Enhanced Expression of Fibroblast Growth Factor Receptor 3 in Human Skin Cancer Cells

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Abstract: Tumor microenvironments cause a wide range of responses in both cancer cells and surrounding host cells, inducing gene expressions of growth factors and their receptors to lead such as angiogenesis and changes of metabolic switch. To examine the possible mechanism by which cancer cells increase growth and survival during tumor progression, we used human squamous cell carcinoma derived cell line DJM1 as a malignant tumor model. Here we report that the cancer cells in the avasculature area in the mouse xenografts were able to induce the expression of fibroblast growth factor receptor 3 (FGFR3) known as an oncogene. *In vitro* experiments confirmed that serum-starvation induced the marked increased expression of FGFR3 in DJM1 cells. As a significant finding, two alternatively spliced isoforms of FGFR3, FGFR3IIIb and FGFR3IIIc, expressed in normal epithelial cells or in mesenchymal cells respectively, were both increased in DJM1 cells under the serum-starved conditions. Moreover, ectopic expression of FGFR3IIIc in DJM1 cells *in vitro* greatly enhanced anchorage-dependent and -independent growth in response to FGF2, suggesting that dysregulation of FGFR3 expression has a role in tumor growth and survival *in vivo*. These findings provide an insight into the mechanism of FGFR3-dependent tumor progression and a basis for the development of cancer therapies.

Keywords: FGFR3, metastatic skin cancer, serum starvation, hypoxia, tumor environment.

INTRODUCTION

Hypoxic and low-nutrient environments are commonly found in the central region of solid tumors [1]. These microenvironments cause a wide range of responses at both systemic and cellular levels including gene expressions, angiogenesis, changes of metabolic switch, and apoptosis [2, 3]. Since solid tumors endure hypoxia before the formation of microvessels, adaptive responses to hypoxia and insufficient nutrients are crucial steps in tumor progression [4, 5]. However, little is known about how tumors can overcome growth factor depletion and manage to proliferate. Aberrant expression of fibroblast growth factors (FGFs) and their receptors (FGFRs) are found in multiple cancers, providing a strong indication of their role in cancer development [6-8]. Of the four different human FGFRs, activating mutations of FGFR3 involved in skeletal malformation syndromes have been identified as a causal gene in multiple myeloma (MM) [9, 10]. FGFR3 mutations have also been identified in gastric and colorectal cancers [11], in virus-associated squamous cell carcinomas [12], and in bladder and cervical carcinoma [13, 14]. These findings suggested that FGFR3 plays an important role in cancer development, and as a result particular attention has been given to FGFR3. The expression of FGFR isoforms is temporally and spatially regulated in embryos and in normal adult organs. FGFR3 has two different transmembrane-type isoforms, FGFR3IIIb and FGFR3IIIc, which have distinctive ligand-specificities. Both FGFR3IIIb and FGFR3IIIc bind to FGF1, however, FGFR3IIIc has higher affinity to FGF2 than FGF1, and FGFR3IIIb does not bind to FGF2 [15, 16]. They are produced by the alternative usage of exons, exon 8 (IIIb) or exon 9 (IIIc), which encode the C-terminal half of the IgIII domain [17]. In the previous studies, FGFR3IIIc was present in non-epithelial cell populations, whereas FGFR3IIIb was restricted to epithelial cell populations [18, 19].

To examine the possible mechanism by which cancer cells increase growth and survival during tumor progression, we used the human squamous cell carcinoma derived cell line DJM1 as a malignant tumor model. We found that the cancer cells in the avasculature area in the mouse xenografts were able to induce the expression of FGFR3 known as an oncogene [20-24]. FGFR3 was expressed in the intratumoral area of the xenografts where a transcription factor, hypoxia-inducible factor-1α (HIF-1α), regulated by low oxygen tension and nutrients [5] was also expressed. However, the molecular mechanism that causes the enhanced expression of FGFR3 in the skin cancer cells and the role of the FGFR3 expression, remains unclear. Therefore, we examined whether FGFR3 expression at both mRNA and protein levels is

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1877-3826/09

enhanced *in vitro* in response to HIF-1α. *In vitro* experiments showed that FGFR3 expression under hypoxic conditions was increased at protein levels but not at mRNA levels. However, FGFR3 expression at both protein and mRNA levels was increased under serum-starved conditions. By the analysis of quantitative real time RT-PCR, we found that both alternatively spliced isoforms, FGFR3IIIb and FGFR3IIIc were dramatically induced under serum-starved conditions. Usually, normal epithelial cells express only FGFR3IIIb isoform, therefore in this study, we sought the biological function of FGFR3IIIc in DJM1 cells utilizing an ecdysone-inducible expression system.

