pathway.

5.2.4 ENDOTHELINS

The endothelin (ET) pathway axis, which includes the biological functions of ETs and their receptors (ETAR and ETBR), acts as a modulator of vasomotor tone, tissue differentiation and development, cell proliferation and hormone production. They are also linked to angiogenesis. Three isoforms of endothelins, ET-1, 2, and 3 are secreted by epithelial cells(Kopetz, Nelson et al. 2002). They interact with two membrane-bound receptors, endothelin A receptor (ETAR) and endothelin B receptor (ETBR). ET-1 stimulates ETAR to induce VEGF release leading to an angiogenesis response (Goligorsky, Budzikowski et al. 1999, Salani, Taraboletti et al. 2000). ET-3 interacts with ETBR to promote endothelial cell growth and new blood vessel formation(Lara, Twardowski et al. 2004). In PCa, key molecules of the ET-1 clearance pathway, neutral endopeptidase, are impaired, resulting in an accumulation in local ET-1 concentrations(Bagnato and Rosanò 2008). It has been reported that ET-1 secreted by prostate cancer cells promotes neovascularization via ETAR and ETBR(Kopetz, Nelson et al. 2002). The various mechanisms by which ET-1 regulates angiogenesis include endothelial cell proliferation, migration, invasion, protease production, tube formation, and the production of VEGF.

5.2.5 FIBROBLAST GROWTH FACTOR (FGF)

FGF is another important pro-angiogenic factor which has been shown to mediate angiogenesis bypassing blockage of therapy targeting VEGF and VEGF receptor. FGF has

been found to be overexpressed in prostate cancer(Kwabi-Addo, Ozen et al. 2004). Immunohistochemical analysis on prostate cancer samples revealed FGF2 receptor protein expression was elevated in prostate cancer cells and in endothelial cells. This increase in the expression of FGF receptor was correlated with androgen-independent prostate cancer. Expression of FGF2 and its receptors were significantly higher in androgen-independent and more aggressive prostate cancer cells(Kwabi-Addo, Ozen et al. 2004).

5.2.6 TGF β

In benign prostate epithelium TGF β is expressed in a very low quantity to maintain epithelial homeostasis in a paracrine mode(Russell, Bennett et al. 1998). It has been found to be overexpressed in prostate cancer cells, increasing extracellular matrix production and inducing angiogenesis. It can also inhibit host immune function and promote tumour cells to escape from host immune surveillance. TGF β has also been shown to favour osteoblastic bone metastases in an animal model. TGF β has three isoforms, TGF β1, TGF β2, and TGF β3, with related receptors TGF βRI, TGF βRII, and TGF βRIII respectively. In prostate cancer, high-grade tumours and advanced clinical tumour stage have higher expression of TGF βRI. Additionally, TGF βRI expression correlates with an increase of tumour vascularity and metastasis (Wikstrom, Damber et al. 2001) . On the contrary, TGF βRIII expression is decreased or lost in human prostate cancer. This loss correlates with advancing tumour stage and a higher probability of recurrence, suggesting TGF βRIII expression to play an important negative role in prostate cancer progression.

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5.2.5 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF is the most prominent and important regulator of physiological angiogenesis(Lonser, Glenn et al. 2003). It plays a significant role in angiogenesis. Cells in tumour tissue including cancer cells, fibroblasts and macrophages secrete VEGF to stimulate the formation of new vessels in response to hypoxia (Dvorak, Detmar et al. 1995, Byrne, Bouchier-Hayes et al. 2005). Several studies have shown that PCa cells highly express VEGF in vitro and in vivo (Ferrer, Miller et al. 1997, Ferrer, Miller et al. 1998). VEGF expression by PCa specimens and PCa cell lines (such as LNCaP, PC-3, and DU 145) is far more greater than that by stromal cells of the normal prostate (Harper, Glynne-Jones et al. 1996, Ferrer, Miller et al. 1997, Jackson, Bentel et al. 1997). Besides its critical role in stimulating neovasculature, VEGF is also a survival factor for tumour cells by protecting them from hypoxia, chemotherapy, and radiotherapy (Byrne, Bouchier-Hayes et al. 2005). Thus, VEGF and VEGF pathway have been chosen as the first and the most important target in anti-angiogenic therapy.

Clinical studies comparing PCa with benign prostate hyperplasia (BPH) revealed that VEGF expression was higher in PCa, especially in high grade PCa, and correlated with increased levels of angiogenesis (Stefanou, Batistatou et al. 2004). In prostate cancer, serum levels of the humoral ligand VEGF were found to be significantly higher in those patients with metastatic disease (Duque, Loughlin et al. 1999). Plasma VEGF levels have also been reported to be an independent prognostic factor in patients with metastatic prostate cancer (George, Halabi et al. 2001). Peyromaure et al compared 17 patients who developed bone metastases after RP with 23 patients who remained disease free and found the expression of VEGF was significantly higher in those who developed bone metastases after RP(Peyromaure, Camparo et al. 2007). The levels of VEGF in serum, plasma, or urine are correlated with patient outcome in both localized as well as

disseminated PCa (Bok, Halabi et al. 2001). In a study of 50 patients with locally advanced disease treated with radical radiotherapy, Green and co-workers reported a significant correlation between higher VEGF expression and worse disease-specific survival (Green, Hiley et al. 2007). In addition, levels of the VEGFR were correlated with a poorer grade of tumour differentiation and prognosis in PCa(Huss, Hanrahan et al. 2001). Based on these findings, VEGF and VEGF pathway have been targeted as a hot strategy to treat prostate cancer.

5.2.6 OTHERS

There are many other angiogenic stimulators also have been implicated in PCa angiogenesis, including Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) (Trojan, Thomas et al. 2004), Transforming growth factor-beta (TGF-b) (Zhang, Lee et al. 2005), Platelet-derived endothelial cell growth factor (PD-ECGF) (Okada, Yokoyama et al. 2001, Sivridis, Giatromanolaki et al. 2002), Hepatocyte growth factor (HGF) (Zhu and Humphrey 2000, Davies, Mason et al. 2003), Interleukin-8 (IL-8) (Inoue, Slaton et al. 2000, Kim, Uehara et al. 2001, Murphy, McGurk et al. 2005) etc.

Role of vasculogenic mimicry has also been explored in prostate cancer. An in vitro study to assess vasculogenic mimicry by prostatic tumour cell lines has been performed (Sharma, Seftor et al. 2002). It revealed that prostate tumour cells can form perfusable vasculogenic-like networks and express various vascular markers in vitro. Prostatic tumour cell-lined channels were also observed in vivo in high grade tumours in PCa patients.

5.3 ANGIOGENIC INHIBITORS

Angiogenic inhibitors, together with angiogenic stimulators, control and maintain the balance of angiogenesis in physical conditions. While in cancer micro-envirement, there are always abundant angiogenic stimulators either secreted by cancer cells or the other cells such as fibroblasts, inflammatory cells in the reactive stroma, overtake the effects of angiogenic inhibitors, leading to neovasculature in tumour. They seem less significant in tumour angiogenesis.

5.3.1 THROMBOSPONDIN-1 (TSP1)

TSP1, the family of extracellular matrix proteins, is the first anti-angiogenic molecule that has been characterized (Good, Polverini et al. 1990). TSP1 inhibits the migration of endothelial cells and induces their apoptosis in vitro (Jimenez, Volpert et al. 2000). TSP1 expression is inhibited in many tumours, including prostate cancer. In androgen dependent prostate cancer, TSP1 expression is negatively correlated with MVD (Kwak, Jin et al. 2002). But in CRPC, TSP1 was no longer correlated with vascularization (Colombel, Filleur et al. 2005).

5.3.2 TISSUE INHIBITOR OF METALLOPROTEINASES (TIMPS)

Tissue inhibitors of metalloproteinases(TIMPs) negatively regulate the activities of MMPs. MMPs are key proteins in remodelling the ECM during angiogenesis as well as tumour invasion and metastasis. They can activate many pro-angiogenic factors by cleavage of their precursor proteins to increase their bioactivities(Shuman Moss, Jensen-Taubman et al. 2012). Escaff et al shown that both MMPs and TIMPs are significantly elevated in PCa samples (Escaff, Fernandez et al. 2010). But clinical trials of a variety of MMP inhibitors showed significant adverse effects with no therapeutic benefit for cancer patients (Coussens, Fingleton et al. 2002).

6 ANTI-ANGIOGENIC THERAPY IN PROSTATE CANCER

The growth of PCa, as with the growth of the other solid tumours, depends on angiogenesis. Therapeutic strategies aimed at preventing the growth of new blood vessels to supply tumours have yielded clinical benefits for patients with many different types of cancers, most notably renal cell carcinoma. There is a strong preclinical basis for studying inhibitors of angiogenesis in prostate cancer, as this process appears to play an important role in prostate carcinogenesis and maintenance. However, it has become increasingly apparent that current anti-angiogenic therapy targeting angiogenesis has only a modest effect in the clinical setting.

As VEGF pathway is the most important one in cancer angiogenesis, many drugs that inhibit VEGF signaling have been tested in prostate cancer. Antibody against VEGF has been developed and tested in PCa, including preclinical and clinical studies. Bevacizumab is the most well-known one and has been approved as a treatment for several cancer since 2008. It is a humanized MAb that binds the VEGF ligand and prevents receptor binding and signal transduction. Recently a phase-III Cancer and Leukemia Group B (CALGB) 90401 trial of bevasizumab in combination with docetaxel and prednisone failed to show significant improvement in OS in men with mCRPC(Kelly, Halabi et al. 2012). Isayeva et al demonstrated that inhibitors of the VEGFR-2 delayed tumour progression only when administered in the early stages of PCa in a TRAMP model, while it was ineffective if administered during the late stages of PCa(Isayeva, Chanda et al. 2007). The other strategy targeting angiogenesis has also been explored extensively, including small molecule inhibitors for VEGFR tyrosine kinase activity, Platelet-Derived Growth Factor Alpha (PDGF-a) and Endogenous Angiogenesis Inhibitors(Wozney and Antonarakis 2014). Only few of them entered phase III clinical trial.

The antitumour effects of anti-angiogenic therapy are rapidly become resistant, notably due to increased invasiveness and accelerated metastasis. These treatments, mostly targeting the VEGF pathway, can lead to disease stabilization and longer periods of progression free survival, but did not lead to prolonged overall survival (Bergers and Hanahan 2008). Some studies in preclinical models have revealed that anti-VEGF therapies promote tumour invasiveness and metastasis (Ebos, Lee et al. 2009, Paez-Ribes, Allen et al. 2009). Thus the anti-angiogenic therapy combined with anti-invasion and anti-metastasis therapy can be a prosperous strategy.

7 INVASION IN CANCER DEVELOPMENT

Cell migration and invasion are critical parameters of tumour progress and the characteristics of higher pathological grades cancer. Migratory cancer cells undergo profound molecular and cellular changes in their cell-cell and cell-matrix adhesion and their actin cytoskeleton, molecular processes that involve the activity of various signalling networks. In most epithelial cancers, alteration of cell adhesion molecules and adherent junctions, conversion from a polygonal to a spindly morphology, expression of matrix-degrading enzymes, increase of cell motility and higher resistance to apoptosis take place. These alterations are characterized by loss of the cell-cell adhesion molecule E-cadherin and obtain of mesenchymal markers N-cadherin and the conversion of epithelial cells to mesenchymal, migratory and invasive cells, a process called the epithelial-mesenchymal transition (EMT). Several studies of solid tumours have proved the association of increased EMT with the capability of cancer cells migration, invasion and metastasis (Gotzmann, Mikula et al. 2004, Brabletz, Hlubek et al. 2005, Huber, Kraut et al. 2005).

Normal benign epithelial cells express “epithelial” adhesion molecules, such as E-cadherin, which helps them to adhere to nearby cells and extracellular matrix. This makes them fix in the constant position and helps to keep their normal shape. It is also considered to be an inhibitor of oncogenesis. Loss of their expression may lead to the alteration of cell shape and mobility, thus the cells are less confined by surrounding compartments and easier to move, which have been frequently observed in human cancer(Cavallaro and Christofori 2004). Epithelial mesenchymal transition also associates with induction of “mesenchymal” cadherins, such as N-cadherin(Berx and van Roy 2009). This type of adhesion molecules normally expressed in mesenchymal cells during organogenesis. They may help cancer cells to form temporary adhesion to extracellular matrix while migrating.

Under most circumstances, cancer cells mimic morphogenic developmental programs to migrate and invade. The reorganization of the actin cytoskeleton are involved in these processes, which lead to the formation of membrane protrusions for cell motility in the tumour micro-environment, including lamellipodia, filopodia, podosomes and invadopodia(Wang, Eddy et al. 2007) (Gupton and Gertler 2007) (Vignjevic and Montagnac 2008) (Olson and Sahai 2009) . Members of the Rho GTPase family play a critical role in transmitting signals from growth factor and cell adhesion receptors to effector proteins of actin cytoskeleton remodelling(Sahai and Marshall 2002, Valderrama and Ridley 2008, Vega and Ridley 2008, Narumiya, Tanji et al. 2009). New imaging technologies facilitated the visualization of the activities of Rho GTPases and the homeostasis of adherent junctions in live cells in a temporal and spatial manner, and have provided new insights into the coordinating control of actin cytoskeleton assembly and disassembly during cell mobility(Sahai 2007, Cavey, Rauzi et al. 2008).

In mesenchymal migration, cells have high levels of focal adhesions and cytoskeletal contractility, interact with focalized matrix and move in a fibroblast-like manner(Grinnell 2008). Besides this individually movement, there is also another mode of cell migration, collectively movement. The tumour cells migrate in sheets, tubes, and clusters, either still connecting with their original tissue or separating as independently migrating clusters (Friedl and Gilmour 2009). This procedure may contribute to local cancer invasion (Alexander, Koehl et al. 2008).

Cells migrating in collective mode also form membrane protrusions, ruffles and pseudopods. They connect cell-matrix adhesion receptors to the actin cytoskeleton to form focal adhesions. In this case, the maintenance of cell-cell adhesions result in partial or complete silence of migration activity inside the mass yet supports cytoskeletal activity at outside edges (Friedl, Noble et al. 1995, Farooqui and Fenteany 2005).They pass through the matrix scaffold by directly proteolyse the extracellular matrix. Actin-myosin contraction is involved in local contraction and cell movement.

8 INVASION IN PROSTATE CANCER

EMT is the most important mechanism of invasion and metastasis in many cancers. Its importance has also been proved in PCa. Several studies have revealed that epithelial-mesenchymal transition markers, E-Cadherin, was down-regulated in prostate cancer. Its expression was negatively correlation with Gleason score and prognosis (Umbas, Isaacs et al. 1994, Contreras, Ledezma et al. 2010) (Putzke, Ventura et al. 2011). Another EMT marker, N-cadherin, which is normally absent in benign prostate gland cells, had also been found to express in prostate cancer and its expression was associated with high Gleason grade and tumour progress (Tomita, van Bokhoven et al.

2000, Jaggi, Nazemi et al. 2006). Monoclonal antibody targeting of N-cadherin inhibited prostate cancer growth, metastasis (Tanaka, Kono et al. 2010).

In prostate cancer of high and intermediate differentiation, collective-cell movement is also a putative mechanism. In highly differentiated prostate cancer, glandular structures are retained in the invading foci. With the loss of apical-basal polarity and dedifferentiation, amorphous cell strands and clusters that with or without an inner lumen extend into the surrounding tissue.

9 RHOA/ROCK SIGNALING PATHWAY

9.1 MOLECULAR STRUCTURE AND EXPRESSION OF RHOA

RhoA is a small guanosine triphosphate hydrolase (GTPase) belonging to the Ras homology (Rho) family. The Rho family of GTPases comprise at least 23 members, including Cdc42, Rac1, and RhoA, which are the three most well studied members. (Wennerberg, Rossman et al. 2005, Bustelo, Sauzeau et al. 2007).

In humans, RhoA protein is encoded by the gene RhoA. This gene locates at Chromosome 3 in the cytogenetic band of 3p21.31. Its sequence contains 5 exons. The transcript length is 1919 bps, which translated to a protein of 193 residues.

Rho protein structures comprise of a single domain with eight helices and six-stranded beta-sheet. Rho proteins contain three insertions and one deletion. The 13-residue insertion (Asp 124 to Gln 136 in RhoA) is the most distinctive feature as compared with other Ras-related GTPases. Different Rho proteins have distinctive amino acid sequence in this insert, resulting in different functional features. RhoA have two specific regions,

called switch I and switch II, distinguishing the conformational differences between RhoA-GDP and RhoA-GTP. Another important difference between the GDP-bound and GTP-bound RhoA is the mode of Mg ion binding. The removal of Mg2+ can be caused by GEFs. GEFs can also cause a displacement in the switch I region, which makes GDP binding site open for GEF interaction, replacing GDP with GTP. A small displacement between at Pro36 and Phe39 is induced in switch I when RhoA bound to GTP instead of GDP. This cause three hydrophobic residues, Val35, Val38, and Phe39, become exposed, which involve in target binding after activation. Both switch I and switch II are involved in the binding with its substrates (eg. ROCKs). RhoA has a ubiquitous tissue distribution(Fig. 4), indicating its versatile function in cell biology. It also has been found to increase in many malignant tumour(Fig. 5).

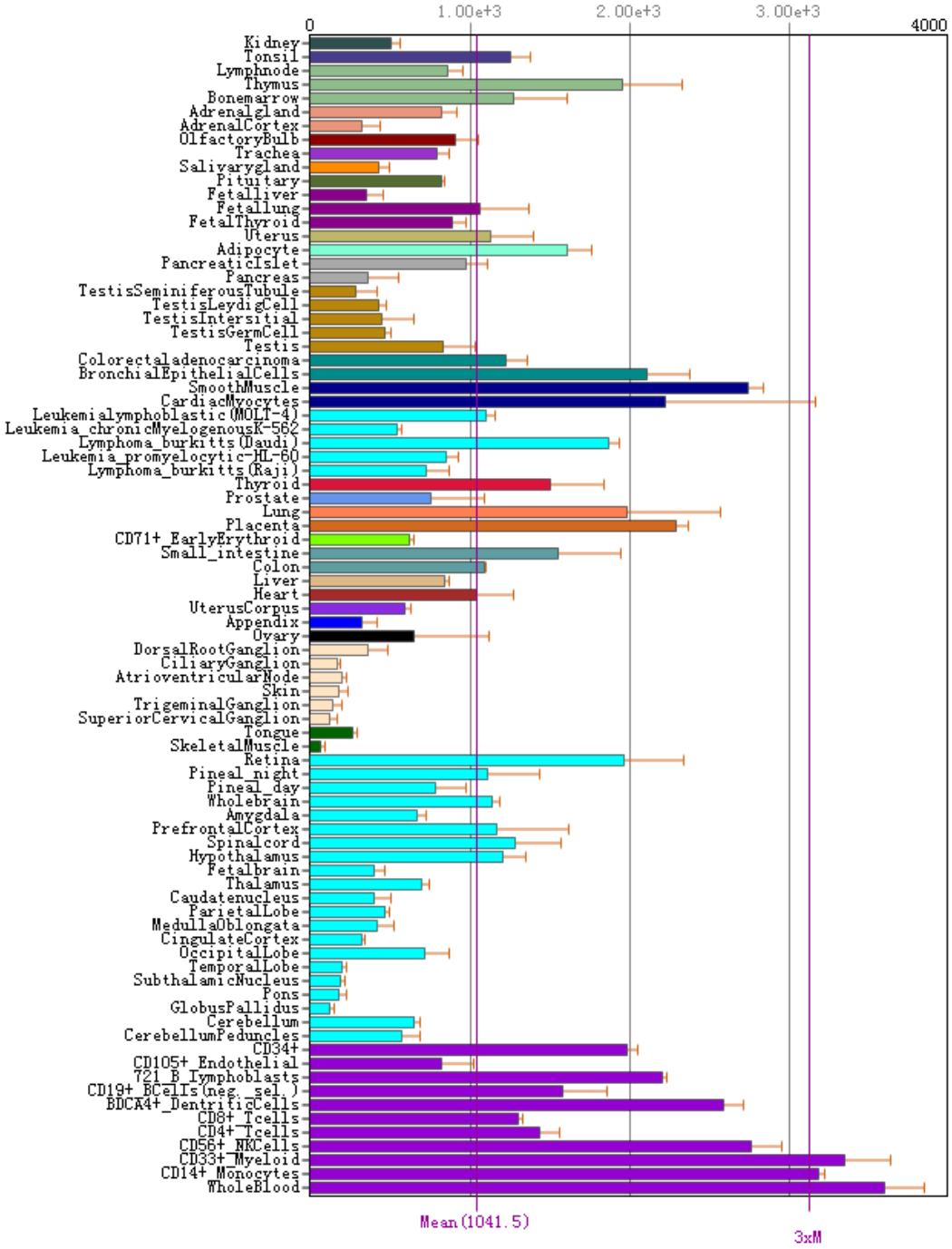

Figure 4 Expression of RhoA in various tissues and cells in human (BioGPS. 2014).

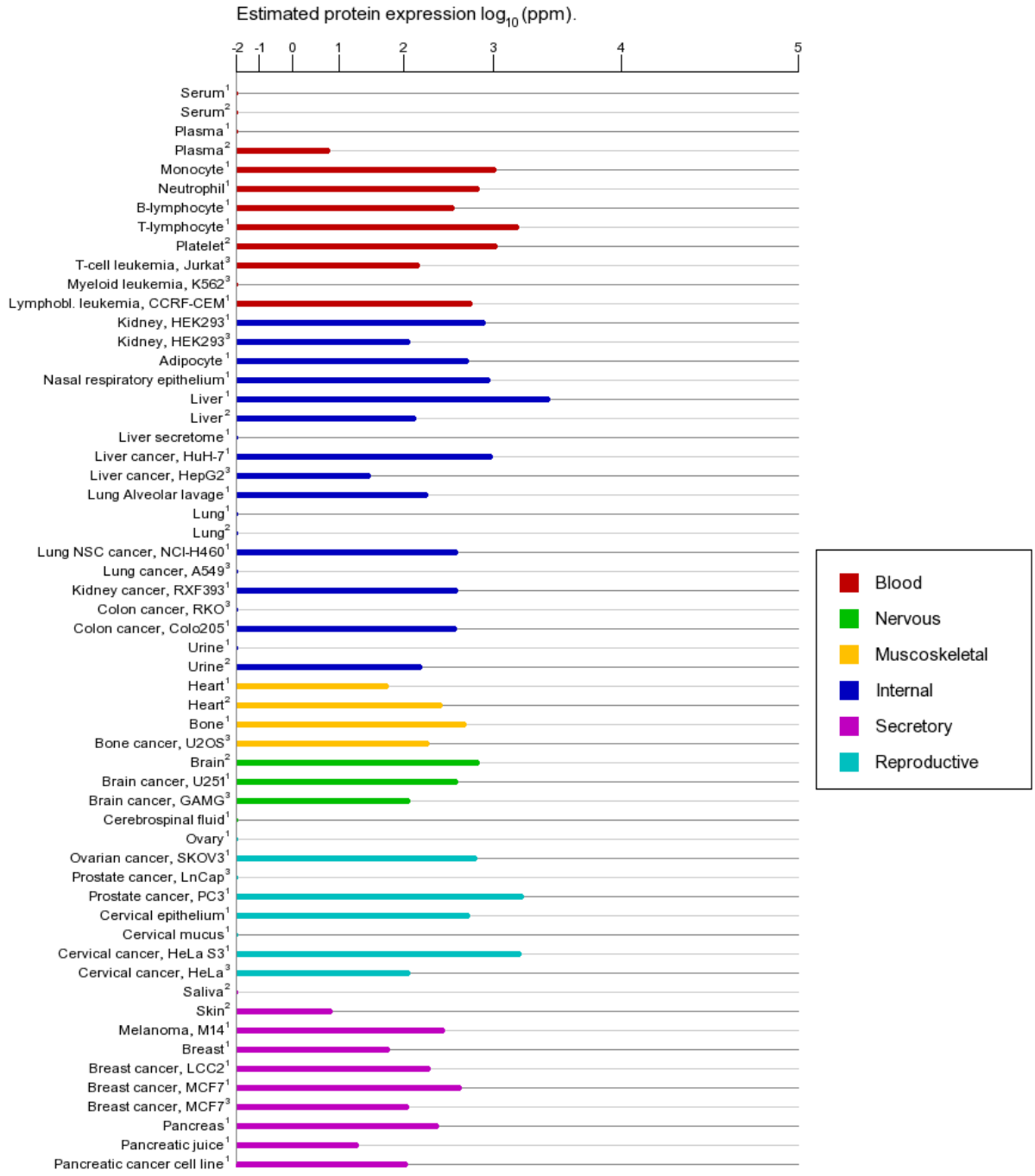

Figure 5 Expression of RhoA in human cancer cells and tissues (GeneCards. 2014).