MATERIALS AND METHODS

Materials-Human recombinant FGF1 and FGF2 were generous gifts from Drs. R. Sasada and T. Kurokawa (Takeda Chemical Industries, Ltd., Osaka).

Cell Culture, Hypoxic and Serum-Starved Conditions

The human skin-derived squamous cell carcinoma cell line was from Dr. H. Katayama. DJM1 cells were grown in high glucose Dulbecco's Modified Eagle medium (DMEM, Nissui Pharmaceutical Co.) containing 10% (v/v) fetal bovine serum (FBS, JRH Biosciences Co.), 2mM L-glutamine, 50U/ml penicillin and 50μg/ml streptomycin (10%FBS-DMEM) at 37°C in a humidified atmosphere of 95% air -5% CO_2. The cells were seeded at a density of $1x10_6$ cells/100 mm culture dishes. After 24h, the plates were replaced with 10 ml of media with or without FBS (serum-starved) and incubated under normoxic (5% CO_2, balanced with air) or hypoxic (1% O_2, 5% CO_2, balanced with N_2) condition in a humidified incubator (Model 9200E, Wakenyaku Co.) at 37°C.

Xenograft Model

Female athymic nude mice (4 to 6-weeks old) were obtained from Shimizu Laboratory Supplies Co. Mice were anesthetized and xenografts were established by injection of $1.0x10_6$ DJM-1 cells per 200μl sterile phosphate-buffer saline (PBS) under the dorsal skin of the mice. 6 weeks after inoculation, xenografts were excised, fixed in Zinc Fixative (BD Biosciences) overnight, processed and embedded in paraffin wax.

Immunohistochemistry

Tumor sections (5μm) were stained with primary antibodies, that were 5μg/ml anti-CD31 antibody (BD Bioscience), 20μg/ml anti-keratin-14 antibody (Lab Vision), 2μg/ml anti-HIF-1α antibody and 2μg/ml anti-FGFR3 antibody (Santa Cruz Biotechnology). All of the slides were subsequently washed several times in PBS with 0.1% Tween 20 and were incubated with Histofine Simple Stain Mouse MAX-PO (R) or Histofine Simple Stain Mouse MAX-PO (Rat) (Nichirei) at room temperature for 45min. Immunoreactive species were detected with the DAB Substrate Kit for peroxidase (Vector Laboratories). Sections were counterstained with hematoxylin.

RNA Extraction and Real Time RT-PCR

Total RNA was extracted using Isogen (Nippon Gene) and first strand cDNA was synthesized using SuperScript™ First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR was performed using Power SYBR Green PCR Master Mix & RT-PCR and Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The calculation method used for relative quantitation is the standard curve method. Detection of human 18S rRNA (NM022551), used as internal control, was performed using RPS18 primers from Takara Bio, Inc. The other primers used for amplification were: FGFR2IIIb forward: 5'-TGG ATC AAG CAC GTG GAA AAG AAC GG-3', FGFR2IIIb reverse: 5'-CAG AGC CAG CAC TTC TGC ATT GGA AC-3', FGFR3IIIb&IIIc forward: 5'-CGG ACG GCA CAC CCT ACG TT-3', FGFR3IIIb reverse: 5'-TCG GCC ACG CCT ATG AAA TTG GT-3', FGFR3IIIc reverse: 5'-CAA AGG TGA CGT TGT GCA AGG AGA GA-3'.

Ectopic Expression of FGFR3IIIc

To construct pIND-FGFR3IIIc, human FGFR3IIIc cDNA was cloned into pIND ecdysone-inducible expression vector (Invitrogen). DJM1 cells were cotransfected with pIND-FGFR3IIIc and pVgRXR by the lipofection method. Stable expression clones were first selected with 200μg/ml of Zeocin (InvivoGen) and 400μg/ml of G418 (Nacalai tesque). Clones were then screened for expression of FGFR3IIIc by incubating with 5μM of Ponasterone A (PA) for 30h followed by immunoblotting analysis. PA was dissolved in ethanol.

Anchorage-Dependent Cell Growth Assay

DJM1-IIIc cells were seeded into 24-well plates ($1.0x10^4$ cells/well) and grown in DMEM containing 1% bovine serum albumin (BSA, serum-free medium). The expression of FGFR3IIIc was induced by 5μM of PA for 30h. Then, the cells were stimulated by FGF1 or FGF2 (10ng/ml) or not stimulated and incubated for 7 days. Cell numbers were counted with a Coulter Counter (Beckman Coulter Inc.). All experiments were performed in three wells and the results are expressed as means ± standard deviations (S.D.). These experiments were performed at least three times.

Anchorage-Independent Soft-Agar Colony Formation Assay

Colony formation assays were carried out in 35 mm dishes. Each dish contained 2ml of 0.72% agar in 10% FBS-DMEM on the bottom layer. The top layer consisted of 2ml of 0.36% agar in 10% FBS-DMEM containing $5x10^4$ DJM1-IIIc cells. Treatment with 5μM of PA, 10ng/ml of FGF1, or FGF2 was initiated at the same time when cells were seeded. After incubated for 25 days, their colonies were stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) at 37°C quantify living cells. The next day, microphotographs were taken under phase contrast microscopy (Eclipse TE300 and IP Lab, Nikon) and stained colonies were counted.

Preparation of Cell Lysates

DJM1 cells were grown in 10% FBS-DMEM or serum-free medium under normoxic or hypoxic condition for the indicated period. Then the cells were washed with cold PBS twice and solubilized in lysis buffer (1% Nonidet P-40, 10mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 10μg/ml aprotinin, 1mM phenylmethylsulfonyl fluoride). After 10min on ice, cell lysates were cleared by

centrifugation at 15,000rpm for 10min. The protein concentration in each sample was quantified by the DC protein assay (Bio-Rad).

Western Blot Analysis

Cell lysates were analyzed by immunoblotting with an anti-FGFR3 antibody or an anti-HIF-1α antibody (Santa Cruz Biotechnology). Samples were electrophoresed through a 7.5% polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes. After saturation in blocking buffer (5% dried non-fat milk in 1xTris-buffered saline, TBS), the blots were incubated for 1h at room temperature with primary antibodies diluted with blocking buffer containing 0.05% Tween 20. After washing, incubation with a horseradish peroxidase conjugated secondary antibody was performed for 1h at room temperature, and reactive proteins were visualized by enhanced chemiluminescence (Super Signal West Pico, Thermo Fisher Scientific Inc.). Some immunoblots were stripped with stripping buffer (Thermo) and reprobed.

RESULTS

Up-Regulation of FGFR3 Expression in DJM1 Cells *in vivo*.

Human squamous cell carcinoma derived cell line DJM1 was established from the dermal metastases in the malignant trichilemmal cyst of the cancer patient [25, 26]. Therefore, DJM1 cells were analyzed for their tumorigenic potential *in vivo*. We implanted DJM1 cells subdermally into the back of nude mice. Tumorigenicity was 100% for all the mice examined. After 6 weeks of inoculation, the xenografts were excised and the histological analysis of the tumor sections was performed, using antibodies against keratin 14, hypoxia inducible factor-1α (HIF-1α), a blood vascular endothelial marker CD31, and FGFR3 (Fig. **1**). Mouse epidermis and tumor were stained with an anti-keratin 14 antibody (Fig. **1B**). Blood vessels that invaded into the tumor were stained with an anti-CD31 antibody, indicating that DJM1 cells induced angiogenesis *in vivo* (Fig. **1C**, arrows). Stromal cells that were not stained with an anti-keratin 14 antibody infiltrated into the tumor tissue, surrounding the blood vessels (Fig. **1B**, arrow heads). Furthermore, increased expression of HIF-1α, a transcription factor induced by low oxygen tension or low nutritional conditions, was detected in the intratumoral area apart from the blood vessels (Fig. **1D**). In the hypoxic region of the tumor where HIF-1α was expressed, the intense expression of FGFR3 was observed (Fig. **1E**). These results suggest that the tumor environmental factors such as low oxygen tension or low nutrition may have induced the increased expression of FGFR3.