9.2 REGULATION OF RHOA ACTIVITY

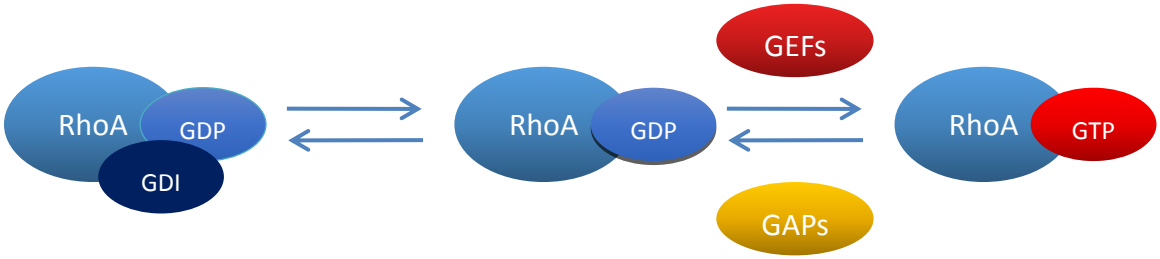

Figure 6 Regulation of RhoA activity.

Rho GTPases function as molecular switches, cycling between an active GTP-bound conformation and an inactive guanosine diphosphate (GDP)-bound conformation by the exchange of GDP to GTP(Bourne, Sanders et al. 1990, Bourne, Sanders et al. 1991). These switches are conducted via guanine nucleotide exchange factors (GEFs) and GTPase activating proteins(GAPs)(Fig. 6). Intrinsic phosphatase activity hydrolyzes GTP to GDP, deactivating RhoA function, and this process is accelerated by interaction with GTPase-activating proteins. Conversely, interaction with guanine-nucleotide exchange factors facilitates the exchange of GDP to GTP, which restores the activation of RhoA. A large number of GEFs are discovered to enable this activation and involved in various specific signalling pathways. The relative affinity difference of the effector molecules between the GTP- and GDP-bound states of the Rho GTPase can be as much as 100-fold, resulting in a highly-specific interaction only with the GTP-bound activated state. When binding with GTP, they interact with downstream effectors to propagate signal transduction (Spiering and Hodgson 2011). In addition, Rho proteins are also regulated by guanine nucleotide dissociation inhibitors (GDIs), which can inhibit both the exchange of GDP to GTP and the hydrolysis of bound GTP. In most cases, Rho proteins are post-translationally modified at their C-termini by prenylation of a conserved

cysteine, facilitating their attachment to cell membranes, which is prerequisite to its activation and essential for its role in cytoskeleton modulation.

9.3 FUNCTION OF RHOA

Several downstream effectors of RhoA have been discovered, such as ROCKs, diaphanous-related formins (DRFs), citron kinase, PRK1/PKN etc. These effectors conduct versatile function in cell biological function, including cytoskeleton organization, cytokinesis, cell cycle regulation, embryotic development, transcriptional control etc.

RhoA plays a central role in modulating cell shape, polarity and motility by its effects on actin polymerization, actomyosin contractility, cell adhesion, and microtubule dynamics. RhoA activates its downstream effector DRFs to promote actin polymerization by addition of actin monomers to the growing end of actin filaments. Meanwhile activation LIMK by ROCKs and consequent inhibition of cofilin, an actin-binding proteins regulating disassembly of actin filaments, contributes to the increase in actin filaments in response to RhoA. Thus DRFs act together with ROCKs to regulate Rho-induced stress fiber formation. In addition, RhoA activates ROCKs and subsequent phosphorylate several proteins involved in regulating myosins and other actin-binding proteins to induce actomyosin-based contractility, which is crucial in migrating cells. Microtubules are critical for determining cell polarity and for vesicular locomotion and intracellular transport. The coordinating action of ROCKs and DRFs is essential in the organization of microtubules. ROCK phosphorylates TAU and MAP2, proteins that regulate microtubule stability. DRFs directly participate in generating and orienting stable microtubules. RhoA also plays a key role in modulating the adhesion between neighbouring cells and

between cell and extracellular matrix. ROCKs and DRFs act reciprocally to regulate the migration of epithelial cells by reorganizing these adhesions.

Cytokinesis requires actomyosin-based contraction. RhoA's effectors ROCKs, citron kinase and DRFs are all implicated in this process. These effectors were found to localize in the cleavage furrow during cytokinesis. Their concerted actions stimulate local actin polymerization and coordinate microtubules with actin filaments at the contractile ring.

RhoA also plays an important role in G1 cell cycle progression, primarily through regulation of the expressions of cyclin D1 and cyclin-dependent kinase inhibitors p21 and p27. Multiple pathways were found to be involved in RhoA-dependent cyclin D1 expression, by activation of downstream protein kinases, resulting in the subsequent modulation of transcription factor activity. RhoA suppresses p21 expression in multiple normal and cancer cell lines, by other transcriptional regulators independent of p53. RhoA also regulates the levels of p27 in a ROCKs dependent manner.

RhoA also has a role in processes involving cell migration in development, such as neurite growth, dorsal closure, bone formation, and myogenesis. Rho-loss of function is lethal in mouse embryo development, resulting from failure in gastrulation and an inability of cell migration.

Many other transcriptional regulations mediated by RhoA has also been described, such as SRF , NF-kappaB, c/EBPb , Stat3 , Stat5 , FHL-2 , PAX6 , GATA-4 , E2F, Oestrogen Receptor alpha , Oestrogen Receptor beta , CREB , and transcription factors that depend on the JNK and p38 MAP kinase pathways. Substrates to these kinases include c-Jun, ELK, PEA3, ATF2, MEF2A, Max and CHOP/2GADD153.

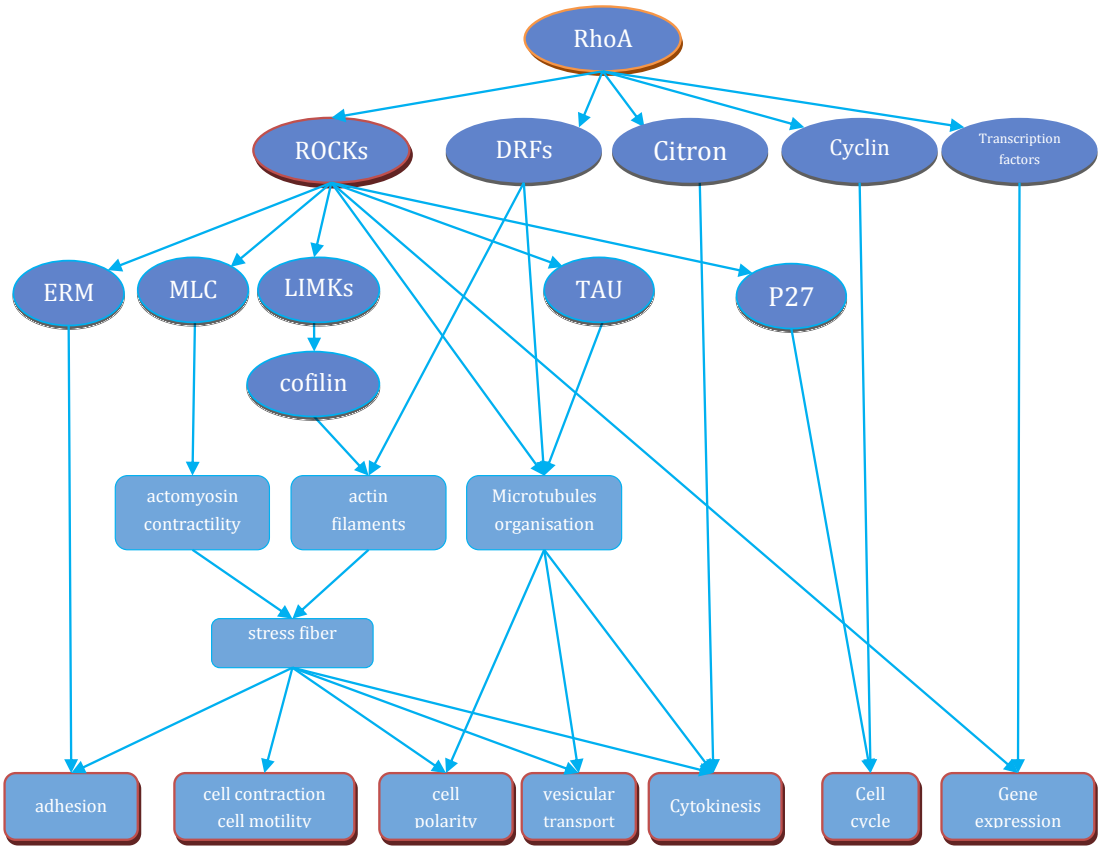

Figure 7 Illustraction of RhoA functioin and the main signaling molecules involved.

9.4 MOLECULAR STRUCTURE AND EXPRESSION OF ROCK

ROCK(also called Rho-associated kinase) is a serine/threonine kinase with a molecular mass of about 160 kDa, which has been shown to be the principle downstream target of RhoA(Ishizaki, Maekawa et al. 1996). It is identified in 1996 as one of the effectors of Rho families (Ishizaki, Maekawa et al. 1996). ROCK displays the greatest homology to other downstream effectors of Rho kinase families such as myotonic dystrophy kinase (DMPK), DMPK-related cell division control protein 42 (Cdc42) binding kinase (MRCK), and citron kinase (CRIK).

There are two ROCK isoforms: ROCK1 (also known as p160ROCK and ROCKβ) and ROCK2 (also known as Rho-kinase and ROCKα). In human, ROCK-1 and ROCK-2 are encoded by two different genes localizing on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively. Two isoforms of Rho-kinase in human have the homologue structure with about 65% of amino acid and 58% of RBD. The highest similarity (92%) is presented at kinase domain. These kinases are formed by parallel homodimers including a kinase domain in its amino-terminus (N-terminal domain), a coiled-coil in its middle dimerization portion, and a putative Pleckstrin-homology (PH) domain in its cysteine-riche domain (C-terminal domain, CRD) (Fig. 8) (Ishizaki, Maekawa et al. 1996, Matsui, Amano et al. 1996). These carboxyl terminal domains constitute an autoinhibitory region that reduces the kinase activity of ROCKs (Amano, Chihara et al. 1999). The Rho-binding domain of ROCKs is localized in the C-terminal portion of the coiled-coil region, and it shows sequence homology to the Rho-interaction domain of kinectin which is a regulating protein of microtubule-based organelle motility (Alberts, Bouquin et al. 1998) (Fig. 9). The coiled-coil region of ROCKs is showed to interact with other α-helical proteins, whereas the PH domain is involved in protein localization (Riento and Ridley 2003).

In human, ROCKs are also widely expressed in various normal and pathological tissues (Fig. 10), including vascular endothelial cells (ECs)(Ming, Barandier et al. 2004, Horowitz, Binion et al. 2007) and many cancer cells (Morgan-Fisher, Wewer et al. 2013). Both ROCK-1 and ROCK-2 are expressed in vascular smooth muscle and in heart. Cell-fractionation studies show that ROCKs are mainly distributed in the cytoplasm fraction but a small amount of ROCKs is also found in the membrane fraction. However, ROCKs are also found in subcellular localization at the vimentin intermediate-filament network and at actin stress fibers.

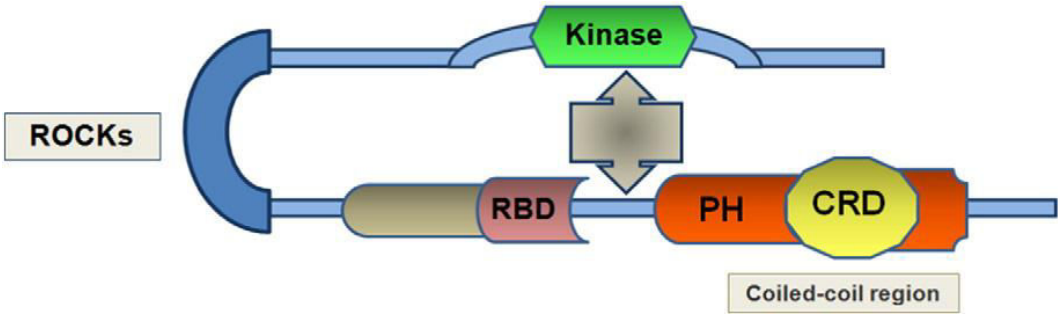

Figure 8 ROCK structure.

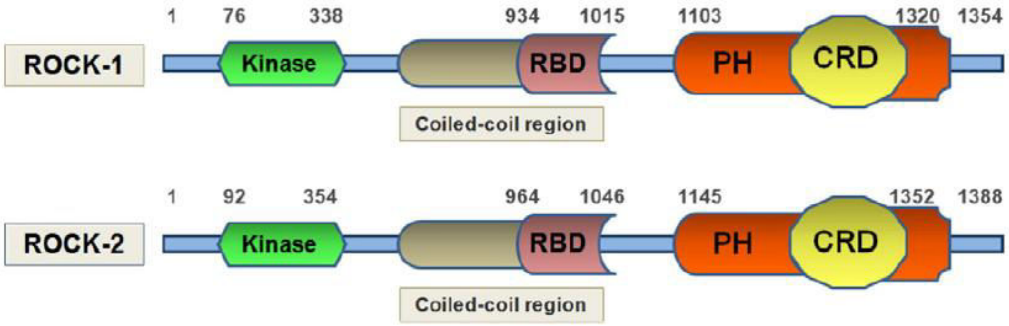

Figure 9 The molecular structure of ROCK1 and ROCK2.

The kinase domain is located at the amino terminus (N-terminus) of the protein, followed by coiled-coil region containing the Rho-binding domain (RBD). The Pleckstrin-homology (PH) with an internal cysteine-rich domain (CRD) is situated in the carboxyl terminus (C-terminus)(Duong-Quy, Bei et al. 2013).

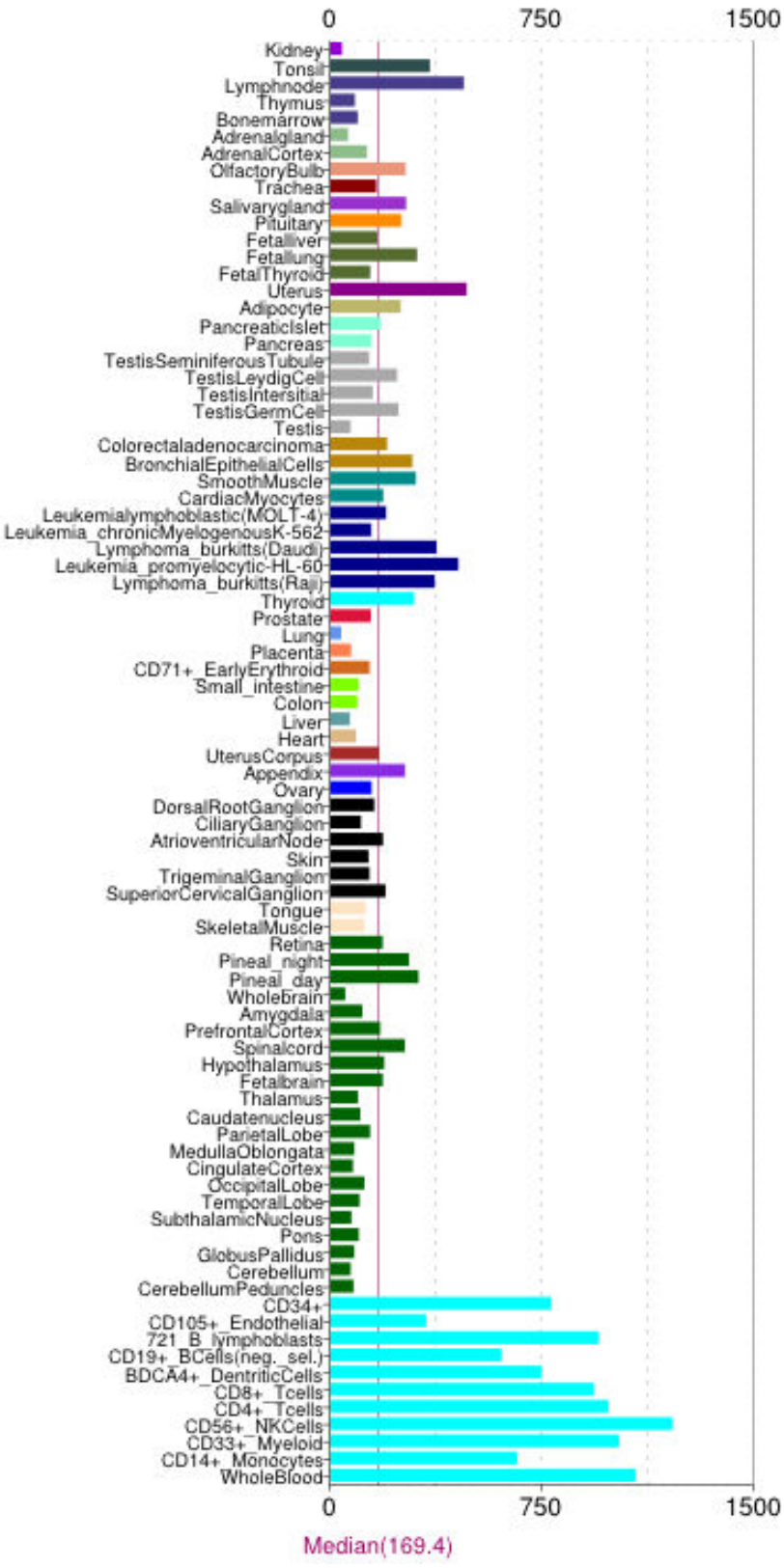

Figure 10 ROCK1 expression in human tissues (BioGPS. 2014)

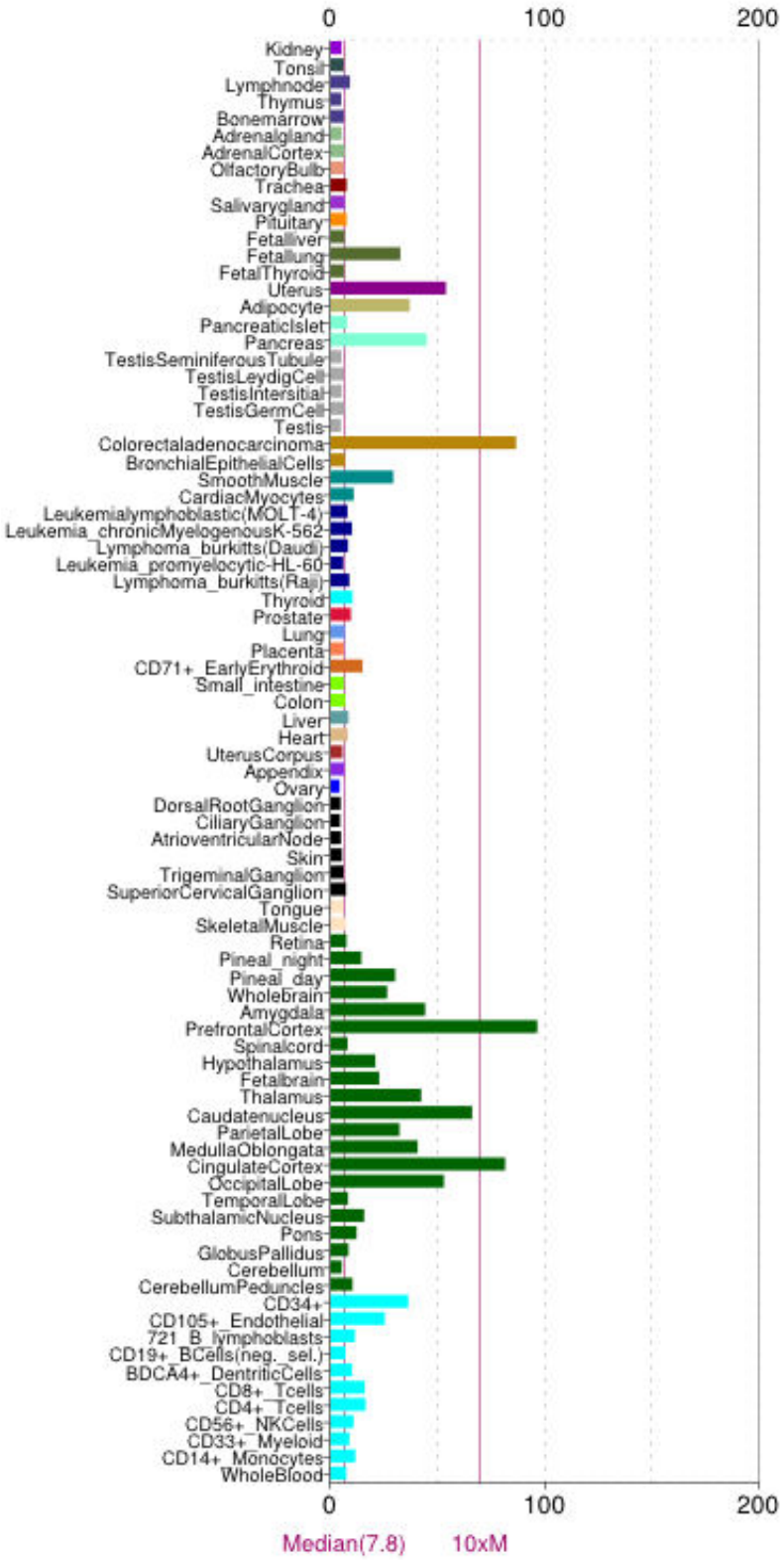

Figure 11 ROCK2 expression in human tissues (BioGPS. 2014)

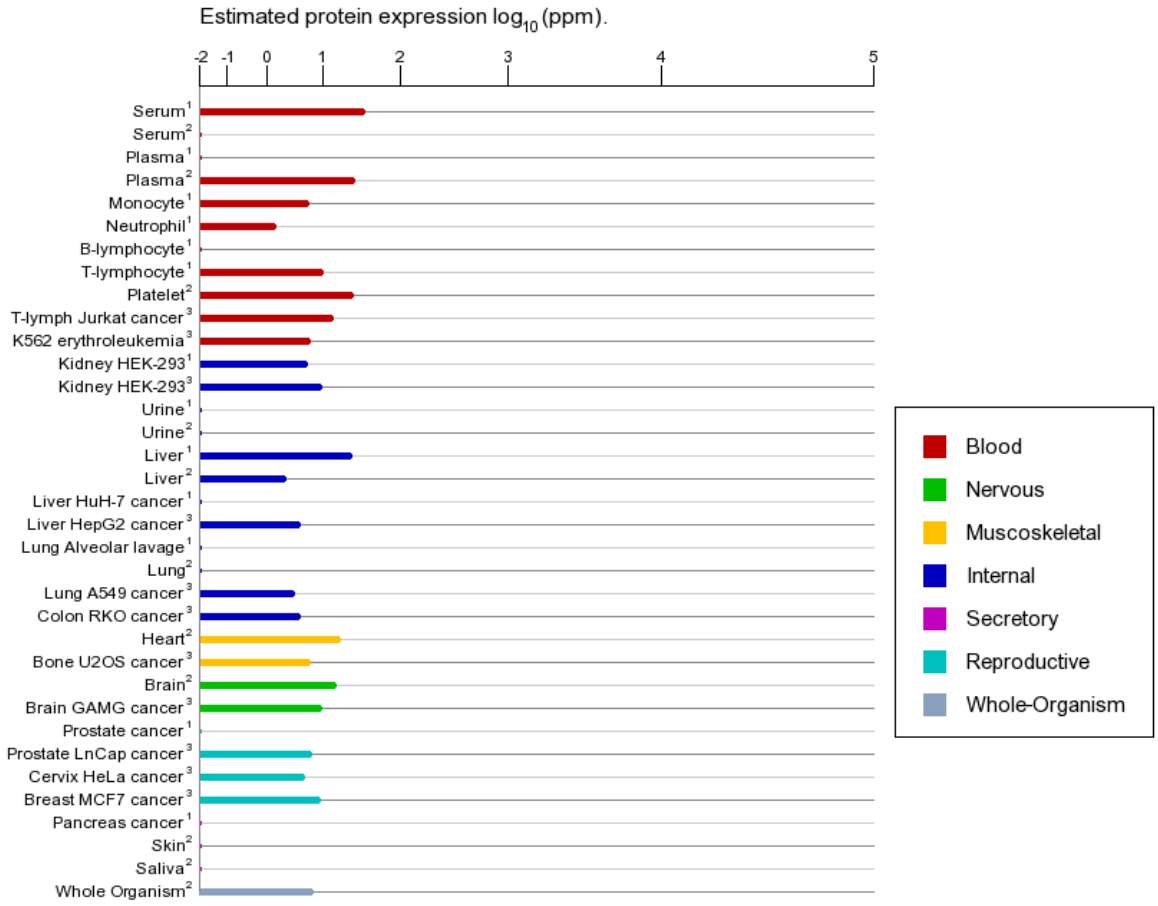

Figure 12 ROCK1 expression in human cancer (GeneCards. 2014)

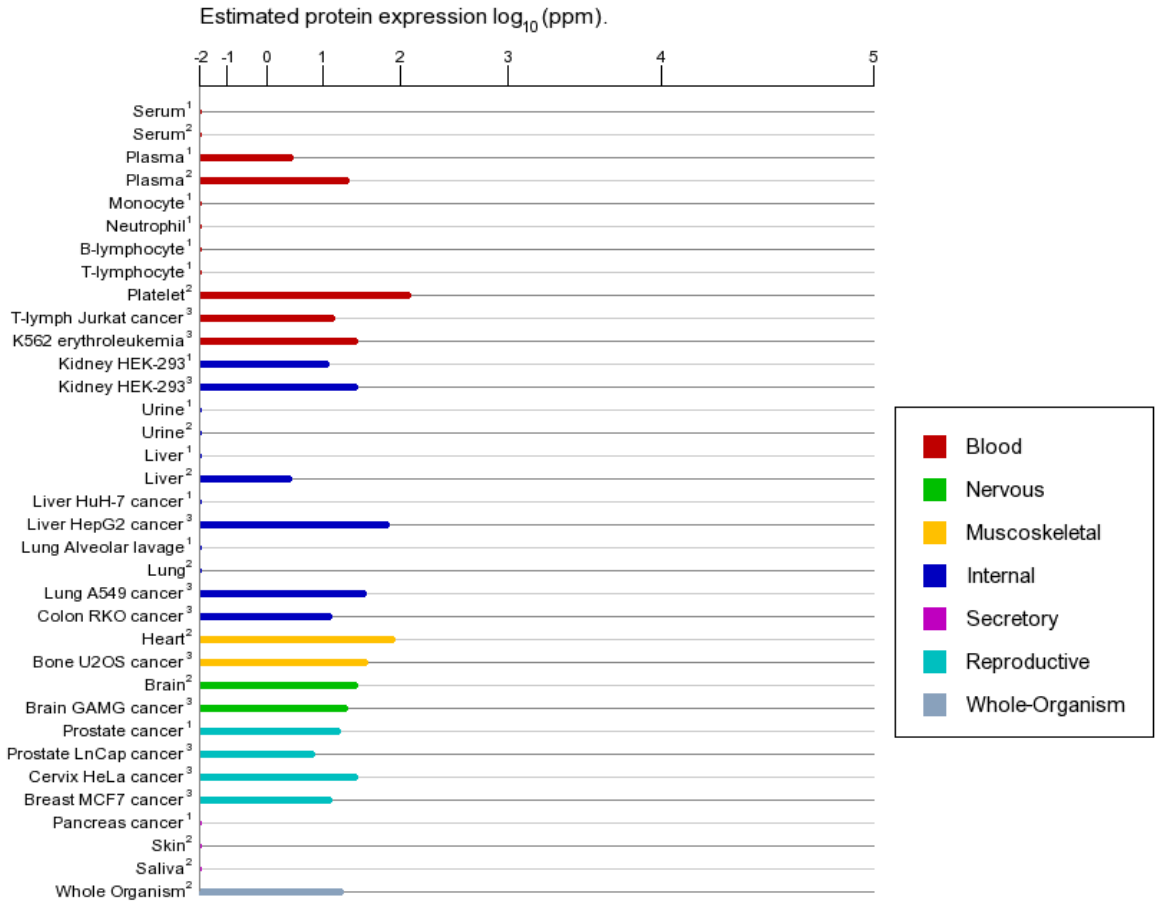

Figure 13 ROCK2 expression in human cancer (GeneCards. 2014)

9.5 REGULATION OF ROCK ACTIVITY

In the structure of ROCKs, the C-terminus (Rho-binding domain and PH domain) plays a role as a dominant-negative autoinhibitor (Fig. 14) due to its independent interaction with the catalytic domain (N-terminus). Lacking of the C-terminus of ROCKs (truncated forms) are constitutively formed the active form kinase. Beside of the self-associative autoinhibition, the activity of ROCKs is also influenced by its affinity for ATP which is regulated by the dimerization of kinases. The relative affinity difference of ROCK between the GTP- and GDP-bound states of the Rho GTPase can be as much as 100-fold, resulting in a highly-specific interaction only with the GTP-bound activated state. Binding of GTPase-RhoA to ROCKs at Rho-binding domain induces conformational

changes of ROCKs, resulting in relieve of autoinhibitory blockage of kinase activity. This binding is believed to stimulate the phosphor−transferase activity of ROCKs (positive regulation) by disrupting the interaction between catalytic and the C-terminal region of proteins, which thereby frees the kinase activity (Fig.14). Independently of RhoA, ROCK activity might be activated by other stimulators such as arachidonic acid (AA), sphingosine phosphorylcholine (SPC), caspase-3 or granzyme B. AA and SPC interact with the negative regulatory region at PH domain, thus disrupting its inhibitory property on the catalytic activity of ROCKs. Caspase-3 cleaves ROCK-1 at the cleavage site DETD1113 whereas granzyme B cleaves the ROCK-2 at the C-terminus at IGLD1131, thus removing an inhibitory region (Fig.14). The activity of ROCKs is also negatively regulated by other small G-binding proteins such as RhoE, Gem, and Rad. RhoE binds to the N-terminal region of kinase domain of ROCK-1 (amino acids 1–420) and therefore interferes with the kinase activity and prevents GTPase-RhoA binding to Rho-binding domain (Fig.14). Overexpression of Gem and Rad might inhibit respectively the downstream responses of ROCKs, but the mechanism is not clearly demonstrated. In addition, the negative regulation of ROCK-mediated target effects by these small G-binding proteins are localized at the different cellular structure, such as RhoE in the Golgi and Gem and Rad in the cytoskeleton.

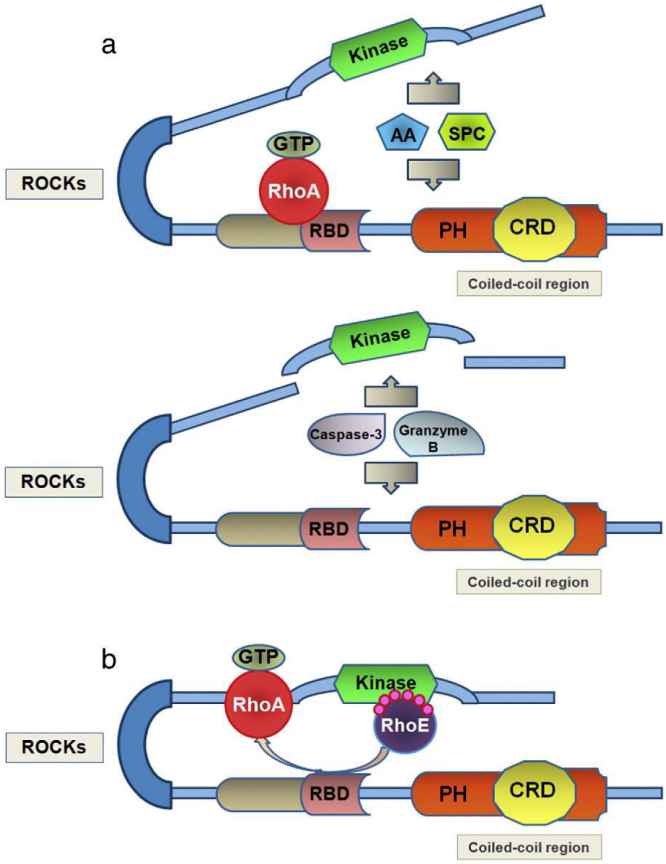

Figure 14 The mechanism of positive regulation of ROCKs activity(Duong-Quy, Bei et al. 2013).

9.6 FUNCTION OF ROCKS

ROCKs phosphorylate a large numbers of targets and mediate a broad range of cellular responses. The most well-studied and important role is a key regulator of actin cytoskeleton, involving in actin filament assembly and contraction via several substrates such as MBS, LIM Kinases, MLCK, ERM proteins.

ROCK is the key molecule required for actin–myosin-based contractile force generation. When bound and activated by RhoA, ROCKs translocate from the cytoplasm to the cell membrane where it increases phosphorylation of the myosin light chain (MLC) of myosin II. This is achieved either by direct phosphorylation, or by phosphorylation of the regulatory myosin-binding subunit of myosin phosphatase (also known as the

phosphatase-targeting subunit), which inhibits the phosphatase activity of this molecule (Somlyo and Somlyo 2003). This in turn, enhances actin binding and the actin-induced adenosine triphosphatase activity of myosin, facilitating the interaction of myosin with F-actin, and ultimately cell contractility and mobility. Moreover, ROCK phosphorylates LIM kinase-1 and 2 (LIMK1/2) (Riento and Ridley 2003). The phosphorylation of LIMK1/2 promotes their activity and subsequently phosphorylates and inhibits cofilin protein's activity to disassemble actin filaments. Thus it facilitates the organization of F-actin into stress fibers and re-arrangement of the actin cytoskeleton (Spiering and Hodgson 2011). The net effect of increased ROCK activity is to elevate force generation, and then facilitate cell adhesion, motility, and invasion. Thus, they play an important role in the regulation of smooth muscle contraction, neurite retraction.

ROCKs phosphorylate ERM to mediate anchoring of actin filaments to integral proteins of the plasma membrane (Matsui, Maeda et al. 1998). ERM proteins contain both a C-terminal actin-binding subunit and an N-terminal 4.1/ezrin/radixin/moesin (FERM) domain, which interacts with plasma membrane proteins. Phosphorylation of ERM leads to the disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization, thus dissociate the cell-cell and cell-matrix adhesion and facilitate cell mobility.

Also, they show scaffolding properties that function to polymerize actin and affect the formation of microtubules, which is required for centrosome positioning and centrosome-dependent exit from mitosis.

Other downstream targets of the Rho kinases include signal transduction molecules and transcription factors such as IRS-1 and PI 3-K/AKT, PTEN c-Ras, SRF, resulting in a variety modulation of gene expression and cell biology.

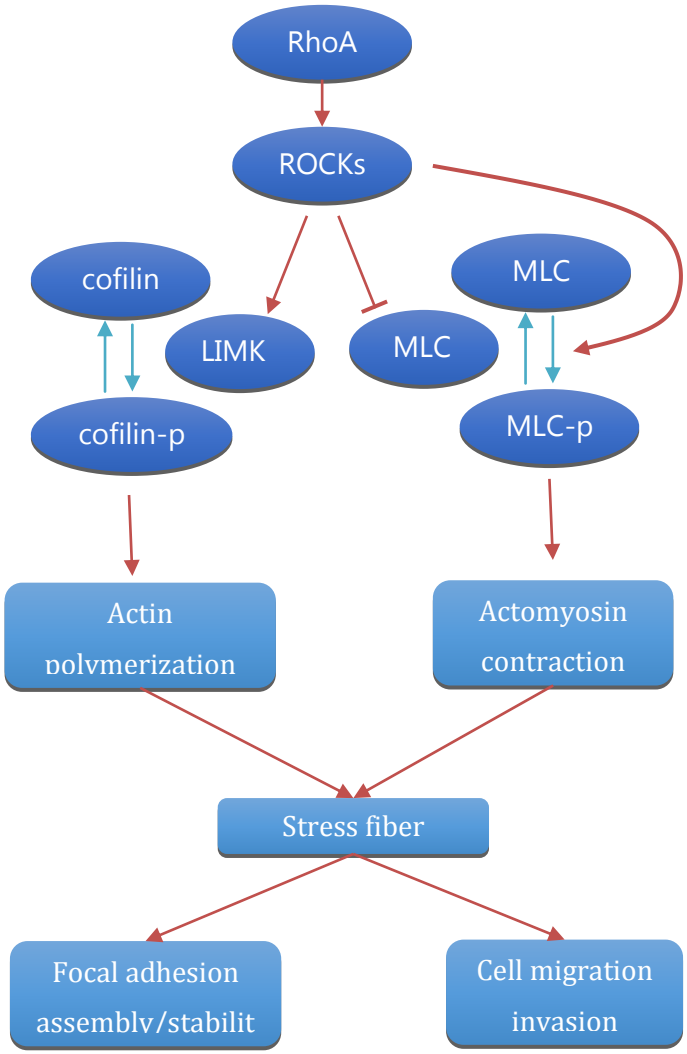

Figure 15 Role of RhoA/ROCK pathway in the actin–myosin-based contractile force generation

9.7 DIFFERENCE BETWEEN ROCK1 AND ROCK2

The differences between ROCK1 and ROCK2 are still poorly understood. Their different tissue distribution and several different roles have been found. ROCK1 and ROCK2 both have ubiquitous tissue distributions. There are still some different patterns of tissue expressions of ROCK1 and ROCK2. A preferential protein expression of ROCK2 can be found in brain and muscle, and that of ROCK1 in blood cells. Subcellularly, ROCK2 is

found primarily in the cytoplasm (Leung, Manser et al. 1995, Matsui, Amano et al. 1996) in association with vimentin (Sin, Chen et al. 1998) and actin stress fibers (Chen, Tan et al. 2002). It also localizes to the plasma membrane (Kimura, Fukata et al. 1998) with an association with its C-terminal region (Kher and Worthylake 2011). It can also be found at the cleavage furrow during late mitosis (Inada, Togashi et al. 1999). In contrast, the intra-cellular distribution of ROCK1 is still not well established. ROCK1 may predominantly associate with the plasma membrane at the apical junctions in endothelial cells (Ishiuchi and Takeichi 2011). Specifically, it indirectly associates with epithelial-cadherin (E-cadherin) complexes through its interaction with the E-cadherin scaffold protein p120-catenin (Smith, Dohn et al. 2012). ROCK1 additionally localizes to the microtubule organizing centre (Chevrier, Piel et al. 2002).

10 RHOA/ROCK PATHWAY IN CANCER ANGIOGENESIS

10.1 ANGIOGENESIS MECHANISM

Angiogenesis is a five-step process involving a complex series of events. Firstly, an increase in the permeability of the basement membrane allows a new capillary to sprout. Next, ECs activated by angiogenic factors migrate through the basement membrane into the extracellular matrix, towards the angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of ECs. This is then followed by re-organization of ECs to form tubules with a central lumen, together with the recruitment of peri-endothelial cells (pericytes) and vascular smooth muscle cells for new capillary stabilization (Sakamoto, Ryan et al. 2008). The RhoA/ROCK pathway plays a role in each of these key steps.

10.2 PERMEABILITY

The endothelium is a semi-permeable barrier that lines the vasculature, comprising ECs that are connected to each other by inter-endothelial junctions, consisting of protein complexes organized as tight junctions and adherent junctions. The latter are in the majority(Mehta and Malik 2006), and are composed of vascular endothelial (VE) cadherin that associates homotypically with VE-cadherin on adjacent cells. VE-cadherin binds to the actin cytoskeleton. Actin-mediated EC contraction occurs as a result of MLC phosphorylation, and this can cause dysfunction of the endothelial barrier by inducing the formation of small gaps between neighbouring cells.(Dudek and Garcia 2001). RhoA, through its downstream effector ROCK, plays a role in endothelial barrier dysfunction by potentiating MLC phosphorylation via inhibition of MLC phosphatase activity. Studies have also confirmed that RhoA contributes to VEGF-induced hyper-permeability in the endothelium(Sun, Breslin et al. 2006).

10.3 MIGRATION

The formation of stress fibers and cellular contraction is essential for EC migration, and these processes are mediated by Rho GTPases(Kiosses, Daniels et al. 1999). Van Nieuw Amerongen et al demonstrated in vitro that VEGF induces the activation of RhoA and this increase in RhoA activity is necessary for VEGF-induced reorganization of the F-actin cytoskeleton. This process can be inhibited by transfection of ECs with a RhoA dominant-negative mutant vector or by a RhoA inhibitor C3 (van Nieuw Amerongen 2002). Zhao et al. showed that increased expression of RhoA in human umbilical vein ECs significantly enhanced cytoskeletal reorganization of transfected cells, cell migration and angiogenic capacity, which suggests that RhoA plays a key part in these processes in vitro(Zhao, Xu et al. 2006).

10.4 PROLIFERATION

Several lines of evidence suggest that Rho proteins play an important role in normal and cancerous cell growth processes, including G1 phase cell cycle progression and mitogenesis(Van Aelst and D'Souza-Schorey 1997). Cytokinesis is a step in mitogenesis which is critical within the cell cycle. In eukaryotic cells, cytokinesis requires an actin and myosin contractile ring, which constricts and cleaves the cell, forming two daughter cells. Inhibition of Rho GTPases prevents the assembly of this contractile ring in a variety of mammalian cells. Expression of constitutively activated Rho GTPases also blocks cytokinesis, suggesting that cycling between the active and inactive forms is required for its function (Glotzer 2001).

The role of RhoA signalling in cell survival has been evaluated in several non-EC cell types. Results showed that inhibition of Rho signalling leads to apoptosis via alterations in cell adhesion and the induction of p53 and other pro-apoptotic proteins, or via ceramide up-regulation leading to caspase cleavage and subsequent activation(Bobak, Moorman et al. 1997, Petrache, Crow et al. 2003). Studies have shown that the ROCK inhibitors, fasudil and Y-27632, not only inhibit VEGF-induced cell proliferation, but also reverse the protective effect of VEGF on apoptosis, which results in a decrease in viability of VEGF-stimulated ECs(Yin, Morishige et al. 2007, Bryan, Dennstedt et al. 2010). Data obtained with these inhibitors have revealed the important role of the RhoA/ROCK pathway in EC proliferation and cell viability.

10.5 MORPHOGENESIS

Cultured ECs can undergo marked changes in shape and tube formation that closely imitate pre-capillary cord formation in vivo(Montesano, Orci et al. 1983). In vitro

angiogenesis assays found that the mean tube length of the capillary-like tubular structures formed by ECs was reduced by transfection of a RhoA dominant-negative mutant vector, the RhoA inhibitor C3, or the ROCK inhibitor Y-27632(van Nieuw Amerongen 2002). In another study, Zhao et al. demonstrated that overexpression of RhoA increased tube length in transfected ECs(Zhao, Xu et al. 2006).

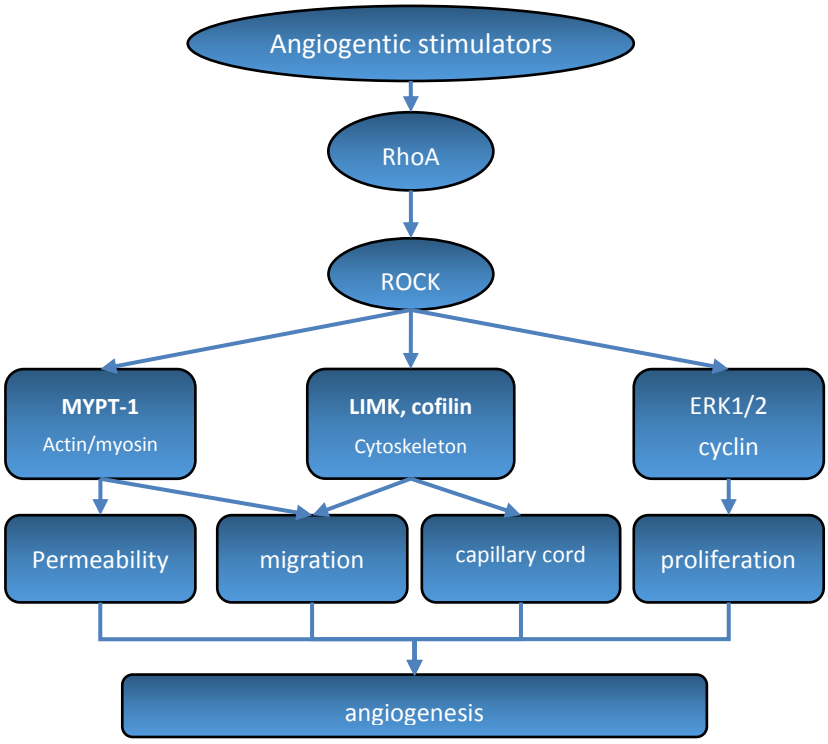

Figure 16 Illustration of the RhoA/ROCK pathway in the angiogenesis.

Pro-angiogenic factors can activate the RhoA/ROCK pathway then subsequently activate the downstream molecules which take part in the multistep of angiogenesis. Firstly, MYPT-1 can be activated by ROCK and cause MLC phosphorylation then actin-mediated EC contraction, which leads to an increase in the permeability of the basement membrane allowing a new capillary to sprout. Other molecules involved in cytoskeleton organization such as LIMK, cofilin are activated and cause ECs to migrate into the extracellular matrix towards the angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of ECs in which ERK1/2, cyclin may play a role when activated by ROCK. This is followed by re-organization of ECs to form tubules with a central lumen and finally reorganize to new capillary stabilization.

11 RHOA/ROCK PATHWAY IN PROSTATE CANCER ANGIOGENESIS

As discussed previously, the RhoA/ROCK pathway participates in the process of angiogenesis in many cancers, including PCa. Tumour blood vessels always exhibit abnormal structure and function. A study investigating the ECs of mice carrying the transgenic adenocarcinoma of the mouse prostate (TRAMP) transgene revealed the aberrant extracellular matrix cues and abnormal responses in these cells correlated with a constitutively high level of baseline activity of Rho GTPase and its downstream effector, ROCK(Ghosh, Thodeti et al. 2008). These findings highlighted the important role of the RhoA/ROCK pathway in the angiogenesis of PCa. Many other studies have also demonstrated that RhoA/ROCK pathway inhibitors decrease angiogenesis and cell growth in PCa (Somlyo, Bradshaw et al. 2000, Somlyo, Phelps et al. 2003, Wu, He et al. 2011).

In an earlier in vitro study, Y-27632 inhibited metastatic growth of highly invasive PC3 cells in immune-compromised mice(Somlyo, Bradshaw et al. 2000). Another ROCK inhibitor, Wf-536, greatly enhanced the in vitro inhibition of EC migration, vacuolation, lumen and cord formation, and VEGF- and hepatocyte growth factor-stimulated endothelial sprout formation, when combined with the matrix metalloproteinase inhibitor, marimastat(Somlyo, Phelps et al. 2003). Early treatment with a combination of Wf-536 plus marimastat, with or without paclitaxel, of immune-compromised mice bearing xenotransplants of PC3 cells was associated with significant inhibition of tumour growth and increased tumour necrosis(Somlyo, Phelps et al. 2003). Some potential anti-angiogenic medications, such as anacardic acid, have been found to inhibit human prostate tumour xenograft angiogenesis by targeting the Rho GTPase signalling pathway(Wu, He et al. 2011).