Up-Regulation of FGFR3 Expression in DJM1 Cells *In Vitro*

To examine whether the low oxygen or nutrition/growth factor-deprivation regulates the expression of FGFR3, DJM1 cells were incubated *in vitro* under three different cultural conditions, such as normoxia, hypoxia, and serum-starved conditions. After the indicated time of incubation, FGFR3 protein expression was analyzed with SDS-PAGE followed by Western blotting. As shown in Fig. (**2**), expression of FGFR3 protein was increased under hypoxic condition (1% O_2) by 24h incubation, whereas induction of FGFR3 in

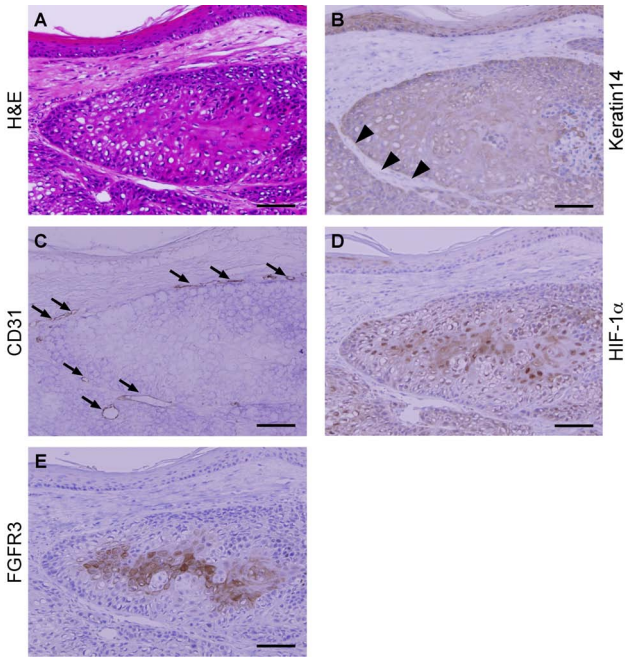

Fig. (1). Up-regulation of FGFR3 expression in DJM1 cells *in vivo*. DJM1 xenografts stained with hematoxylin and eosin (**A**) or the epithelial marker keratin-14 (**B**, brown). The arrowheads point to the invasion of stromal cells into the tumor tissue (**B**). The arrows point to the blood vessels stained with CD31 (**C**, brown). The hypoxic area was stained with HIF-1α (**D**, brown). FGFR3 was expressed in the hypoxic area of tumor tissue (**E**, brown). Scale bars; 100μm.

normoxic condition was weaker than that in hypoxic condition (Fig. **2A**). The several bands of FGFR3 are consistent with variable N-terminal glycosylation. Although HIF-1α was induced in the hypoxic condition after 8h incubation, the delayed induction of FGFR3 suggests that HIF-1α may not be a direct inducer for FGFR3 up-regulation.

Next, we analyzed FGFR3 mRNA expression during hypoxic and normoxic conditions by quantitative real time RT-PCR. FGFR3 has the IIIb and IIIc isoform which are produced by alternative splicing of the carboxy-terminal half of the third Ig domain. Expression of each FGFR3IIIb and IIIc was normalized with that of ribosomal RNA RPS18. As shown in Fig. (**2B**), both FGFR3 isoforms were not significantly induced at mRNA levels during normoxia or hypoxia for the indicated time periods. Further, we analyzed FGFR2IIIb mRNA and protein expression (data not shown), but neither mRNA level nor protein level of FGFR2 was changed significantly. FGFR2IIIc mRNA was not detected (data not shown). These results suggest that the up-regulation of FGFR3 protein during hypoxia might be due to increased translation or protein stabilization.

Next, we examined whether FGFR3 expression is up-regulated under serum-starved conditions. For serum-starved condition, cells were grown in DMEM containing 1% BSA. After 24h incubation, FGFR3 protein expression was analyzed by Western blotting (Fig. **2C**). Compared to that in the presence of 10% FBS, the expression of FGFR3 protein was strongly up-regulated under serum-starved conditions. We next analyzed mRNA levels of FGFR3 isoforms by

Real-time RT-PCR (Fig. **2D**). DJM1 cells were incubated under serum-starved conditions for the indicated periods and total RNA was extracted. As shown in Fig. (**2D**), mRNA of FGFR3IIIb was induced 4.5-fold of control level after 8h and kept high levels for 48h under serum-starved condition. Similarly, FGFR3IIIc mRNA was induced as high as 4-fold of control level after 8h and kept high levels. The increase of mRNA levels in both FGFR3 isoforms was correlated with the increased expression of FGFR3 protein (Fig. **2C**). The mRNA of FGFR2IIIb was not increased significantly.

Taken together, these data show that FGFR3 expression is induced in response to the change in the environment *in vitro* such as serum-starvation and hypoxic conditions. In addition, these results suggest that the serum-starved induction of FGFR3IIIc, which is not usually expressed in normal epithelial cells, may allow DJM1 cells to respond to new types of FGFs such as FGF2 and to mediate new signals into the cells.