12 RHOA/ROCK PATHWAY IN CANCER INVASION

Transformation of benign tumours to malignant cancers results from several events in hyperplasic cells including the reduce of cell adhesion, increased cell motility and invasion as well as the growth of metastatic colonies at locations other than the cells origin. RhoA/ROCK pathway causes actomyosin contraction, transformation, and transcription of the SRF mediated genes. Also, it shows scaffolding properties that function to polymerize actin and affect the formation of microtubules. These effectors suggest its involvement in cell motility of both mesenchymal and collective migration. Dysregulation of this pathway has been shown to be implicated in increasing cell migration during tumour cell invasion and metastasis. The effects of RhoA/ROCK on these functions would seem to be critical for the formation and progress of tumours.

RhoA activation by somatic mutations have been found in several different cancers, including breast, lung, ovary, and intestine (Forbes, Bhamra et al. 2008, Forbes, Tang et al. 2010). RhoA can also be activated by the disorders of its regulator. For example, p27 is often inactivated in cancer and loses its inhibition on RhoA activity, thus promotes RhoA induced invadopodia and tumour progress in breast cancer(Wu, Wang et al. 2006). Elevated RhoA expressions in cancer tissue have also been found in many studies. Overexpression of RhoA conferred rat hepatoma MM1 cells increased invasive ability both in vitro and in vivo (Yoshioka, Nakamori et al. 1999).

ROCK expression and activity have also been found to be elevated in many cancers and correlated with tumour aggressiveness and prognosis. Somatic mutations in ROCK genes have been identified in several cancer cell lines and human tumour tissues, some of which encode the constitutively active form (Greenman, Stephens et al. 2007) (Forbes, Bhamra et al. 2008). Elevated ROCK protein levels have also been reported in several human cancers. Both ROCKs has been reported to be elevated in several cancer cell lines

such as invasive sarcoma cell lines (Rosel, Brabek et al. 2008), as well as in human cancer samples including breast cancer (Lane, Martin et al. 2008), testicular cancer, bladder cancer and oesophageal cancer (Kamai, Arai et al. 2002, Kamai, Tsujii et al. 2003, Zhou, Zhao et al. 2003). High ROCK I expression was correlated with poor overall survival in breast cancer (Lane, Martin et al. 2008), osteosarcoma (Liu, Choy et al. 2011). Elevated ROCK II protein expression levels have also been reported to be associated with shorter disease-free survival in patients with bladder cancer in colon and bladder cancers (Kamai, Tsujii et al. 2003, Vishnubhotla, Sun et al. 2007). Expression of constant activated ROCKs results in increased cell invasion in three dimensional collagen matrices(Izawa, Amano et al. 1998, Pawlak and Helfman 2002) and metastasis in an in vitro experimental model(Rosel, Brabek et al. 2008). Ectopic ROCK expression in mouse epidermis has also been reported to induce hyperplasia and cancer formation (Samuel, Lopez et al. 2011).

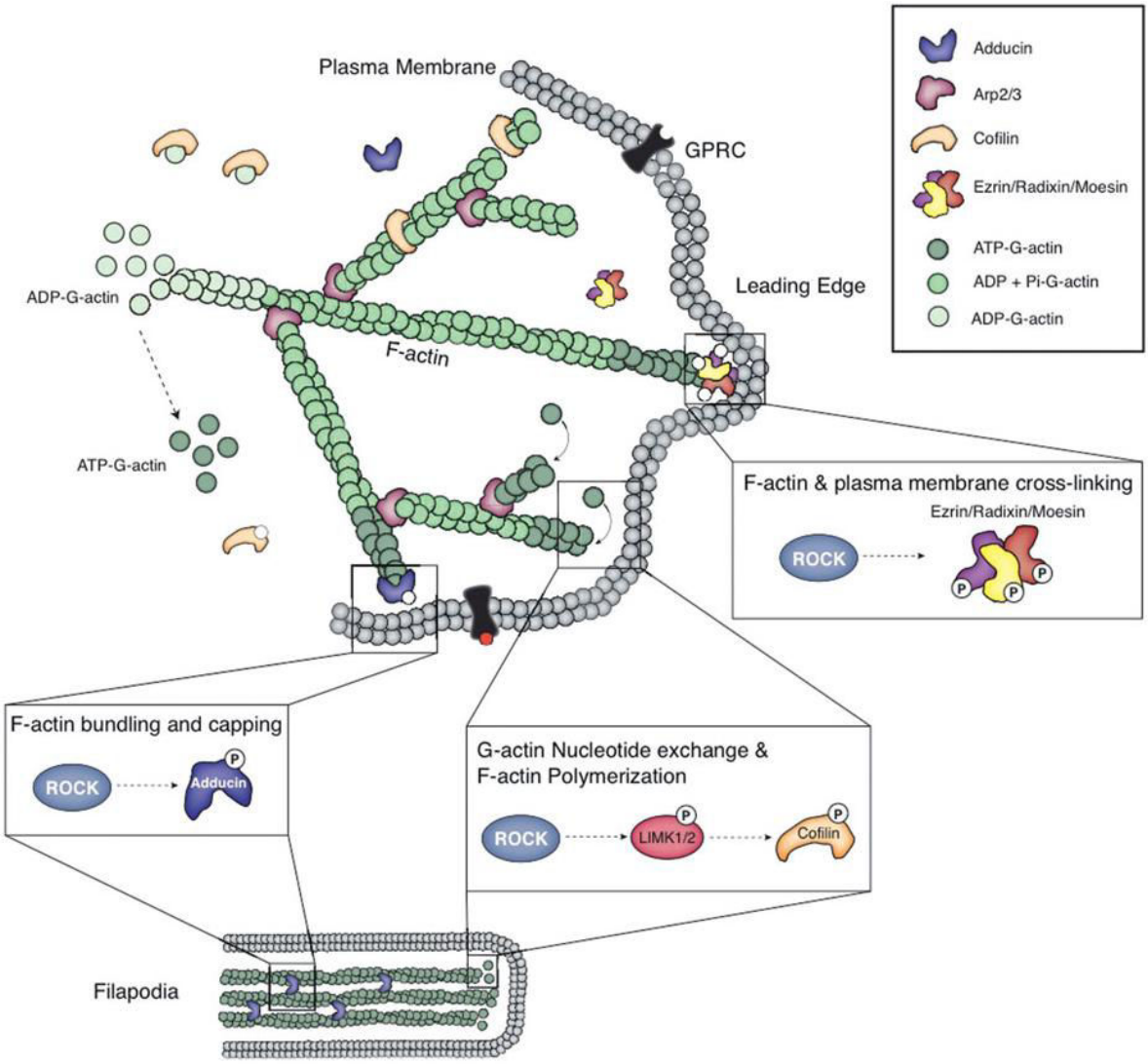

Figure 17 ROCK signaling and the leading edge(Schofield and Bernard 2013).

13 RHOA/ROCK PATHWAY IN PROSTATE CANCER INVASION

The important roles of RhoA in prostate cancer invasion have also been confirmed. Hodge, JC et al reported higher RhoA expression in high invasive variant of PC-3 prostate cancer cells than in low invasive variants and its action through the motility component of the invasion process(Hodge, Bub et al. 2003). Neuropeptide-stimulated migration in prostate cancer cells was shown to be mediated by RhoA (Zheng, Iwase et al. 2006). RhoA was also reported to induce migration towards monocyte chemo-attractant

protein 1 (MCP-1; CCL2) in PC-3 cells (Loberg, Tantivejkul et al. 2007). Several inhibitors of prostate cancer cell migration and inhibition, such as TMEFF2(Chen, Corbin et al. 2014), miR-34a(Yamamura, Saini et al. 2012), Epac (Grandoch, Rose et al. 2009), FTY720(Zhou, Ling et al. 2006), WIN55212 (Nithipatikom, Gomez-Granados et al. 2012) were discovered to act via RhoA in prostate cancer cell lines. In prostate cancer patients, RhoA was found to be overexpressed in cancer tissues where it correlated with increased PCa aggressiveness and poor outcome after surgery(Schmidt, Duncan et al. 2012).

ROCKs have also been reported to involved in prostate cancer invasion in several studies. Hu et al found a significantly elevated ROCK1 expression in prostate cancer tissue(Bu, Tang et al. 2011). The Rho-kinase inhibitor, Y-27632, has also been shown to inhibite prostate cancer cell line PC-3 migration in in vitro chemotactic migration, improve median survival significantly in a prostate cancer mice model(Somlyo, Bradshaw et al. 2000). Another Rho-kinase inhibitor Wf-536 has also been reported to significantly inhibite tumor growth and increased survival in prostate cancer mice model when combined with matrix metalloproteinase inhibitor Marimastat(Somlyo, Phelps et al. 2003).

AIMS OF THE WORK

RhoA/ROCK pathway is a key regulator of the cytoskeleton and has been implicated in angiogenesis, invasion process and aggressiveness of prostate cancer. There are only sparse data on the RhoA expression pattern and activity in localized prostate cancer. The effects of inhibition of RhoA/ROCK on the PCa-induced angiogenesis are still not well evaluated. Thus the aims of the two parts of this study are as following:

Study I: To investigate the anti-angiogenic effects of fasudil, a ROCK inhibitor, on PCa-induced angiogenesis in vitro, including proliferation, migration, tube formation and spheroid sprouting of PCa-conditioned HUVECs.

Study II: To evaluate RhoA/ROCK expression pattern and RhoA activity in prostate specimen from radical prostatectomy for clinical localized prostate cancer by Immunohistochemical staining, Western Blotting, G-LISA assay.

RESULTS

PART I ARTICLE I

FASUDIL INHIBITS PROSTATE CANCER-INDUCED ANGIOGENESIS IN VITRO

Fasudil inhibits prostate cancer-induced angiogenesis *in vitro*

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Abstract. Inhibition of angiogenesis is an important therapeutic strategy for advanced stage prostate cancer (PCa). RhoA/Rho-associated protein kinases (ROCK) are key regulators of the cytoskeleton and have been implicated in PCa angiogenesis. We investigated the anti-angiogenic effects of fasudil, a ROCK inhibitor, on PCa-induced angiogenesis *in vitro*. Proliferation of PCa-conditioned human umbilical vein endothelial cells (HUVECs) was assessed using a bromodeoxyuridine (BrdU) assay, and migration was assessed with a wound healing assay. *In vitro* angiogenesis of PCa-conditioned HUVECs was evaluated by tube formation and a spheroid sprouting assay. Fasudil inhibited PCa-induced endothelial cell proliferation at a concentration of 100 μM, and also decreased PCa-induced endothelial cell migration at a concentration of 30 μM. In the *in vitro* angiogenesis assay, fasudil exerted a more significant effect. Tube formation was significantly inhibited at fasudil concentrations exceeding 3 μM, and spheroid sprouts were significantly thinner and shorter (at fasudil concentrations of 10 and 30 μM, respectively). Western blotting results showed that expression of phosphorylated myosin phosphatase target subunit 1 (MYPT-1) was significantly lower after fasudil treatment, confirming that fasudil inhibited ROCK activity in these model systems. These data suggest that fasudil may be a useful anti-angiogenic agent for PCa.

Introduction

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer-related death in men in Western countries. Advanced and metastatic stages of the disease are found in 35% of patients with PCa diagnosed at autopsy (1). Among patients with localized cancer who are eligible for radical prostatectomy, ~35% will develop recurrence (metastatic disease) within 10 years of surgery (2,3).

Androgen deprivation therapy (ADT) can be effective in patients who present with or progress to advanced or metastatic disease. Unfortunately, the median duration of response to ADT is limited to between 8 months and 3 years (4), and these patients will eventually become castration resistant. Chemotherapy is an effective treatment for castration-resistant PCa, but the median duration of response is only 10.3 months (5). There is clearly an urgent need to develop additional systemic interventions for patients with progressive PCa. Angiogenesis plays a crucial role in PCa progression and metastasis. Microvessel density (MVD) has been found to be more prominent in PCa than in benign prostatic hyperplasia (BPH) and normal tissue (6,7). It has been reported that MVD increases with increased Gleason's score, particularly in poorly differentiated PCa (8). MVD was also significantly correlated with cancer-specific survival in 221 patients with PCa followed up for a median of 15 years (9).

Vascular endothelial growth factor (VEGF) is the most prominent regulator of physiological angiogenesis and has been correlated with increased levels of angiogenesis in clinical studies comparing PCa with BPH (7). Higher VEGF expression and serum levels have also been found in patients with metastasis or poorly differentiated tumors, as well as in those with a poor prognosis (10-13). However, it has become increasingly apparent that current anti-angiogenic therapy targeting VEGF has only a modest effect in the clinical setting.

RhoA and its downstream effector, Rho-associated protein kinase (ROCK), serve as key regulators of extracellular stimulus-mediated signaling networks that are involved in various cellular processes, including motility, mitosis, proliferation and apoptosis (14). Suppression of the RhoA/ROCK signaling pathway with the ROCK inhibitor, Y-27632, was found to inhibit VEGF-induced angiogenesis *in vitro* (15). Another ROCK inhibitor, fasudil, has been shown to inhibit VEGF-induced angiogenesis *in vitro* and *in vivo* (16). A study carried out on endothelial cells from transgenic adenocarcinoma of the mouse prostate (TRAMP) mice revealed that their behavior correlated with a constitutively high level of baseline activity of Rho GTPase and ROCK (17). This suggests that the

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Key words: fasudil, ROCK, angiogenesis, prostate cancer

RhoA/ROCK pathway has an important role in PCa angiogenesis. However, the anti-angiogenic effects of ROCK inhibitors in PCa are unclear. We investigated the role of fadusil, a ROCK inhibitor, that has been approved for clinical use for pulmonary arterial hypertension, on PCa-induced angiogenesis *in vitro*.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (C-12200; Heidelberg, Germany) and cultured in endothelial cell growth medium (C-22010; PromoCell). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Subcultures were obtained by trypsinization and were used for experiments at passages 3 to 9. Before performing the experiments, the cells were made quiescent by incubating overnight in endothelial cell basal medium (C-22210) containing 0.5% (w/v) fetal bovine serum (FBS). The PCa cell line, PC-3, was purchased from The European Collection of Cell Cultures and grown in F-12K (Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% (w/v) FBS. PC-3 cells were seeded at a concentration of $6x10^6$ cells/T75 flask. On the following day, the medium was replaced with basal medium without FBS, and the supernatants were harvested after a 24-h incubation to serve as conditioned medium (PC3CM). Recombinant human VEGF 165 was purchased from R&D Systems (293-VE; Minneapolis, MN, USA). HUVECs were cultured in endothelial cell basal medium plus 2% (w/v) FBS (control group), in PC3CM plus 2% (w/v) FBS (PC3CM group), or in basal medium plus 2% (w/v) FBS and 30 ng/ml VEGF (VEGF group).

Cell proliferation assay. HUVEC proliferation was evaluated using a BrdU incorporation assay kit (Amersham; Cell Proliferation Biotrak ELISA System; RPN250; GE Healthcare, Little Chalfont, UK), according to the manufacturers' instructions. In brief, HUVECs were plated in 96-well microculture plates ($3x10^3$ cells/well). After a 48-h incubation at 37˚C in a 5% CO_2 atmosphere, with or without fasudil (1-100 μM), 10 μl BrdU labeling reagent was added, and the cells were cultured for a further 2 h. Cells were washed twice with Dulbecco's PBS (D8537; Sigma-Aldrich, St. Louis, MO, USA), fixed with fixative solution and then blocked with blocking buffer. BrdU incorporation was revealed by incubation with 100 μl/well horseradish peroxidase (HRP)-labeled anti-BrdU working solution for ~ 90 min. Tetramethylbenzidine (TMB) substrate at room temperature was added at 100 μl/well for 20 min. Absorbance was measured at 450 nm using a microplate reader. All determinations were performed in octuplicate, and each experiment was repeated three times.

Cell migration assay. Cell motility was assessed using a wound-healing migration assay. HUVECs were seeded to full confluency in 6-well plates. The following day, a uniform scratch was made down the centre of the well using a 100-μl micropipette tip, and the cells were washed twice with PBS. After incubation for 24 h with or without 30 μM fasudil in the control, PC3CM and VEGF groups, the cells were fixed and photographed. Photographic imaging was performed using a Leica inverted microscope. Cell migration was quantified by measuring the ratio of the migration area to the total area of the wound gap. Each experiment was repeated three times.

Tube formation assay. Ninety-six-well plates were chilled to 4˚C and coated with 50 μl of Matrigel (354234; BD Biosciences, Oxford, UK) per well. Freshly passaged HUVECs were seeded onto the gel. Endothelial tube morphogenesis was carried out in the presence or absence of fasudil (3-30 μM). Endothelial tube formation was observed after 16 h and photographed under phase contrast microscopy using a Leica inverted microscope. Quantification of the digital images was performed by counting the total number of tubes in five 40x fields, and total tube length was quantified using ImageJ™ software (NIH, Bethesda, MD, USA). Tube formation was expressed as fold change or percentage, compared to the controls. All determinations were performed three times, and each experiment was repeated three times.

Spheroid sprouting assay. HUVECs were suspended in culture medium containing 0.2% (w/v) methylcellulose (Sigma-Aldrich) and seeded in non-adherent round-bottom 96-well plates (Greiner, Frickenhausen, Germany). All suspended cells formed a single spheroid in each well of defined size and cell number (~400 cells/spheroid). Spheroids were left to form for 24 h and then embedded in 1.5 mg/ml collagen gel. The spheroid-containing gel was rapidly transferred to pre-warmed 24-well plates and allowed to polymerize for 30 min. Endothelial basal medium or PC3CM with or without fasudil (1-100 μM) was then added to the surface of the gel (500 μl/well). After 16 h, images were captured using a Leica inverted microscope. Sprouting was quantified using NIH ImageJ software by measuring the cumulative sprout length, which consisted of every sprout from 10 spheroids in each group.

Western blot assay. Protein was extracted on ice from the cultured HUVECs with cold RIPA lysis buffer (9806; Cell Signaling Technology, Boston, MA, USA) containing Pierce™ Protease and Phosphatase Inhibitor (88669; Thermo Scientific, Rockford, IL, USA). Lysates were centrifuged at 12,000 x g for 20 min at 4˚C, and the supernatant was collected. Total protein concentrations were determined using a bicinchoninic acid assay (BCA) protein assay kit (23250; Thermo Scientific). Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrically transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with 5% (w/v) non-fat milk in PBS-0.1% (v/v) Tween-20 (PBST) and incubated with primary antibodies against MYPT-1 (1:1,000; sc-25618; Santa Cruz Biotechnology, Dallas, TX, USA), phospho-MYPT-1 (1:500; ABS45; Millipore, Billerica, MA, USA), anti-ROCK1 (1:500; sc-6055), anti-ROCK2 (1:1,000; sc-1851; both from Santa Cruz Biotechnology) and β-actin (1:500; ab8229; Abcam, Cambridge, UK) overnight at 4˚C. Finally, the membrane was incubated with HRP-conjugated secondary antibodies as follows: goat anti-mouse IgG-HRP (1:5,000; sc-2005; Santa Cruz Biotechnology), rabbit anti-goat IgG-HRP (1:10,000; sc-2768; Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (1:10,000; sc-2004; Santa Cruz Biotechnology) for 1 h at room temperature. After washing three times with PBST, proteins

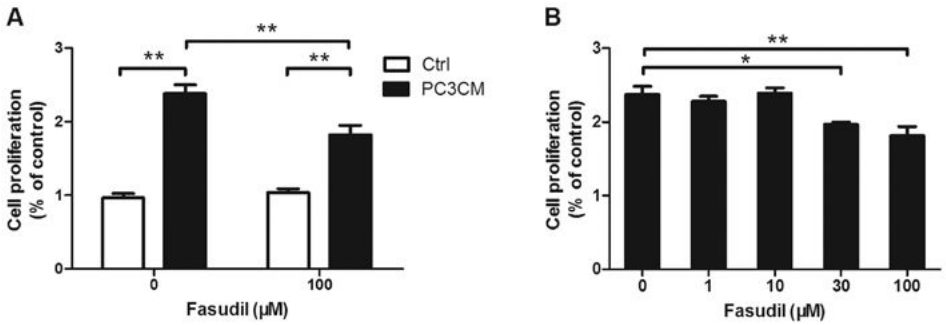

Figure 1. Effects of fasudil on PCa-induced HUVEC proliferation. HUVECs were cultured in endothelial culture medium or PC3CM for 48 h, with or without various concentrations of fasudil. A BrdU assay was used to assess the effects of fasudil on cell proliferation. (A) Cell proliferation was significantly decreased in the PC3CM group treated with 100 μM fasudil (P<0.01). (B) The inhibitory effect of fasudil on PC3CM-induced HUVEC proliferation was dose-dependent. The results shown are the mean ± SEM of three independent experiments. *P<0.05, **P<0.01. HUVECs, human umbilical vein endothelial cells; PCa, prostate cancer; PC3CM, PCa cell line PC3-conditioned medium.

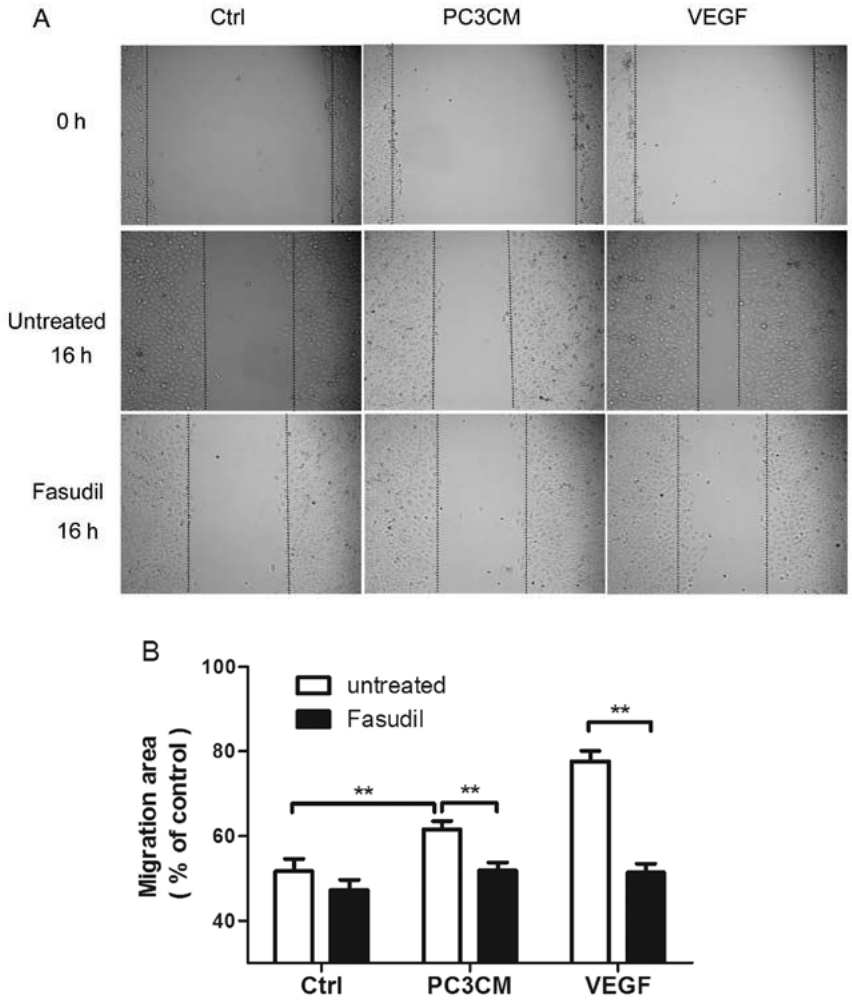

Figure 2. Fasudil inhibits PC3CM-induced HUVEC migration. (A) Fasudil (30 μM) significantly retarded PC3CM-cultured HUVEC migration into the scratched gap. (B) Quantification of wound healing. HUVEC migration was quantified by counting the percentage of the migration area 16 h after fasudil treatment. Values are the mean ± SEM from 4 cultures each in triplicate experiments. **P<0.01. HUVECs, human umbilical vein endothelial cells; PCa, prostate cancer; PC3CM, PCa cell line PC3-conditioned media, SEM, standard error of the mean.

were visualized using an ECL Prime Western blotting detection kit (GE Healthcare). Photographs of the protein bands were captured using a digital imaging system (ImageQuant LAS; GE Healthcare), and densitometric measurements of band intensity in the western blotting were performed using NIH ImageJ software. The results shown are representative of three or more independent experiments.