FGF2 Enhances Anchorage-Dependent and -Independent Growth of DJM1-IIIc Cells Expressing FGFR3IIIc

Therefore, we wanted to investigate whether the induced expression of FGFR3IIIc has an effect on growth stimulation or cell survival in DJM1 cells. To gain insight into the biological function, we employed the ecdysone-inducible gene expression system to see the effects of FGFR3IIIc expression. As described in the "Material and methods section", the cells expressing FGFR3IIIc by the addition of Ponasterone A (PA) were cloned. DJM1-IIIc, one of the clones selected was treated with the indicated concentrations (0.1-10μM) of PA for 30h. PA induces FGFR3IIIc expression in a concentration-dependent manner (Fig. **3A**).

First, we tested the effect of the induced FGFR3IIIc expression on the anchorage-dependent growth of DJM1-IIIc cells treated with or without PA (Fig. **3B**). DJM1-IIIc cells not treated with PA, do not express FGFR3IIIc. Addition of FGF1 stimulated anchorage-dependent growth as well as it did in the cells expressing FGFR3IIIc (-, FGF1: 153±10%). However, stimulation with FGF2 was similar to FGF1 in cells not treated with PA (-, FGF2: 175±7%). On the other hand, PA-treated DJM1-IIIc cells expressed FGFR3IIIc and addition of FGF1 or FGF2 stimulated cell growth of DJM1-IIIc cells expressing FGFR3IIIc compared to no addition as control (100%) (Fig. **3B**) (+, FGF1: 156±21%, FGF2: 269±10%). Increased cell number with FGF2 stimulation was much greater than that with FGF1 (1.7 fold). These