Statistical analysis. Data are expressed as means ± standard deviation. Significance of differences was determined by the

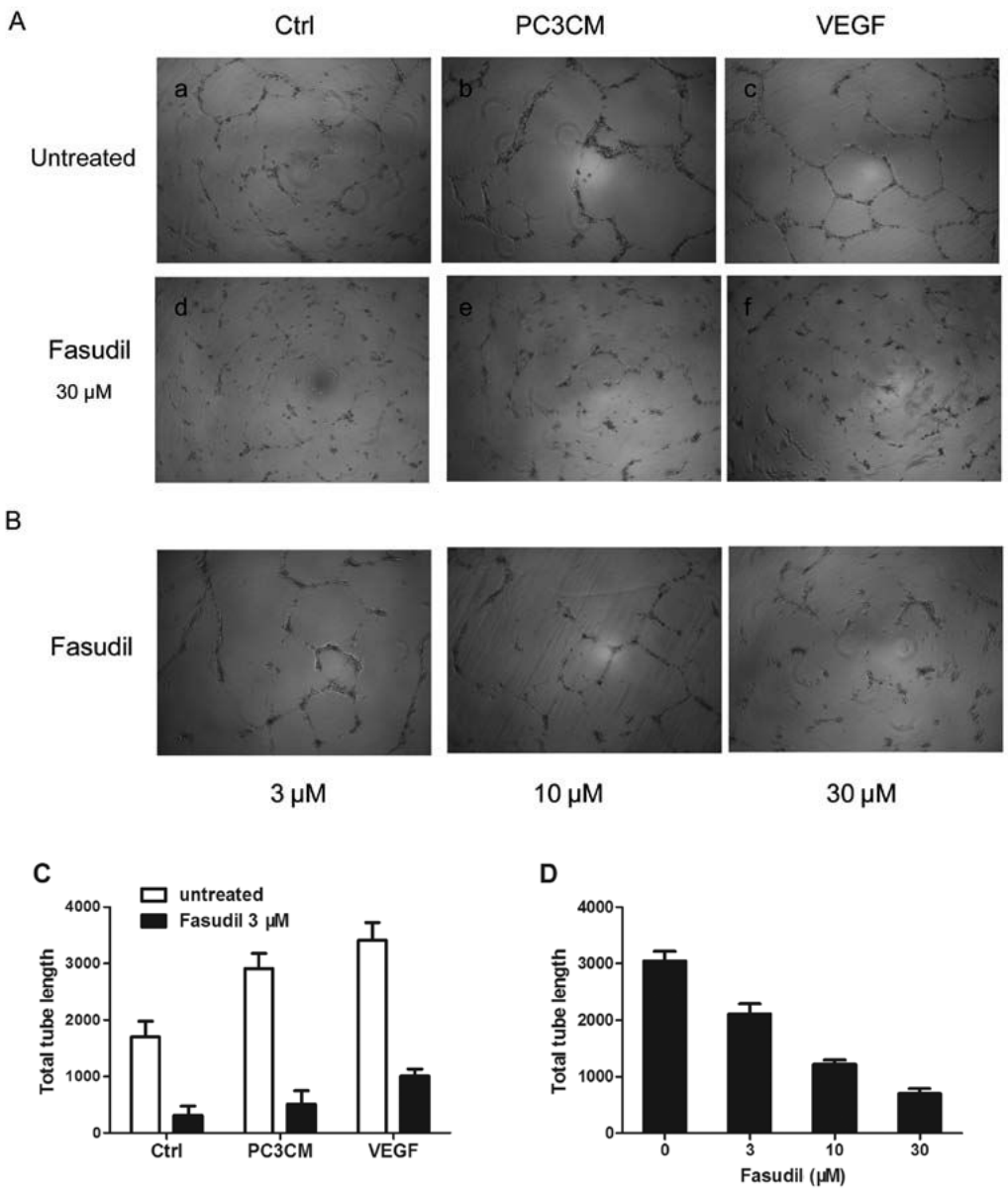

Figure 3. PC3CM-induced capillary-like tube formation is reduced by fasudil. (A) HUVECs were cultured on Matrigel with basal medium (a), PC3CM (b), or basal medium containing 30 ng/ml VEGF (c) either in the absence (a-c), or presence of 30 μM fasudil (d-f). Images were captured after 16 h under a Leica inverted phase-contrast microscope. Representative images are shown above. PC3CM (b) increased the total tube length compared to the control (a), whereas fasudil strongly inhibited tube formation (e). (B) Dose-dependent effects of fasudil on total tube length in PC3CM-cultured HUVECs. As the fasudil concentration increased, the total tube length gradually decreased. (C and D) Total tube length was quantified by evaluating five fields in each experiment, and data were verified by three independent experiments. Values are expressed as the mean ± SEM. HUVECs, human umbilical vein endothelial cells; PCa, prostate cancer; PC3CM, PCa cell line PC3-conditioned media; SEM, standard error of the mean; VEGF, vascular endothelial growth factor.

two-tailed Student's t-test or the analysis of variance least significant difference (ANOVA LSD) test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Fasudil inhibits PC3CM-induced HUVEC proliferation. Endothelial cell proliferation is crucial for angiogenesis. PC3CM-treated HUVECs were exposed to fasudil concentrations ranging from 1 to 100 μM, and HUVEC proliferation was examined using a BrdU assay. Fasudil concentrations of ≥30 μM had a significant inhibitory effect on PC3CM-induced cell proliferation, while proliferation in the control group was unchanged (Fig. 1).

Fasudil inhibits PC3CM-induced HUVEC migration. The inhibitory effects of fasudil on endothelial cell motility were assessed using a wound-healing migration assay. Fasudil (30 μM) significantly decreased the number of cells migrating into the scratched gap in the control, PC3CM and VEGF groups, indicating the potent inhibitory effect of fasudil on HUVEC movement and migration. VEGF increased HUVEC migration significantly more than PC3CM-induced HUVEC migration. After treatment with 30 μM fasudil, all migrations decreased to similar levels (Fig. 2).

Fasudil inhibits PC3CM-induced HUVEC tube formation. The effect of fasudil on capillary-like structure formation *in vitro* was examined using a 3-dimensional (3D) Matrigel

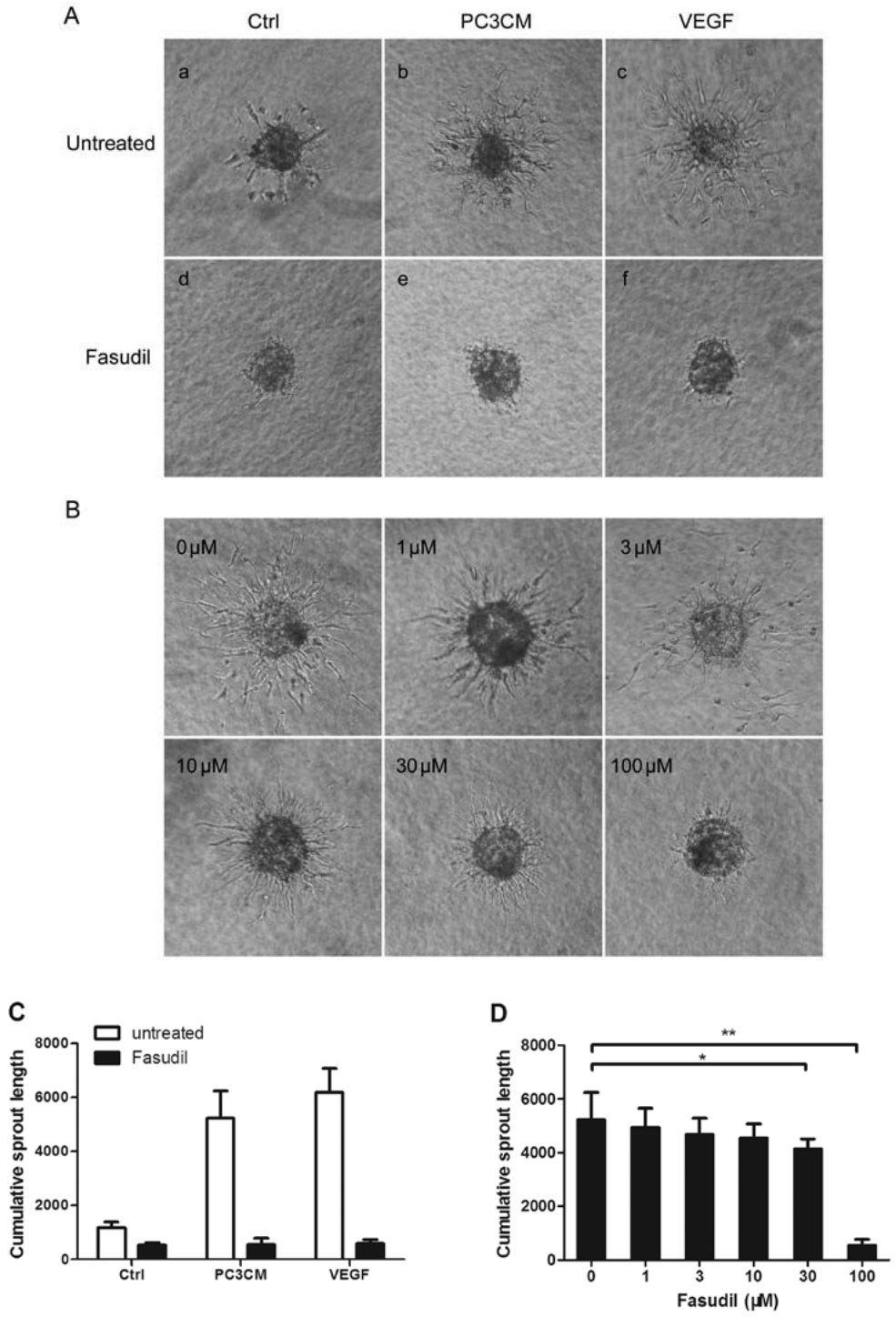

Figure 4. PC3CM-induced HUVEC spheroid sprouting is decreased by fasudil. (A) The sprouting of capillary-like structures from collagen-embedded HUVEC spheroids treated with fasudil (100 μM) or left untreated was determined in basal medium (Ctrl), PC3CM, or basal medium containing 30 ng/ml VEGF. After 16 h, sprouting was digitally recorded using a phase-contrast microscope. Sprouting from HUVEC spheroids stimulated with PC3CM (b) was almost completely inhibited by fasudil (e). (B) Fasudil decreased HUVEC sprouting in a dose-dependent manner, and 100 μM fasudil inhibited sprouting almost completely. With increasing concentration of fasudil from 10-30 μM, the sprouts became thinner and more abundant compared with the ordered architecture of the untreated HUVEC spheroid sprouts. These sprouts were more like cell protrusions. (C and D) Quantification of sprouts from 10 spheroids (mean ± SEM) was assessed by evaluating the cumulative sprout length per spheroid, and data were verified by three independent experiments. *P<0.05; **P<0.01. HUVECs, human umbilical vein endothelial cells; PCa, prostate cancer; PC3CM, PCa cell line PC3-conditioned media; SEM, standard error of the mean; VEGF, vascular endothelial growth factor.

assay. When seeded onto Matrigel, HUVECs form tube structures and connect with each other, mimicking the *in vivo* process of angiogenesis. Sixteen hours after seeding, untreated HUVECs exhibited a clear capillary-like network formation. However, fasudil treatment dramatically decreased the capillary-like network formation in a dose-dependent manner. As fasudil concentration increased, total tube length gradually decreased (Fig. 3).

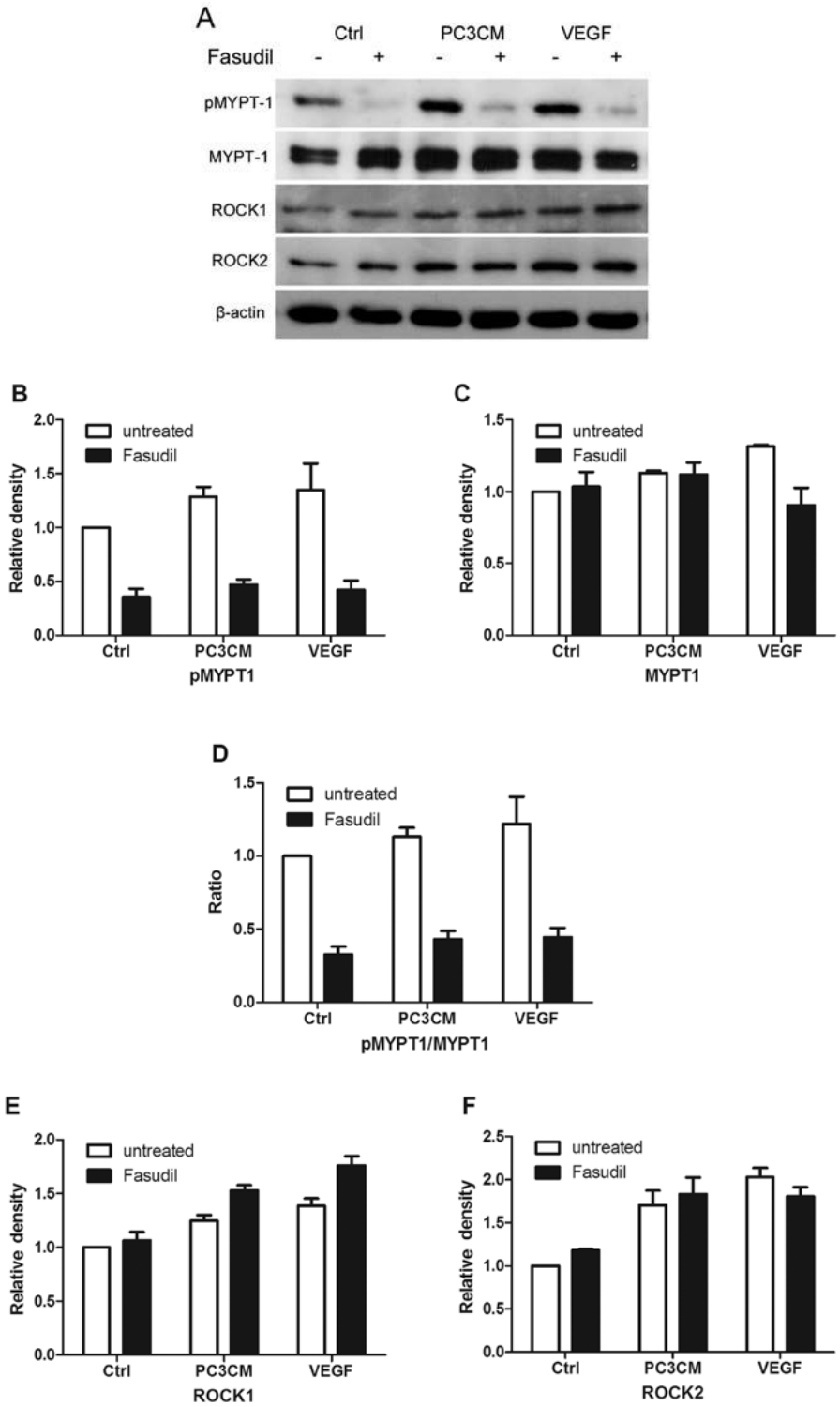

Figure 5. Fasudil inhibits PC3CM-induced ROCK activity. HUVECs were cultured in either basal medium (Ctrl), PC3CM, or basal medium containing VEGF (30 ng/ml) and treated with 30 μM fasudil or left untreated. ROCK expression was detected by immunoblotting with anti-ROCK1 and anti-ROCK2 antibodies. ROCK activity was detected by phosphorylation of the downstream effector, MYPT-1, with anti-phospho-MYPT-1 and anti-MYPT-1 antibodies. (A) Representative images of western blots are shown. The relative density of each blot was quantified as fold-expression relative to the control. The data shown are the mean ± SEM of 6 independent experiments. PC3CM increased pMYPT-1 expression and fasudil decreased pMYPT-1 expression (B) without any significant changes in MYPT-1 (C), resulting in an increase in the pMYPT-1/MYPT-1 ratio (D) in the PC3CM group and a decrease in this ratio after fasudil treatment. (E and F) PC3CM increased ROCK1 and ROCK2 expression while fasudil had no effect on ROCK expression. HUVECs, human umbilical vein endothelial cell; MYPT, myosin phosphatase target subunit 1; PCa, prostate cancer; PC3CM, PCa cell line PC3-conditioned media; ROCK, Rho-associated protein kinase; SEM, standard error of the mean; VEGF, vascular endothelial growth factor.

Fasudil inhibits PC3CM-induced HUVEC spheroid sprouting. In the sprout formation assay, HUVECs seeded in non-adhesive conditions in round bottom 96-well plates contributed to the formation of a single spheroid with a quiescent, non-proliferating surface monolayer within 24 h. The spheroids were then embedded in a 3D collagen matrix.

In the untreated control group, baseline sprouting was low (Fig. 4Aa). When cultured with PC3CM (Fig. 4Ab), baseline sprouting increased dramatically, although it was still less than that in the cells cultured with basal medium containing 30 ng/ml VEGF (Fig. 4Ac). Sprouting was almost completely inhibited by treatment with 100 μM fasudil (Fig. 4Ad-f). We then examined the dose-dependent response of fasudil on PCa-induced HUVEC sprouting. As shown in Fig. 4B, fasudil decreased HUVEC sprouting in a dose-dependent manner and 100 μM fasudil again inhibited sprouting almost completely.

Furthermore, when treated with increasing concentrations of fasudil, the sprouts became thinner and the HUVEC nucleus seldom emerged from the spheroids. These sprouts resembled cell protrusions, were markedly thinner compared with the untreated HUVEC sprouts, and were more abundant compared with the ordered architecture of the single HUVEC spheroid sprouts (Fig. 4B).

Fasudil inhibits PC3CM-induced HUVEC ROCK activation. MYPT-1 is one of the most crucial downstream effectors of ROCK. As fasudil is a ROCK inhibitor, we examined the inhibitory effects of fasudil on ROCK by measuring phospho-MYPT-1 (pMYPT-1), the active form of MYPT-1.

As shown in Fig. 5, when cultured with PC3CM or basal medium containing VEGF, expression of both MYPT-1 and pMYPT-1 was increased in the HUVECs, resulting in a moderate increase in the pMYPT-1/MYPT-1 ratio, indicating ROCK activation. Fasudil treatment lead to a significant decrease in pMYPT-1 and a slight decrease in MYPT-1, resulting in a significant decrease in the pMYPT-1/MYPT-1 ratio (Fig. 5A-D). A moderate increase in ROCK1 and ROCK2 expression was also detected, but ROCK expression was not altered significantly by fasudil treatment (Fig. 5E and F).

Discussion

To our knowledge, there have been no previous reports on the effects of fasudil on PCa-induced angiogenesis. In this study, HUVECs were cultured with the PCa cell line PC3CM to mimic endothelial cells in PCa tissue. Fasudil was then added to examine its effects on PC3CM-induced HUVECs using *in vitro* angiogenesis assays.

When cultured with PC3CM, ROCK1 and ROCK2 expression increased in the HUVECs, as did pMYPT-1 and total MYPT-1 expression. The pMYPT-1/MYPT-1 ratio was also increased. This indicates activation of the RhoA/ROCK pathway in PC3CM-stimulated HUVECs. It has been reported that endothelial cells in PCa tissue from TRAMP mice, a spontaneous PCa mouse model, have a constitutively high baseline level of activity of Rho GTPase and its downstream effector ROCK (17). This suggests that the RhoA/ROCK pathway plays a crucial role in PCa angiogenesis. HUVECs cultured in PC3CM share some of the characteristics of PCa endothelium and can therefore be used to represent it.

Angiogenesis involves a complex series of events that take place in a multi-step process. Endothelial cells migrate through the basement membrane toward an angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of endothelial cells, followed by the formation of capillary tubes via endothelial cell organization. The RhoA/ROCK pathway plays a role in each of these steps.

We evaluated the effects of fasudil on each of these steps in PCa-induced HUVECs. Fasudil was found to inhibit PC3CM-induced HUVEC proliferation, migration, tube formation and spheroid sprouting. This is in accordance with previous studies on VEGF-induced endothelial cell proliferation, migration and tube formation after treatment with the RhoA inhibitor, C3, or ROCK inhibitors, Y-27632 and fasudil (15,16,18).

It is interesting to note the morphological changes that occurred in the spheroid sprouting assay after treatment with fasudil. After treatment with 10 μM fasudil, the sprouts were much thinner than those on untreated cells. However, the HUVEC nucleus was observed less frequently moving out of the spheroids than in the controls. The movement of the nucleus decreased as the fasudil concentration increased, whereas sprouting was not affected until the concentration of fasudil exceeded 30 μM. These sprouts were more akin to cell protrusions, were markedly thinner compared with PC3CM-induced HUVEC sprouts, and were more abundant and disorganized compared with the ordered architecture of single HUVEC spheroid sprouting.

In conclusion, fasudil significantly inhibits the key steps of endothelial cell angiogenesis, including proliferation, migration and capillary tube formation, in a dose-dependent manner. These effects may be due to inhibition of ROCK activity induced by PCa cell secretions. Fasudil may be a useful anti-angiogenic agent and should be investigated further for its potential role in the anti-angiogenic treatment of PCa.

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PART II ARTICLE II

HIGH RHOA EXPRESSION AT THE TUMOUR FRONT BUT NOT IN THE CENTER OF PROSTATE CANCER

High RhoA expression at the tumor front but not in the centre of prostate cancer

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Abstract Objective: To evaluate RhoA expression and activity in different regions of clinically localized pro-state cancer tissue. **Patients and methods:** A total of 34 paraffin embedded and 20 frozen prostate specimens were obtained from 45 patients treated with radical pro-statectomy for clinically localized cancer. The expression patterns of RhoA were tested by immune-histochemical staining and Western blotting, and further compared between the tumour centre, tumour front and distant peritumoral tissue. RhoA activity was assessed by G-LISA. The association of RhoA expression and clini-cal features and outcome were also analysed. **Results:** We found an increasing gradient of expression from the centre to the periphery of index tumour foci. RhoA ex-pression was indeed significantly higher at the tumour front as compared to tumour centre, using immunehistochemistry (p=0.001). Also, Gleason scores were significantly higher in both tumour front and tumour centre of these patients (p=0.044 and 0.039, respect-tively). After a median follow-up of 52 months, the rate of PSA relapse was higher in patients with a higher RhoA expression at the tumour front (62.5% vs 35%), although the difference was not significant (p=0.089). There was no association between RhoA expression and PSA, pathological stage. **Conclusions:** RhoA expression was found to be higher at tumor front and associated with high Gleason score, suggesting the potential role of RhoA in prostate cancer invasion and may serve as a potential therapeutic target.

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Introduction

Prostate cancer (PCa) is the most prevalent malignancy in men in Western countries [1]. It is the second leading cause of cancer-related death in this area. Several autopsy studies have shown however that most of cancers remain latent [2]. These small and well differentiated cancers, so-called clinically insignificant [3] , may not be of any threat. If detected with PSA screening and extended biopsy, they should therefore not be treated with radical and morbid treatments [4] and be managed with active surveillance. Distinguishing these latent cancers from those aggressive cancers that will spread and develop distant metastasis remains currently a critical issue.

The molecular mechanisms that differentiate those cancers with invasive phenotype from those that will remain latent within the gland remain widely unknown. Many factors have been implicated in the process of prostate cancer invasion and metastasis, but the initial molecular transforming events occurring in prostatic epithelial cells and their local microenvironment are unknown.The capacity of the tumour cells to migrate and invade the prostate tissue is crucial in the initial steps of tumour progression. RhoA GTPase is a well-established regulator of rearrangements of cell cytoskeleton dynamically and spatiotemporally during cell migration. Previous reports showed that RhoA was active at the rear of the cells to promote tail retraction, and also localized at the leading edge of migrating cells to regulate protrusion at the front of the cell[5-7]. RhoA can promote the invasive behaviour of tumour cells through invadopodia-driven and bleb-driven amoeboid invasion[8-10]. It has been shown to be up regulated and to participate in the tumour invasion process in many solid tumours, such as Ovarian Carcinoma [11], ameloblastoma [12], breast cancer [13,14].

Although recent data suggested an over expression of RhoA in prostate cancer tissue [15], its activity has been poorly studied in localized and locally advanced prostate cancer. More importantly, the pattern of RhoA expression has never been evaluated in distinct tumor and peritumoral

areas. Given its potential role in local tumor invasion, RhoA expression might differ throughout the prostate gland. The aim of the present study was therefore to evaluate and compare the expression and activity of RhoA in radical prostatectomy specimen from patients treated for localized or locally advanced prostate cancer, and to assess their value as a prognostic marker of aggressiveness. We compared three zones: tumor center, tumor front and peritumoral tissue.

Patients and methods

Patients All human tissues were obtained from the department of Surgical Pathology, Cochin hospital, Paris, France, in accordance with the ethical policy and procedure of the hospital's Institutional Review Board. Written consent was obtained from each patient. Tumor tissues were graded according to the modified Gleason grading system. Pathologic stage was *determined* according to the 2009 tumor-node-metastasis classification for prostate cancer.

Immunohistochemistry We selected 34 cases of patients diagnosed with presumed clinically localized prostate cancer and treated by radical prostatectomy. Median follow-up after RP was of 52 months. Clinical data including age, PSA, pathological stage, Gleason score and follow-up were recorded. Fresh-cut sections were selected from Formalin-fixed/paraffin-embedded tissue blocks containing tissue with the highest density of tumor cells and the highest Gleason score (index tumor). Sections were deparaffinized, rehydrated through alcohol gradient, heated for 40 min at 96°C in Sodium Citrate Solution (10 mM, pH 6.0) for antigen retrieval. Slides were then incubated with goat polyclonal anti-RhoA antibody (SANTA CRUZ, sc-32039, dilution 1:50) overnight at 4°C and biotinylated rabbit anti-goat secondary antibody(Vector Laboratories, BA-5000, dilution 1:200, Burlingame, CA) at room temperature for 60 minutes. This was followed by incubation with VECTASTAIN Elite ABC Reagent (VECTOR LABORATORIES, PK-7100, Burlingame, CA) for 30 min. Peroxidase activity was examined with the DAB (Diaminobenzidine Tablets, Sigma, D4418). Sections were counterstained using hemalum and mounted using Permount (Fischer Scientific, Pittsburgh, PA). Two pathologists independently scored the tissue staining for RhoA. The central area fully constituted of cancerous glands were defined as "tumor center". The edges of tumor foci were indicated as "tumor front". Peritumoral benign prostate glands without PIN in the same slide serve as control and were labeled "peritumoral prostatic tissue". An ordinate, categorical (semi-quantitative) method was used to evaluate staining across each area. RhoA expressions were graded as no staining (0), or weak (1), or moderate(2) or strong staining(3).

All human tissues were obtained in accordance with the ethical policy of the hospital's Institutional Review Board. Written inform consent was taken from each patient. The individual in this manuscript has given written informed consent to publish these case details.

Western blot analysis A total of 20 tissue samples were obtained from 11 patients who underwent radical prostatectomy for clinically localized or locally advanced prostate cancer. Immediately after surgical removal, small pieces of tissues were gross dissected by the pathologist, snap-frozen and stored in liquid nitrogen. Histological analysis of radical prostatectomy frozen sections was performed by the same pathologist. The percentage of tumour cells in each sample was evaluated. 12 samples contained 50 to 90% of cancerous glands and were labelled as "tumour tissue". 8 samples without cancerous tissue were labelled "peritumoral prostatic tissue" (P). Then these samples were subjected to Western blot for RhoA expression and G-LISA analysis for RhoA activity. Proteins were extracted on ice with cold RIPA lysis buffer (#9806, Cell Signalling Technology, Boston, MA) containing Pierce™ Protease and Phosphatase Inhibitor (#88669, Thermo Scientific, IL, USA). Lysates were centrifuged at 12 000 g for 20 min at 4°C, and the supernatant was collected. Total protein concentrations were determined using a bicinchoninic acid assay (BCA) protein assay kit (#23250, Thermo Scientific). Equal amounts of protein were subjected to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis and then electrically transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with 5% (w/v) non-fat milk in PBS–0.1% (v/v) Tween 20 (PBST) and incubated with goat primary antibodies against RhoA (SANTA CRUZ, sc-32039, 1:250) and β-actin (SANTA CRUZ, SC47778, 1:500) overnight at 4°C. Finally, the membrane was incubated with HRP-conjugated secondary antibodies as following: rabbit anti-goat IgG-HRP (1:5000; SC2768, Santa Cruz Biotechnology) and goat anti-mouse IgG-HRP (1:5000; SC2005, Santa Cruz Biotechnology) for 1 h at room temperature. After washing three times with PBST, proteins were visualized using an ECL Prime Western blotting detection kit (GE Healthcare). Photographs of protein bands were taken using a digital imaging system (Image Quant LAS, GE Healthcare Bio-Sciences, Piscataway, NJ, USA), And densitometric measurements of band intensity in the Western blots were performed using NIH ImageJ software. The results shown are representative of three independent experiments.

GLISA analysis RhoA activity was assayed using a G-LISA® RhoA Activation Assay Biochem Kit™ (Cytoskeleton, Cat. # BK124, Denver, CO, USA) performed according to manufacturer's prescription. Briefly, prostate samples were homogenized in ice-cold lysis buffer with protease inhibitor cocktail in the kit, then centrifuged at 15,000 rpm at 4°C for 1 minute. Supernatants were harvested and protein concentrations were measured by Precision Red Advanced Protein Assay Reagent (Cytoskeleton, ADV02) and were finally equalized with ice cold lysis buffer to 1.0 mg/ml. Equalized prostate tissue protein extractions were transferred to a Rho-GTP-binding protein pre-coated plate. The plate was placed on an orbital shaker at 200 rpm for 30 min at 4 °C, and then incubated with anti-RhoA primary antibody, followed by secondary antibody on a microplate shaker at 200 rpm at room temperature for 45 min each. The plate was then incubated with HRP detection reagent at 37 °C for 15 min. After HRP stop buffer was added, absorbance was read at 490nm.

Statistical analysis

Comparison between proportions was performed utilizing the $\chi 2$ test with Yates continuity correction for independent samples, Mcnemar test for two paired samples. Continuous data from two independent groups were compared with Mann–Whitney U test. All the tests were performed for two tails. P<0.05 was set as the significant level.

Table 1. Patient demographics

	IHC cohort	WB & G-LISA cohort
All patients(n)	34	11
Age(years)		
Median (range)	62 (50-73)	61 (49-71)
PSA(ng/ml)		
Median (range)	8.66 (2.5-30)	9.2 (4.4-30)
Tumor stage		
T2	17 (50%)	6 (55%)
T3	17 (50%)	5 (45%)
Gleason Score		
3+3	15 (44%)	5 (45%)
3+4	11 (32%)	3 (27%)
4+3	6 (18%)	2 (18%)
4+4	2 (6%)	1 (9%)
Followup		
(months,range)	52(7-112)	N/A
PSA relapse(n,%)	16(47.1%)	N/A

PSA relapse is defined as a rise of serum total PSA>0.2 ng/mL on two consecutive measurements

Results

Clinicopathologic features of the study population

Clinicopathologic features of the study population were summarized in Table 1.

RhoA expression and activity in prostate tissues involved or not with cancer

RhoA protein expression was first assessed by Western blotting in 20 frozen samples obtained from radical prostatectomy specimen, as previously described. As shown in Fig.1, RhoA expression was significantly lower in tissues involved with cancer as compared to tissues with no evidence of cancer, considered as peritumoral tissues (p<0.05). As activation of RhoA is a prerequisite to execute its effects, we evaluated its activity by GLISA in the same samples. Although RhoA activity was higher in tissues involved with cancer, the difference was not statistically significant (0.20 vs 0.12, p=0.22).

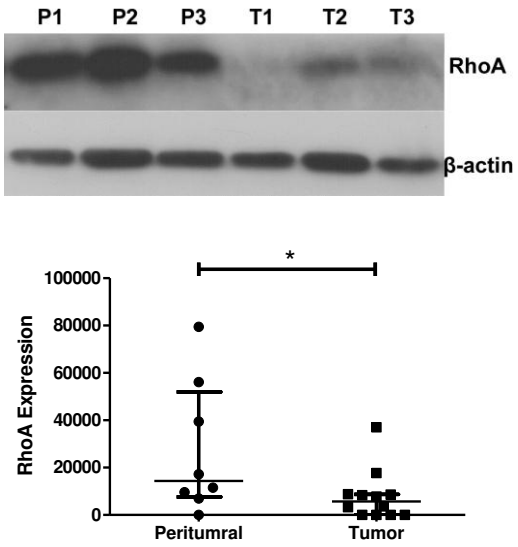

Figure 1 RhoA expressions in prostate cancer by Western blotting.

(a)Representative western blots showing RhoA expression in three prostatic tumor tissues(T1-3) and three peritumoral tissues(P1-3). (b)Quantitative analysis of RhoA expression. The expression of RhoA in prostate cancer tissue is significantly lower than in the peritumoral prostatic tissues(* p<0.05).

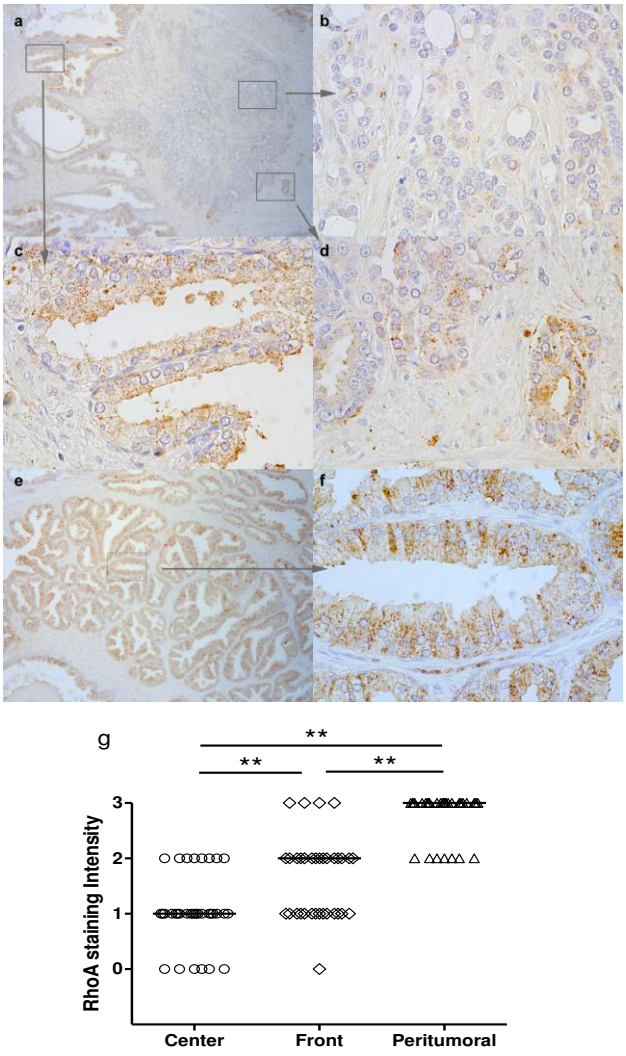

Figure 2 RhoA expression in prostate cancer by immunohistochemistry.

(a) Showed an increasing gradient of expression from the center to the periphery of index tumor foci. The expression of RhoA in prostate cancer front(d) is significantly higher than in the cancer center(b), while the adjacent(c) or distal(e,f) peritumoral prostate gland has the highest expression. Individual intensities of each sample and median intensities in each group were demonstrated in (g). Magnification X50 for a,e; X400 for b,c,d,f. (** p<0.01).

RhoA expression patterns throughout cancerous prostate glands

To further evaluate patterns of RhoA expression in prostatic tissues, immunohistochemistry was performed in 34 paraffin embedded samples of radical prostatectomy specimen. Analysis of staining intensity enabled to detect different patterns of expression throughout the tissue. In the distant peritumoral tissue, immnunohistological analysis showed moderate or strong staining for RhoA expression in all cases. At the tumour centre, RhoA expression was significantly lower, with moderate staining observed in only 8 cases (23%, p<0.001). We did not observe strong staining in this area in any of the cases. At the tumour front, staining was moderate or strong in 19 cases (56%), which was significantly higher than in the tumour centre (p<0.01).

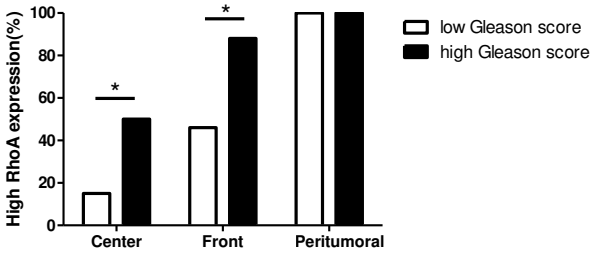

Figure 3 RhoA expressions increased in the patients with a higher Gleason score.

In both cancer centre and cancer front, RhoA expressions were significantly higher, as indicated by the percentage of RhoA stain score 2 and above, in patients with Gleason score 4+3 or higher(15% vs 50%, p=0.044 and 46% vs 88%, p=0.039 respectively).

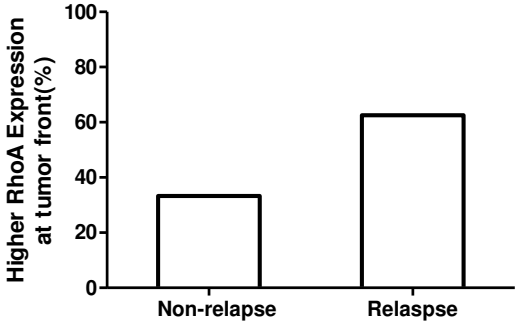

Figure 4 Higher RhoA expression in patients with PSA relapse.

Patients who developed relapse disease have higher RhoA expression in tumor front than those without relapse, but this did not reach statistically significant (62.5% vs 35%, p=0.089).

Association of RhoA expression with adverse oncologic features and outcomes

In areas involved with cancer, RhoA expression was significantly higher in high grade tumours (Gleason score >3+4), whatever the location in the tumour centre or front (fig 4). In the tumour front, RhoA expression was higher in patients who experienced PSA relapse after

surgery, although the difference was not statistically significant (Fig 4, p=0.09). No statistical association was observed between RhoA expression and PSA level before surgery or pathological stage.

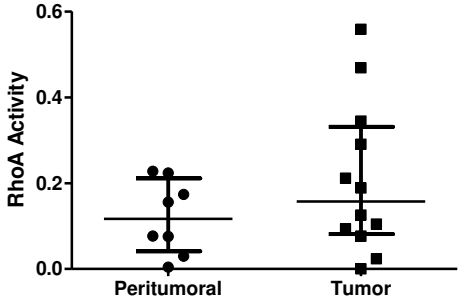

Figure 5 RhoA activities were higher in the tumor than in peritumoral tissues.
Higher RhoA activity in the tumor than in peritumoral prostatic tissues, but this did not reach statistical significant difference(p=0.35).

Discussion

To our knowledge, our study is the first evaluating RhoA expression not only in the centre of tumours but also at the tumour front and in peritumoral tissue. We have demonstrated significantly increased RhoA expression at the tumour front as compared to tumour centre.
RhoA has been reported to play a critical role in cancer cell migration and invasion. RhoA is a small guanosine triphosphate hydrolase, serves as key regulators of extracellular stimulus-mediated signalling networks involved in a diversity of cellular processes including motility, mitosis, proliferation, and apoptosis[16]. During cell movement, RhoA is active at the trailing edge of the cell to promote retraction, as well as at the leading edge of migrating cells to regulate protrusion [5,7,6]. Increases in RhoA activity were found to be correlated with increases in protrusion rates, and were synchronous with cell-edge advancement [6]. Silencing RhoA expression inhibits the progression of breast cancer [17] and gastric cancer [18] in vitro and in vivo.
Recent reports suggested the potential involvement of RhoA in prostate cancer invasion. Hodge et al reported a higher expression of RhoA in highly invasive variants of PC-3 prostate cancer cells as compared to low invasive variants, and that RhoA promoted cancer invasion through the activation of cell motility[19]. Neuropeptide-stimulated migration in prostate cancer cells was shown to be mediated by RhoA [20]. RhoA was also reported to induce migration towards monocyte chemoattractant protein 1 (MCP-1; CCL2) in PC-3 cells [21]. Several inhibitors of prostate cancer cell migration, such as TMEFF2[22], miR-34a[23], Epac[24], FTY720[25], WIN55212[26] were showed to inhibit RhoA activity in prostate cancer cell lines. RhoA was also reported to be overexpressed in prostate cancer tissue from patients with clinically localized prostate cancer treated with radical prostatectomy, and its expression to be associated with poor outcome after surgery [15].
Most clinical studies evaluating potential new tumour markers now use tissue microarrays, which enable to study a large number of cases, but are only representative of a small amount of tissue, usually in the centre of tumours. The tumour microenvironment is however highly complex and variable from a location to another. Several studies addressing this issue showed that tumour front and tumour centre had different characteristics, resulting in different behaviour [27,28] . In colorectal carcinomas, Cianchi et al showed that cells at the invasive front have a more aggressive behaviour in comparison with central tumor regions [27]. In prostate cancer, cells at the tumor front were also suggested to have a more aggressive phenotype, and to express specific features as compared to the tumor center. CXCR4, which is implicated in tumor invasion through the extra cellular matrix, is specifically expressed at the tumor front of prostate tumors, while its expression in the tumor centre is low[28]. Our own results indicate that RhoA expression in the tumor centre is low and clearly highlight the importance of studying the tumour front, as well as surrounding peritumoral tissue before starting large assays to avoid biased interpretations
As RhoA is implicated in cancer cell invasion, it is reasonable to hypothesize that cancer cells expressing RhoA at the tumour front may have higher mobility and aggressiveness. This hypothesis is also supported by the finding that the probability of PSA relapse after surgery was higher in patients with high RhoA expression at the tumour front, although not statistically significant. Our results also showed that high RhoA expression was associated with high Gleason score in both tumour centre and tumour front, suggesting that poorly differentiated tumours were more likely to express RhoA, which would in return facilitate invasion through the tissue.
While a previous study suggested lower expression of RhoA in benign prostate glands [15], we found the opposite. In the study from Schmidt et al, the authors used biopsy material from 91 patients with localized prostate cancer. They observed a lower expression of RhoA in benign areas as compared to cancer. However, the true location of these so-called benign areas may not be accurately stated on biopsy material, because they may be located closely to

tumour foci unsampled with biopsy. It is also difficult to determine if the tumoral tissue sampled with biopsy is located at the tumour front or centre. Interestingly, we observed a significantly higher RhoA expression in the distant peritumoral area, while its activity, as determined by G-LISA, was lower than in tumour areas. As RhoA is a ubiquitous protein with many different functions, our results may suggest that RhoA expression and activity in the distant peritumoral area was not related with the presence of cancer. RhoA has also a role in secretory granules trafficking and exocytosis [29,30], and may execute these functions in the epithelial cells of the distant tissue. While RhoA expression in the tumour cells was lower, its higher activity may also further consolidate the hypothesis that RhoA has a specific role in the prostate cancer progression. As RhoA has several downstream effectors, ROCK 1 and 2 being the most important, a comparison of their specific activity in the different tissue locations would further improve the comprehension of RhoA pathways and implications in prostate cancer progression.

Our study has several limitations, the most important being the small sample size that may have biased our statistical analysis. A larger sample of patients is required to test reliably the association between RhoA expression and other adverse clinical and pathological features. Another limitation is the absence of whole gland analysis. We focused only on the index tumour, while contiguous or distant tumours may not have the same profile of expression. Further analyses will be conducted in more samples and assess the downstream effectors on the whole prostatic gland.

In conclusion, our work identified higher RhoA expression in prostate tumour front and its correlation with cancer relapse, the correlation of higher RhoA expression with higher Gleason score and the higher RhoA activity in tumour. This indicated the association of RhoA expression with aggressiveness of prostate cancer. The insights described here may provide the foundation for novel therapeutic approaches that inhibit clinically aggressiveness of PCa.

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PART III OTHER RESULTS

EXPRESSION OF RHOA/ROCK PATHWAY MOLECULES IN PROSTATE CANCER TISSUE

NO DIFFERENCE OF THE EXPRESSIONS OF RHOA/ROCKS IN THE CAPILLARY ENDOTHELIAL CELLS OF PROSTATE CANCER TISSUES

As RhoA/ROCK pathway has been indicated to be involved in prostate cancer angiogenesis, therefore it is interesting to investigate the expression of RhoA/ROCK pathway molecules in the vessels of prostate cancer tissue. It has been reported that endothelial cells in PCa tissue from TRAMP mice, a spontaneous PCa mouse model, have a constitutively high baseline level of activity of Rho GTPase and its downstream effector ROCK (Ghosh, Thodeti et al. 2008). But there is still no report on the expression of these molecules in the endothelial cells in human PCa tissue. Thus in this study we assessed the expression of RhoA, ROCK1 and ROCK2 in human PCa tissue.

As it has been mentioned in the Part II, 34 cases of patients with prostate cancer and treated by radical prostatectomy were enrolled. Fresh-cut sections were selected from Formalin-fixed/paraffin-embedded tissue blocks containing tissue with the highest density of tumor cells and the highest Gleason score (index tumor). The sections were subjected to RhoA examination by immunohistochemistry as previously described. Another section from the same patients was subjected to ROCK1 and ROCK2 examination. The procedures were similar to that of RhoA except that slides were incubated with goat polyclonal anti-ROCK1 antibody (SANTA CRUZ, sc-6055, dilution 1:50) and goat polyclonal anti-ROCK2 antibody (SANTA CRUZ, sc-1851, dilution 1:50) overnight at 4°C.). The tissue staining for RhoA, ROCK1 and ROCK 2 expression were evaluated by the same pathologists in the different regions of the tissue as previously defined: tumor center, tumor front and distal peritumoral tissue.

We found no significant difference of the expressions of RhoA, ROCK1 and ROCK2 between tumour foci and distal prostatic tissues (Fig. 30). This may due to the limitation

of immunohistochemistry assay, as their expressions were higher in the prostatic epithelial cells and cancerous cells, thus they were relatively lower in the endothelial cells both in tumour foci and distal prostatic tissues and add difficulty to distinguish the difference between them.

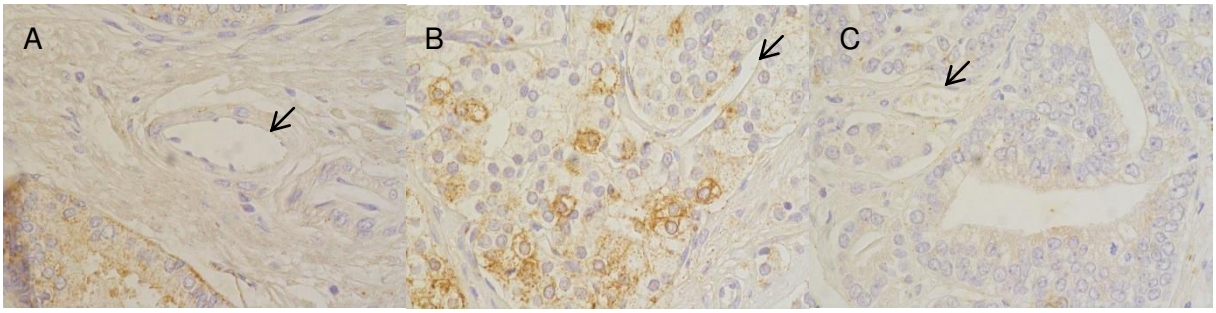


Figure 18 RhoA expression in the capillary endothelial cells in human PCa tissue.

(A) showed a capillary (arrow) in the interstitial tissue near distal peritumoral prostate gland. (B, C) showed capillaries (arrows) in the tumor front(B) and tumor center(C) of prostate cancer foci. The intensities of RhoA in each region were comparable. Magnification X400.

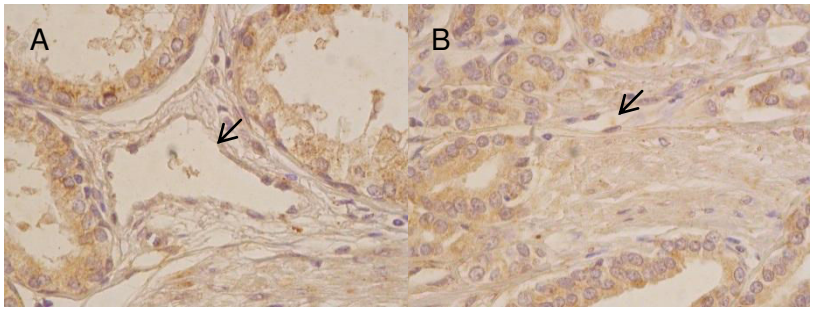


Figure 19 ROCK1 expression in the capillary endothelial cells in human PCa tissues.

(A) showed a capillary (arrow) in the interstitial tissue between distal peritumoral prostate glands. (B) showed capillary (arrow) in prostate cancer foci. The intensities of ROCK1 in each region were comparable. Magnification X400.

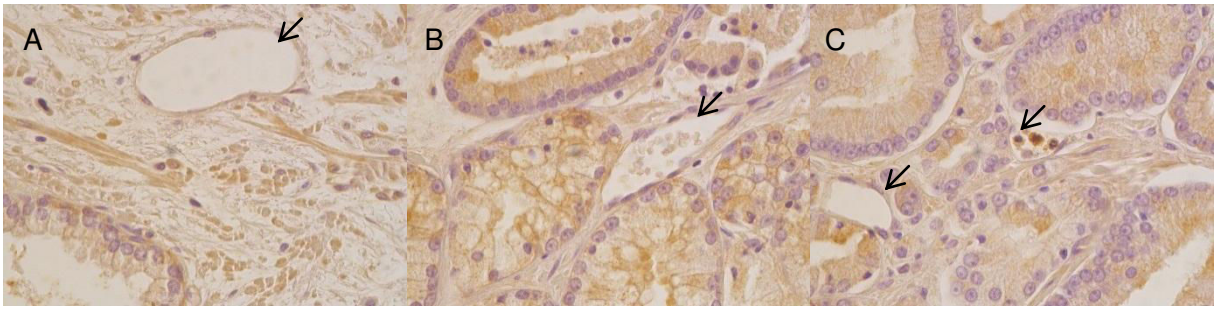


Figure 20 ROCK2 expression in the capillary endothelial cells in human PCa tissues.

(A) showed a capillary (arrow) in the interstitial tissue near distal peritumoral prostate gland. (B,C) showed capillaries (arrows) in the tumor front(B) and tumor center(C) of prostate cancer foci. The intensities of ROCK2 in the capillaries of each region were comparable. Magnification X400.

HIGHER ROCK2 EXPRESSION BUT NOT ROCK1 AT THE TUMOUR FRONT OF PROSTATE CANCER

ROCKs have been shown to be the principle downstream target of RhoA and execute the most important function of RhoA in regulating cytoskeleton (Ishizaki, Maekawa et al. 1996). The expression of RhoA in cancer cells of PCa tissues have been found to be significantly higher in the tumor front and correlated with clinical outcomes, as shown in Part II of this project. It is interesting and valuable to explore the expression of ROCKs, including both isoforms ROCK1 and ROCK2, in the cancer cells of human PCa samples.

The patients and sections have been described in previous studies of this project. The same sections with staining of ROCK1 and ROCK2 were reanalysed for their expression in prostate cancer cells as compared with peritumoral epithelial gland cells.

In the tumour tissue, the expression patterns of ROCK2 were similar to that of RhoA. At the tumour front, ROCK2 expression was significantly higher ($p<0.01$). In the distant peritumoural tissue, immunohistological analysis showed moderate staining for ROCK2 expression in all cases, which had no significant difference with either tumour center or tumour front. There were no significant difference between ROCK2 expressions and clinical features and outcomes, maybe due to the limitation of the sample size ($n=17$). ROCK1 expressions were no difference between the tumour center, tumour front and distant peritumoral tissue.

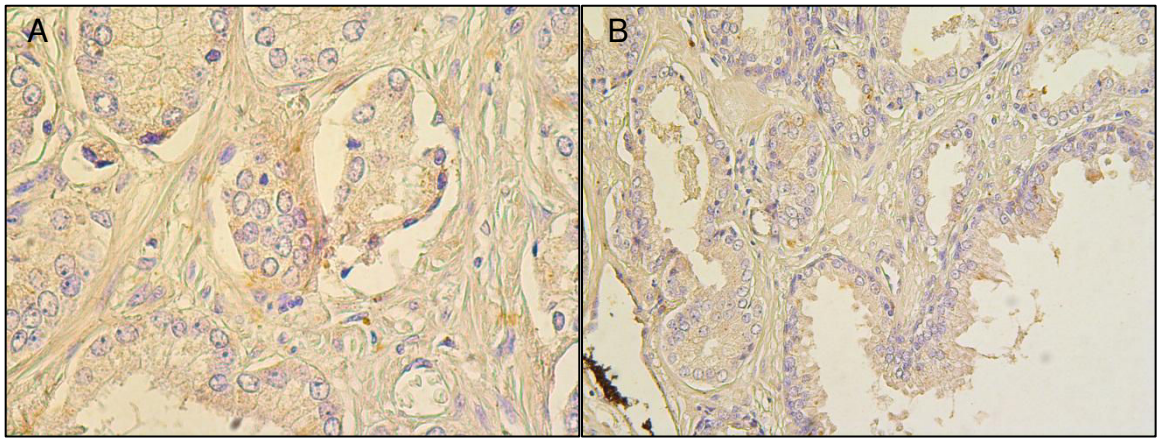
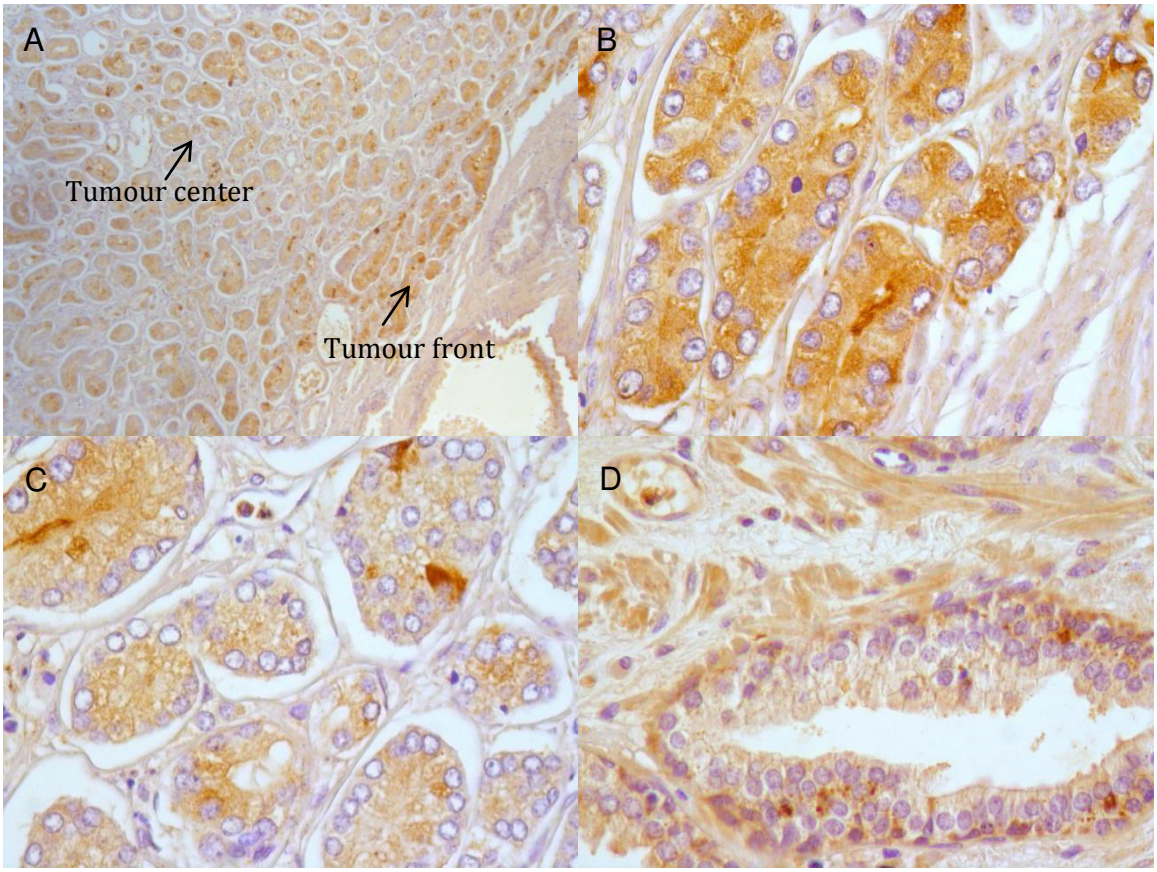


Figure 21 ROCK1 expression in the cancer cells in human PCa tissue.

(A) showed ROCK1 expression in distal peritumoral tissue. (B) showed ROCK1 expression in prostate cancer foci. The intensities of ROCK1 in each region were comparable. Magnification X400.



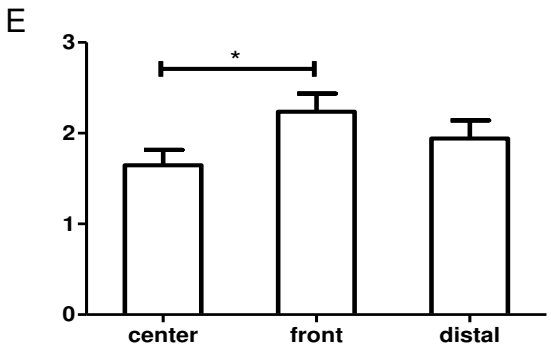


Figure 22 ROCK2 expression in prostate cancer center is significantly lower than cancer front.

(A) showed a gross view of ROCK2 expression in PCa foci. (B, C) showed the stain in the tumor front (B) and tumor centre (C) of prostate cancer foci. (D) showed that in distal peritumoral tissue. The intensities of ROCK2 stain were comparable significantly higher in tumour front than in the tumor centre. No statistical difference can be drawn between distal peritumoral tissue and any region of tumor foci. Magnification X50 for (A) and X400 for (B, C, D).

DISCUSSION

RHOA/ROCK PATHWAY INVOLVED IN PROSTATE CANCER ANGIOGENESIS

RhoA/ROCK pathway play versatile functions in cell biology, including modulating cell shape, polarity and motility by its effects on actin polymerization, actomyosin contractility, cell adhesion and microtubule dynamics. RhoA activates its principle downstream effectors ROCKs, as well as the other effectors like DRFs, to promote the increase in actin filaments and stress fiber formation. In addition, RhoA activates ROCKs and subsequent phosphorylate several proteins, such as MLC, involved in regulating myosins and other actin-binding proteins to induce actomyosin-based contractility, which is crucial in migrating cells. RhoA also plays a key role in modulating the adhesion between neighbouring cells and between cell and extracellular matrix, thus regulating the migration of endothelial cells by reorganizing these adhesions.

Several studies have demonstrated the important role of RhoA/ROCK pathway in the multi-steps of tumour angiogenesis, including permeability, proliferation, migration and tube morphogenesis. RhoA has been shown to contribute to VEGF-induced hyperpermeability in the endothelium (Sun, Breslin et al. 2006). ROCK inhibitors, fasudil and Y-27632, have been reported to inhibit VEGF-induced cell proliferation and reverse the protective effect of VEGF on apoptosis (Yin, Morishige et al. 2007, Bryan, Dennstedt et al. 2010). In vitro angiogenesis assays found that the mean tube length of the capillary-like tubular structures formed by ECs was reduced by transfection of a RhoA dominant-negative mutant vector, the RhoA inhibitor C3, or the ROCK inhibitor Y-27632(van Nieuw Amerongen 2002), while overexpression of RhoA increased tube length in transfected ECs(Zhao, Xu et al. 2006). Endothelial cells migration can be inhibited by transfection of ECs with a RhoA dominant-negative mutant vector or by a RhoA inhibitor C3 (van Nieuw Amerongen 2002). Increased expression of RhoA in human umbilical vein ECs significantly enhanced cell migration (Zhao, Xu et al. 2006). In

vitro angiogenesis assays found that the mean tube length of the capillary-like tubular structures formed by ECs was reduced by transfection of a RhoA dominant-negative mutant vector, the RhoA inhibitor C3, or the ROCK inhibitor Y-27632(van Nieuw Amerongen 2002). Overexpression of RhoA increased tube length and angiogenic capacity of transfected cells, which suggests that RhoA plays a key part in these processes in vitro (Zhao, Xu et al. 2006).

There are still few reports on the role of RhoA/ROCK pathway in PCa. A study investigating the ECs from prostatic adenocarcinoma of TRAMP mice revealed a constitutively high level of baseline activity of Rho GTPase and its downstream effector, ROCK (Ghosh, Thodeti et al. 2008). Several studies have demonstrated that RhoA/ROCK pathway inhibitors decrease angiogenesis and cell growth in prostate cancer animal model, including Y-27632 and Wf-536 (Somlyo, Bradshaw et al. 2000, Somlyo, Phelps et al. 2003). Some potential anti-angiogenic medications, such as anacardic acid, have been found to inhibit human prostate tumour xenograft angiogenesis by targeting the Rho GTPase signalling pathway (Wu, He et al. 2011).

There is still no report on the expression of RhoA/ROCK pathway molecules in the vessels of human prostate cancer. In our study, we tried to assess the expression of RhoA/ROCK pathway molecules in the vessels of human prostate cancer specimen from radical prostatectomy. But we failed to distinguish the difference between their expression in the vessels of cancer foci and distal prostatic tissues by immunohistochemistry assay. We considered this insignificance may due to the limitation of immunohistochemistry technique. Their expression in ECs of prostatic cancer tissues has only been reported in the animal model by separating fresh endothelial cells of tumour foci by magnetic beads. It is difficult to perform this test in human prostate cancer specimen by the same method.

TARGETING RHOA/ROCK PATHWAY INHIBITED PROSTATE CANCER ANGIOGENESIS

Although several inhibitors of RhoA/ROCK pathway have been studied for their inhibitory effects on some cancers, there is still no report on the effects of fasudil on prostate cancer angiogenesis. Fasudil is the only ROCK inhibitor which has been approved to the market. It was first approved in Japan in 1995 for the treatment of cerebral vasospasm. Now there is an on-going Phase III clinical trial evaluating its safety and efficacy on hypertension. It was well tolerated without any serious adverse reactions in the previous clinical trials. Thus we chose fasudil as the inhibitor to target RhoA/ROCK pathway in our study.

To our knowledge, there have been no previous reports on the effects of fasudil on PCa-induced angiogenesis. In this study, HUVECs were cultured with the PCa cell line PC-3 conditioned media PC3CM to mimic endothelial cells in PCa tissue. Fasudil was then added to examine its effects on PC3CM-induced HUVECs using in vitro angiogenesis assays.

When cultured with PC3CM, ROCK1 and ROCK2 expression increased in HUVECs, as did pMYPT-1 and total MYPT-1 expression. The pMYPT-1/MYPT-1 ratio also increased. This indicates activation of the RhoA/ROCK pathway in PC3CM-stimulated HUVECs. It has been reported that endothelial cells in PCa tissue from TRAMP mice, a spontaneous PCa mouse model, have a constitutively high baseline level of activity of Rho GTPase and its downstream effector ROCK (Ghosh, Thodeti et al. 2008). This suggests that the RhoA/ROCK pathway plays a crucial role in PCa angiogenesis. HUVECs cultured in PC3CM share some of the characteristics of PCa endothelium and can therefore be used to represent it.

Angiogenesis involves a complex series of events that take place in a multi-step process. Endothelial cells migrate through the basement membrane toward the angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of endothelial cells, followed by the formation of capillary tubes via endothelial cell organization. The RhoA/ROCK pathway plays a role in each of these steps.

We evaluated the effects of fasudil on each of these steps in PCa-induced HUVECs. Fasudil was found to inhibit PC3CM-induced HUVEC proliferation, migration, tube formation and spheroid sprouting. This is in accordance with previous studies on VEGF-induced endothelial cell proliferation, migration and tube formation after treatment with the RhoA inhibitor, C3, or ROCK inhibitors, Y-27632 and fasudil (van Nieuw Amerongen 2002, Yin, Morishige et al. 2007, Bryan, Dennstedt et al. 2010).

A limitation of this part of study was that we did not perform in vivo experiments. As we have known the inhibitory effects of fasudil on PCa-induced angiogenesis in vitro and the inhibitory effects of other ROCK inhibitors on PCa angiogenesis in vivo, further studies shall be conducted to verify the effects of fadusil in animal model and its potential value in the clinical application.

RHOA/ROCK PATHWAY INVOLVED IN PROSTATE CANCER INVASION

RhoA/ROCK pathway has been shown to be involved not only in tumour angiogenesis but also tumour invasion, which is one of the most important characteristics of malignant cancers. RhoA/ROCK pathway causes actomyosin contraction, transformation, and transcription of the SRF mediated genes. Also, it shows scaffolding properties that function to polymerize actin and affect the formation of microtubules, thus involved in cell motility of both mesenchymal and collective migration. During cell movement, RhoA is active at the trailing edge of the cell to promote retraction, as well as at the leading

edge of migrating cells to regulate protrusion (Kurokawa and Matsuda 2005, Pertz, Hodgson et al. 2006, Machacek, Hodgson et al. 2009). Increases in RhoA activity were found to be correlated with increases in protrusion rates, and were synchronous with cell-edge advancement (Machacek, Hodgson et al. 2009). Silencing RhoA expression inhibits the progression of breast cancer (Pille, Denoyelle et al. 2005) and gastric cancer (Sun, Tong et al. 2007) in vitro and in vivo.

Recent reports suggested the potential involvement of RhoA in prostate cancer invasion. Hodge et al reported a higher expression of RhoA in highly invasive variants of PC-3 prostate cancer cells as compared to low invasive variants, and that RhoA promoted cancer invasion through the activation of cell motility(Hodge, Bub et al. 2003). Neuropeptide-stimulated migration in prostate cancer cells was shown to be mediated by RhoA (Zheng, Iwase et al. 2006). RhoA was also reported to induce migration towards monocyte chemoattractant protein 1 (MCP-1; CCL2) in PC-3 cells (Loberg, Tantivejkul et al. 2007). Several inhibitors of prostate cancer cell migration, such as TMEFF2 (Chen, Corbin et al. 2014), miR-34a (Yamamura, Saini et al. 2012), Epac(Grandoch, Rose et al. 2009), FTY720 (Zhou, Ling et al. 2006), WIN55212 (Nithipatikom, Gomez-Granados et al. 2012) were showed to inhibit RhoA activity in prostate cancer cell lines. RhoA was also reported to be overexpressed in prostate cancer tissue from patients with clinically localized prostate cancer treated with radical prostatectomy, and its expression to be associated with poor outcome after surgery (Schmidt, Duncan et al. 2012).

Most clinical studies evaluating potential new tumour markers now use tissue microarrays, which enable to study a large number of cases, but are only representative of a small amount of tissue, usually in the centre of tumours. The tumor microenvironment is however highly complex and variable from a location to another. Several studies addressing this issue showed that tumour front and tumour centre had

different characteristics, resulting in different behaviour. In colorectal carcinomas, Cianchi et al showed that cells at the invasive front have a more aggressive behaviour in comparison with central tumor regions (Cianchi, Cuzzocrea et al. 2010). In prostate cancer, cells at the tumor front were also suggested to have a more aggressive phenotype, and to express specific features as compared to the tumor center. CXCR4, which is implicated in tumor invasion through the extra cellular matrix, is specifically expressed at the tumor front of prostate tumors (Delongchamps et al, 2014) while its expression in the tumor center is low.

To our knowledge, our study is the first evaluating RhoA expression not only in the centre of tumours but also at the tumour front and in peritumoral tissue. We have demonstrated significantly increased RhoA expression at the tumour front as compared to tumour centre. Our own results indicate that RhoA expression in the tumor center is low and clearly highlight the importance of studying the tumour front, as well as surrounding peritumoral tissue before starting large assays to avoid biased interpretations.

As RhoA is implicated in cancer cell invasion, it is reasonable to hypothesize that cancer cells expressing RhoA at the tumor front may have higher mobility and aggressiveness. This hypothesis is also supported by the finding that the probability of PSA relapse after surgery was higher in patients with high RhoA expression at the tumor front, although not statistically significant. Our results also showed that high RhoA expression was associated with high Gleason score in both tumour centre and tumour front, suggesting that poorly differentiated tumors were more likely to express RhoA, which would in return facilitate invasion through the tissue.

While a previous study suggested lower expression of RhoA in benign prostate glands (Schmidt, Duncan et al. 2012), we found the opposite. In the study from Schmidt et al, the authors used biopsy material from 91 patients with localized prostate cancer. They observed a lower expression of RhoA in benign areas as compared to cancer. However, the true location of these so-called benign areas may not be accurately stated on biopsy material, because they may be located closely to tumor foci unsampled with biopsy. It is also difficult to determine if the tumoral tissue sampled with biopsy is located at the tumour front or centre. Interestingly, we observed a significantly higher RhoA expression in the distant peritumoral area, while its activity, as determined by G-LISA, was lower than in tumor areas. As RhoA is a ubiquitous protein with many different functions, our results may suggest that RhoA expression and activity in the distant peritumoral area was not related with the presence of cancer. RhoA has also a role in secretory granules trafficking and exocytosis (Gasman, Chasserot-Golaz et al. 1998, Frantz, Coppola et al. 2002), and may execute these functions in the epithelial cells of the distant tissue. While RhoA expression in the tumor cells was lower, its higher activity may also further consolidate the hypothesis that RhoA has a specific role in the prostate cancer progression.

As RhoA has several downstream effectors, ROCK 1 and 2 being the most important, we assessed the ROCK1 and ROCK2 expression in the prostate cancer specimen of the same patients. We found that the expression patterns of ROCK2 were similar to that of RhoA in the tumour tissue. ROCK2 expression was significantly higher at the tumour front than in the tumour centre. While ROCK1 expressions were no difference between the tumour center, tumour front and distant peritumoural tissue. These indicated ROCK2, but not ROCK1, may be the downstream effector of RhoA which play an important role in prostate cancer invasion. There were no significant difference between ROCK2

expressions and clinical features and outcomes, maybe due to the limitation of the sample size.

This study has several limitations, the most important being the small sample size that may have biased our statistical analysis. A larger sample of patients is required to test reliably the association between RhoA expression and other adverse clinical and pathological features. Another limitation is the absence of whole gland analysis. We focused only on the index tumour, while contiguous or distant tumors may not have the same profile of expression. Further analyses will be conducted in more samples and assess the downstream effectors on the whole prostatic gland.

TARGETING RHOA/ROCK PATHWAY IN THE TREATMENT OF PROSTATE CANCER

We have demonstrated the critical role of RhoA/ROCK pathway in prostate cancer angiogenesis by inhibiting ROCK activity using fasudil in *in vitro* experiments. The higher expression of RhoA and its downstream effector ROCK2 in tumour front of human prostate cancer specimen indicated its potential role in tumour invasion and aggressiveness. As the role of this pathway in tumour angiogenesis and invasion have also been reported by several studies, our studies further confirmed the multiple roles of RhoA/ROCK pathway in the progress of prostate cancer and their potential value as a target for the treatment of prostate cancer.

CONCLUSIONS AND PERSPECTIVES

Conclusions

The results of our studies clearly demonstrated the multiple roles of RhoA/ROCK pathway in both prostate cancer angiogenesis and aggressiveness. Our main discovers and conclusions are summarized as following:

1. In the first study (Part I), we found fasudil significantly inhibits the key steps of endothelial cell angiogenesis, including proliferation, migration, capillary tube formation and spheroid sprouting in a dose-dependent manner. These effects may due to inhibition of ROCK activity induced by PCa cell secretions. Fasudil may be a useful anti-angiogenic agent and should be investigated further for its potential role in the anti-angiogenic treatment of PCa.

2. In the second study (Part II & III), we identified higher RhoA and ROCK2 expression in prostate tumour front. The correlation of higher RhoA expression with higher Gleason score and higher rate of cancer relapse. This indicated the association of RhoA/ROCK2 pathway with aggressiveness of prostate cancer. The insights described here may provide the foundation for novel therapeutic approaches targeting RhoA/ROCK pathway to inhibit clinically aggressiveness of PCa.

Perspectives

Several studies have implicated the role of RhoA/ROCK pathway in prostate cancer angiogenesis and invasiveness. Our results contributed to confirm their role in these processes. Some of our preliminary results indicated RhoA may play an important role in prostate cancer through ROCK2. But the detailed mechanisms are still unclear and need to be further confirmed. Further studies are still needed to elucidate the dysregulation of upstream regulators and downstream effectors of RhoA/ROCK pathway in prostate cancer as well as their precise actions.

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APPENDIX

ONCOLOGY LETTERS 8: 1907-1911, 2014

The role of the RhoA/Rho kinase pathway in angiogenesis and its potential value in prostate cancer (Review)

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Abstract. Prostate cancer (PCa) remains a major cause of mortality among males in western countries, with little change in mortality rates observed over the past 25 years. Despite recent advances in therapy, treatment options for metastatic castration-resistant disease remain limited. In terms of chemotherapy, only the combination of docetaxel and prednisone has been shown to improve survival in these patients, but duration of response to therapy is short. There is a continuing unmet need for new systemic interventions that act either alone or synergistically with chemotherapy in patients with progressive PCa. Angiogenesis plays a critical role in tumor growth and metastasis in PCa. Several strategies have been used to target angiogenesis; however, it is becoming increasingly apparent that current anti-angiogenic therapies frequently achieve only modest effects in clinical settings. The RhoA/Rho kinase (ROCK) pathway plays a crucial role in the process of angiogenesis in PCa, and studies have demonstrated that ROCK inhibitors decrease VEGF-induced angiogenesis and tumor cell growth. However, further research is required to fully elucidate the molecular mechanisms involved in this pathway, and the potential value of modulating these mechanisms in the treatment of PCa. This study reviews the current understanding of the role of the RhoA/ROCK pathway in the process of angiogenesis in PCa, and the potential of this pathway as a therapeutic target in the future.

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Key words: RhoA, ROCK, angiogenesis, prostate cancer

Contents

1. Introduction

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer-related mortality in males in western countries. Advanced and metastatic stages of the disease were present in 35% of 1,589 patients with PCa diagnosed by autopsy (1). Among those patients with localized cancer who are able to receive radical prostatectomy (RP), ~35% will develop a recurrence (metastatic disease) within the 10 years following surgery (2,3). For those who present at initial diagnosis or progress with advanced or metastatic disease, androgen deprivation therapy (ADT) can be effective. However, the median duration of response to ADT is limited to between 8 months and 3 years (4), and these patients will eventually become castration resistant. Another effective treatment for castration-resistant PCa is chemotherapy, although the median duration of response is only 10.3 months (5). Therefore, there is a clear and urgent need to develop additional systemic interventions for patients with progressive PCa.

2. Role of angiogenesis in prostate cancer

Angiogenic switch. Angiogenesis plays a critical role in PCa progression and metastasis. Without neovascularization, tumor growth will stop at a diameter of 2-3 mm (6). Once tumor cells are able to make their own new blood vessels, they can further expand and metastasize in a process termed the 'angiogenic switch' (7). The angiogenic switch in tumors is due to a shift in the balance towards neovascularization, when pro-angiogenic factors outweigh anti-angiogenic factors (8). Cancer cells and other cells in tumor tissue, such as macrophages and fibroblasts, can secrete pro-angiogenic factors, including vascular

endothelial growth factor (VEGF), basic fibroblast growth factor and interleukin 8, which promote the formation of new blood vessels, causing an increase in microvessel density (MVD) in cancer tissue.

MVD. MVD has been found to be greater in PCa than in benign prostatic hyperplasia (BPH) and normal tissue (9,10). It has been reported that MVD increases with increasing Gleason score in Pca, particularly in the poorly differentiated type (11). The MVD was also significantly correlated with cancer-specific survival in 221 patients with PCa followed for a median of 15 years (12). In other studies, mean MVD was found to correlate with increasing Gleason score and disease progression (from extraprostatic extension to metastasis) in PCa (13,14). Weidner *et al* showed that MVD was significantly higher in PCa samples from patients with metastatic disease, compared with those from patients without metastatic disease (11). Results from another study indicated that PCa angiogenesis correlated with progression after RP in patients with a Gleason score >7 (15). These data highlight the important role of angiogenesis in the progression and metastasis of PCa. MVD counts have also been shown to potentially predict tumor progression from high-grade intraepithelial neoplasia to PCa, as well as outcome in patients undergoing RP (16).

VEGF and angiogenesis in PCa. As tumors undergo an angiogenic switch, many pro-angiogenic growth factors are secreted. Among these, VEGF is the most significant and also the most prominent regulator of physiological angiogenesis. Cells in tumor tissue, including cancer cells, fibroblasts and macrophages, secrete VEGF to stimulate new vessel formation in response to hypoxia (17,18).

Clinical studies comparing PCa with BPH revealed that VEGF expression was correlated with increased levels of angiogenesis, and that this was at least partly mediated by VEGF (10). In PCa, serum levels of the humoral ligand, VEGF, have been found to be significantly higher in patients with metastatic disease, compared with those patients with localized disease or healthy controls (19). Plasma VEGF levels have also been found to be an independent prognostic factor in males with metastatic PCa (20). Peyromaure *et al* compared 17 patients who developed bone metastases after RP with 23 patients who remained disease free and found that the expression of VEGF was significantly higher in the former group (21). Levels of VEGF in serum, plasma or urine are correlated with patient outcome in both localized as well as disseminated PCa (22). In a study of 50 patients with locally advanced disease treated with radical radiotherapy, Green *et al* reported a correlation between higher VEGF expression and worse disease-specific survival (23). In addition, levels of the VEGFR cognate receptor were found to be associated with a poorer grade of tumor differentiation and prognosis in PCa (24). Given these findings, angiogenesis inhibition has been targeted as a strategy to treat PCa. However, it has become increasingly apparent that current anti-angiogenic therapy targeting VEGF elicits only modest effects in clinical settings.

3. Role of the RhoA/Rho kinase pathway in angiogenesis

RhoA and its effector Rho kinase (ROCK)

RhoA and its activation. RhoA is a small guanosine triphosphate hydrolase (GTPase) belonging to the Ras homology (Rho) family. The Rho family of GTPases comprise at least 23 members (25,26), which serve as key regulators of extracellular stimulus-mediated signaling networks involved in a diversity of cellular processes including motility, mitosis, proliferation and apoptosis (27). RhoA promotes actin stress fiber formation and focal adhesion assembly.

Rho GTPases function as molecular switches, cycling between an active GTP-bound conformation and an inactive guanosine diphosphate (GDP)-bound conformation (28,29). When binding with GTP, they interact with downstream effectors to propagate signal transduction (30). Intrinsic phosphatase activity hydrolyzes GTP to GDP, deactivating RhoA function, and this process is accelerated by interaction with GTPase-activating proteins. Conversely, interaction with guanine-nucleotide exchange factors facilitates the exchange of GDP to GTP, which restores the activation of RhoA. The relative affinity difference of the effector molecules between the GTP- and GDP-bound states of the Rho GTPase can be as much as 100-fold, resulting in a highly-specific interaction only with the GTP-bound activated state (Fig. 1). In addition, Rho proteins are also regulated by guanine nucleotide dissociation inhibitors (GDIs), which can inhibit both the exchange of GDP to GTP and the hydrolysis of bound GTP. In the majority of cases, Rho proteins are post-translationally modified at their C-termini by prenylation of a conserved cysteine, and this facilitates their attachment to cell membranes.

ROCK and its function. Rho kinase (ROCK) is a serine/threonine kinase with a molecular mass of ~160 kDa, which has been shown to be the principle downstream target of RhoA. There are two ROCK isoforms: ROCK1 (ROCKβ or p160 ROCK) and ROCK2 (ROCKα or Rho kinase). ROCK1 and ROCK2 show an overall homology of 65% in their amino acid sequence and 92% in their kinase domains. Both ROCK1 and ROCK2 are expressed in vascular endothelial cells (ECs) (31,32).

When bound and activated by RhoA, ROCK translocates from the cytoplasm to the cell membrane, where it increases phosphorylation of the myosin light chain (MLC) of myosin II. This is achieved either by direct phosphorylation, or by phosphorylation of the regulatory myosin-binding subunit of myosin phosphatase (also known as the phosphatase-targeting subunit), which inhibits the phosphatase activity of this molecule (33). This, in turn, enhances actin binding and the actin-induced adenosine triphosphatase activity of myosin, facilitating the interaction of myosin with F-actin, and ultimately cell contractility. ROCK proteins can also phosphorylate cofilin indirectly via LIM kinase, and this facilitates the organization of F-actin into stress fibers and re-arrangement of the actin cytoskeleton (30).

RhoA/ROCK pathway and cell motility. In eukaryotes, organization and reorganization of the cytoskeleton underpins cellular morphology and motility. The actin cytoskeleton is composed of actin filaments and numerous specialized actin-binding proteins. Actin filaments are created by the simple polymerization of actin monomers, regulated dynamically by numerous upstream signaling pathways, notably Rho GTPases (30). A coordinated regulation of the actin network is essential to produce directed cell movement.

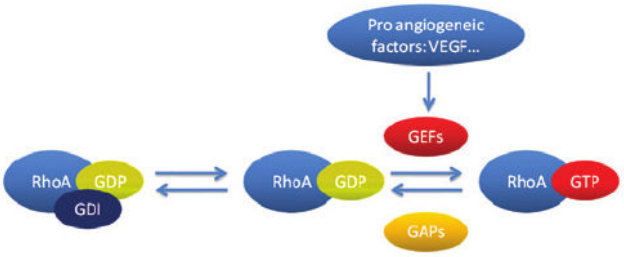

Figure 1. Regulation of RhoA. RhoA functions as a molecular switch, cycling between an active GTP-bound conformation and an inactive GDP-bound conformation. Intrinsic phosphatase activity hydrolyzes GTP to GDP, deactivating RhoA function, and this process is accelerated by interaction with GAPs. Conversely, interaction with GEFs facilitates the exchange of GDP to GTP, which restores the activation of RhoA. RhoA is also regulated by GDIs, which can inhibit the exchange of GDP to GTP. Pro-angiogenic factors may activate RhoA by GEFs. GTP, guanosine triphosphate; GDP, guanosine diphosphate; GAPs, GTPase-activating proteins; GEFs, guanine-nucleotide exchange factors; GDIs, guanine nucleotide dissociation inhibitors; VEGF, vascular endothelial growth factor.

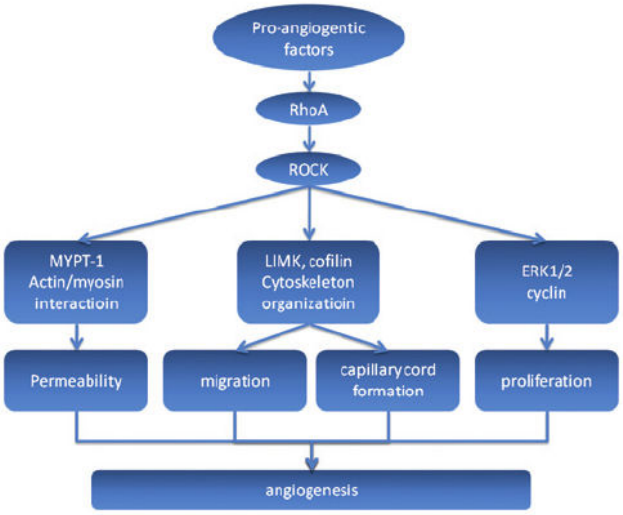

Figure 2. Illustration of the RhoA/ROCK pathway in angiogenesis. Pro-angiogenic factors can activate the RhoA/ROCK pathway then subsequently activate the downstream molecules that take part in the multisteps of angiogenesis. Firstly, MYPT-1 can be activated by ROCK and cause myosin light chain phosphorylation followed by actin-mediated EC contraction, which leads to an increase in the permeability of the basement membrane allowing a new capillary to sprout. Other molecules involved in cytoskeletal organization, such as LIMK and cofilin, are activated and cause ECs to migrate into the extracellular matrix towards the angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of ECs, in which ERK1/2 and cyclin may play a role when activated by ROCK. This is followed by re-organization of ECs to form tubules with a central lumen, which finally reorganize to result in new capillary stabilization. ROCK, Rho kinase; MYPT-1, myosin phosphatase target subunit 1; ECs, endothelial cells; ERK1/2, extracellular signal-regulated kinase 1/2.

During cell movement, RhoA is active at the trailing edge of the cell to promote retraction, while Rac, another member of the Rho family, is active at the leading edge, promoting protrusion. Active RhoA has also been shown to localize at the leading edge of migrating cells (34-36), indicating that RhoA not only acts in retraction, but also regulates protrusion at the front of the cell. Notably, increases in RhoA activity were found to be correlated with increases in protrusion rates, and were synchronous with cell-edge advancement (36). These data highlight the important role of RhoA in cell movement.

Association between the RhoA/ROCK pathway and angiogenesis

Mechanism of angiogenesis. Angiogenesis is a five-step process involving a complex series of events. Firstly, an increase in the permeability of the basement membrane allows a new capillary to sprout. Next, ECs activated by angiogenic factors migrate through the basement membrane into the extracellular matrix, towards the angiogenic stimulus. The leading front of migrating cells is driven by enhanced proliferation of ECs. This is then followed by re-organization of ECs to form tubules with a central lumen, together with the recruitment of periendothelial cells (pericytes) and vascular smooth muscle cells for new capillary stabilization (37). The RhoA/ROCK pathway plays a role in each of these key steps (Fig. 2).

Permeability. The endothelium is a semi-permeable barrier that lines the vasculature, comprising ECs that are connected to each other by interendothelial junctions, consisting of protein complexes organized as tight junctions and adherent junctions. The latter are in the majority (38), and are composed of vascular endothelial (VE) cadherin that associates homotypically with VE-cadherin on adjacent cells. VE-cadherin binds to the actin cytoskeleton. Actin-mediated EC contraction occurs as a result of MLC phosphorylation, and this can cause dysfunction of the endothelial barrier by inducing the formation of small gaps between neighboring cells (39). RhoA, through its downstream effector ROCK, plays a role in endothelial barrier dysfunction by potentiating MLC phosphorylation via inhibition of MLC phosphatase activity. Studies have also confirmed that RhoA contributes to VEGF-induced hyperpermeability in the endothelium (40).

Migration. The formation of stress fibers and cellular contraction is essential for EC migration, and these processes are mediated by Rho GTPases (41). van Nieuw Amerongen *et al* demonstrated *in vitro* that VEGF induces the activation of RhoA and this increase in RhoA activity is necessary for VEGF-induced reorganization of the F-actin cytoskeleton. This process can be inhibited by transfection of ECs with a RhoA dominant-negative mutant vector or by a RhoA inhibitor C3 (42). Zhao *et al* showed that increased expression of RhoA in human umbilical vein ECs significantly enhanced cytoskeletal reorganization of transfected cells, cell migration and angiogenic capacity, which suggests that RhoA plays a key part in these processes *in vitro* (43).

Proliferation. Several lines of evidence suggest that Rho proteins play an important role in normal and cancerous cell growth processes, including G1 phase cell cycle progression and mitogenesis (44). Cytokinesis is a step in mitogenesis which is critical within the cell cycle. In eukaryotic cells, cytokinesis requires an actin and myosin contractile ring, which constricts and cleaves the cell, forming two daughter cells. Inhibition of Rho GTPases prevents the assembly of this contractile ring in a variety of mammalian cells. Expression of constitutively activated Rho GTPases also blocks cytokinesis, suggesting that cycling between the active and inactive forms is required for its function (45).

The role of RhoA signaling in cell survival has been evaluated in several non-EC cell types. Results showed that inhibition of Rho signaling leads to apoptosis via alterations in

cell adhesion and the induction of p53 and other pro-apoptotic proteins, or via ceramide upregulation leading to caspase cleavage and subsequent activation (46,47). Studies have shown that the ROCK inhibitors, fasudil and Y-27632, not only inhibit VEGF-induced cell proliferation, but also reverse the protective effect of VEGF on apoptosis, which results in a decrease in viability of VEGF-stimulated ECs (48,49). Data obtained with these inhibitors have revealed the important role of the RhoA/ROCK pathway in EC proliferation and cell viability.

Morphogenesis. Cultured ECs can undergo marked changes in shape and tube formation that closely imitate pre-capillary cord formation *in vivo* (50). *In vitro* angiogenesis assays found that the mean tube length of the capillary-like tubular structures formed by ECs was reduced by transfection of a RhoA dominant-negative mutant vector, the RhoA inhibitor C3, or the ROCK inhibitor Y-27632 (42). In another study, Zhao *et al* demonstrated that overexpression of RhoA increased the tube length in transfected ECs (43).

4. RhoA/Rho kinase pathway and angiogenesis in prostate cancer

As discussed previously, the RhoA/ROCK pathway participates in the process of angiogenesis in numerous types of cancer, including PCa. Tumor blood vessels always exhibit abnormal structure and function. A study investigating the ECs of mice carrying the transgenic adenocarcinoma of the mouse prostate transgene revealed that the aberrant mechanosensing of extracellular matrix cues and resulting abnormal responses in these cells correlated with a constitutively high level of baseline activity of Rho GTPase and its downstream effector, ROCK (51). These findings highlighted the important role of the RhoA/ROCK pathway in the angiogenesis of PCa. A number of other studies have also demonstrated that RhoA/ROCK pathway inhibitors decrease angiogenesis and cell growth in PCa (52-54).

In an *in vitro* study, Y-27632 inhibited metastatic growth of highly invasive PC3 cells in immunocompromised mice (52). Another ROCK inhibitor, Wf-536, greatly enhanced the *in vitro* inhibition of EC migration, vacuolation, lumen and cord formation, and VEGF- and hepatocyte growth factor-stimulated endothelial sprout formation, when combined with the matrix metalloproteinase inhibitor, marimastat (53). Early treatment with a combination of Wf-536 plus marimastat, with or without paclitaxel, of immunocompromised mice bearing xenotransplants of PC3 cells was associated with significant inhibition of tumor growth and increased tumor necrosis (53). Certain potential anti-angiogenic medications, such as anacardic acid, have been found to inhibit human prostate tumor xenograft angiogenesis by targeting the Rho GTPase signaling pathway (54).

5. Implications for therapy

VEGF antibody bevacizumab has been approved for numerous cancer therapies, such as for colon, lung and kidney cancer. One of its main side effects is hypertension, while ROCK inhibitors have vasodilation effects. For example, fasudil has shown favorable effects in patients with angina (55) and pulmonary hypertension (56) in clinical trials and has been approved for cerebral vasospasm by the inhibition of vessel contraction. Their effects on anti-angiogenesis have also gradually been discovered, as we have reviewed above. It may be feasible to combine the ROCK inhibitor with VEGF antibody in order to enforce their anti-angiogenesis effects while reducing the side effects.

6. Conclusion

Inhibition of angiogenesis has been targeted as a strategy to treat PCa. Until now, the main focus has been on the VEGF pathway. However, it has become increasingly apparent that current anti-angiogenic therapy elicits only modest effects in clinical settings. The RhoA/ROCK pathway plays a crucial role in cancer angiogenesis and should also be a potential target for anti-angiogenic therapy. Additional studies are required to elucidate the molecular mechanisms of the RhoA/ROCK pathway in PCa angiogenesis, and the potential value of modulating these mechanisms for the treatment of PCa.

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Abstract

Prostate cancer remains a major cause of mortality among males in western countries. Treatment options for metastatic castration-resistant disease remain limited. There is a continuing unmet need for new systemic interventions in patients with progressive prostate cancer. RhoA/Rho-associated protein kinases (ROCK) are key regulators of the cytoskeleton and have been implicated in PCa angiogenesis and tumour invasion. In the first study (Part I), we investigated the anti-angiogenic effects of fasudil, a ROCK inhibitor, on PCa-induced angiogenesis in vitro. Proliferation of PCa-conditioned human umbilical vein endothelial cells (HUVECs) was assessed using a bromodeoxyuridine (BrdU) assay, and migration was assessed with a wound healing assay. In vitro angiogenesis of PCa-conditioned HUVECs was evaluated by tube formation and a spheroid sprouting assay. Fasudil inhibited PCa-induced endothelial cell proliferation, and also decreased PCa-induced endothelial cell migration. In the *in vitro* angiogenesis assay, tube formation and spheroid sprouts were significantly inhibited at fasudil in a dose dependent manner. Western blotting results showed that expression of phosphorylated myosin phosphatase target subunit 1 (MYPT-1) was significantly lower after fasudil treatment, confirming that fasudil inhibited ROCK activity in these model systems. In the second study (Part II & III), we evaluated RhoA expression and activity in a total of 34 paraffin embedded and 20 frozen prostate specimens, respectively, obtained from 45 patients treated with radical prostatectomy for clinically localized cancer. The expression patterns of RhoA were tested by immunohistochemical staining and Western blotting, and further compared between the tumour centre, tumour front and distant peritumoral tissue. RhoA activity was assessed by G-LISA. Our results showed an increasing gradient of expression from the centre to the periphery of index tumour foci. RhoA expression was indeed significantly higher at the tumour front as compared to tumour centre, using immunohistochemistry ($p=0.001$). Gleason score was significantly higher in the patients with higher RhoA expression in both the tumour front and tumour centre ($p=0.044$ and 0.039, respectively). After a median follow-up of 52 months, the rate of PSA relapse was higher in patients with a higher RhoA expression at the tumour front (62.5% vs 35%), although the difference was not significant ($p=0.089$). There was no association between RhoA expression and PSA, pathological stage. We also found ROCK2 expression, but not ROCK1 expression, was significantly higher in the prostate cancer tumor front. In conclusion, we found fasudil significantly inhibits the key steps of endothelial cell angiogenesis, including proliferation, migration, capillary tube formation and spheroid sprouting, in a dose-dependent manner. These effects may due to inhibition of ROCK activity induced by PCa cell secretions. We also identified higher RhoA and ROCK2 expression in human prostate tumour front. The correlation of higher RhoA expression with higher Gleason score and higher rate of cancer relapse. This indicated the association of RhoA/ROCK2 pathway with aggressiveness of prostate cancer. The insights described here may provide the foundation for novel therapeutic approaches targeting RhoA/ROCK pathway to inhibit angiogenesis and clinically aggressiveness of PCa. Fasudil may be a useful anti-angiogenic agent and should be investigated further for its potential role in PCa treatment.

Key words: prostate cancer, RhoA, ROCK, angiogenesis, invasion