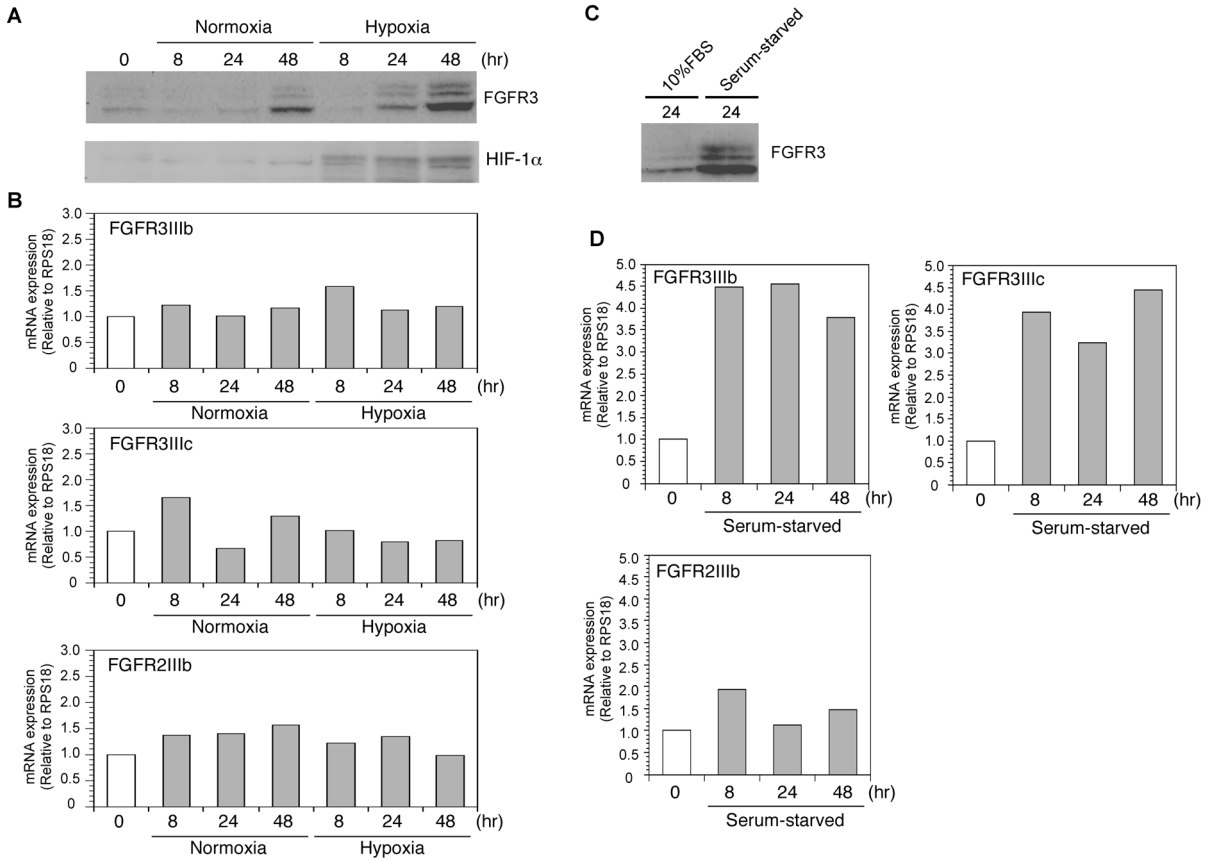

Fig. (2). Up-regulation of FGFR3 expression in DJM1 cells *in vitro*. (**A**) FGFR3 expression in DJM1 cells cultured under normoxic or hypoxic conditions *in vitro* Cell lysates from DJM1 cells grown in normoxic or hypoxic conditions (1% O^2) after incubation for indicated periods, were analyzed with antibodies against FGFR3 or HIF-1α. (**B**) Real-time RT-PCR analysis of FGFRs. DJM1 cells were incubated as described in (**A**). Total RNA was extracted and the expression levels of FGFR3IIIb, FGFR3IIIc, and FGFR2IIIb were determined by quantitative real-time RT-PCR. (**C**) Western blot analysis of FGFR3 in DJM1 cells cultured in the presence of 10% FBS or in serum-starved conditions. After 24h seeding the cells, the medium was changed to DMEM containing 1%BSA (serum-starved) or unchanged (10% FBS). Cell lysates from DJM1 cells grown for 24h were collected and analyzed. (**D**) Real time RT-PCR analysis of FGFRs. DJM1 cells were incubated as in (**C**) for indicated periods, total RNA was extracted and the expression levels of FGFR3IIIb, FGFR3IIIc, and FGFR2IIIb were determined using real time RT-PCR.

results demonstrate that FGFR3IIIc mediates the FGF2 signal to increase the anchorage-dependent growth of DJM1-IIIc cells.

In order to further demonstrate the effects of FGFR3IIIc expression, we utilized an anchorage-independent colony formation soft agar assay (Fig. **3C**). DJM1-IIIc cells were seeded with or without 5μM of PA, and in the presence of 10ng/ml FGF1, FGF2 or the absence in the medium containing 0.36% agar on the top of the bottom agar layer. Subsequently, the cells were incubated for 25 days and the colonies were stained with MTT to detect living cells. Compared to no addition, FGF1 and FGF2 did not increase colony formation of DJM1-IIIc cells not expressing FGFR3IIIc (-, FGF1: 90±19%, FGF2: 91±16%). When DJM1-IIIc cells were treated with PA to induce the expression of FGFR3IIIc, FGF1 slightly stimulated colony formation, and furthermore, FGF2 greatly stimulated colony formation (+, FGF1: 123±21%, FGF2: 276±37%) (Fig. **3C**).

Together, these results demonstrate that expression of FGFR3IIIc induces greater response to FGF2 in anchorage-dependent and -independent cell growth in DJM1 cells.

DISCUSSION

Overexpression of FGFRs occurs frequently in human tumors and correlates with a poor prognosis and shorter survival time for the patient [7, 8]. DJM1 cells were originally established from a patient who died from metastasis of malignant skin squamous cell carcinoma [25, 26]. As shown in Fig. (**1**), DJM1 xenografts induced tumor angiogenesis. In the present study, we found that FGFR3 protein expression is induced in the central area of DJM1 xenografts. Since solid tumors endure hypoxia before the formation of microvessels, adaptive responses to hypoxia and insufficient nutrients are crucial steps in tumor progression [5]. We hypothesized that FGFR3 expression is induced in the DJM1 tumor environment and may play a role in tumor progression.

To clarify first whether FGFR3 expression is regulated in an HIF-1α-dependent manner, we incubated DJM1 cells under hypoxia and normoxia *in vitro* and analyzed FGFR3 expression at both protein and mRNA levels (Fig. **2**). Under hypoxia, HIF-1α protein was up-regulated by 8h incubation. On the other hand, FGFR3 protein was also up-regulated by 24h and continued to increase by 48h. Next we analyzed FGFR3 mRNA using quantitative real time RT-PCR.

In the present study, DJM1 cells induced FGFR3 expression under hypoxic, serum-starved conditions *in vitro*. Under hypoxia, FGFR3 protein was increased, whereas mRNA of FGFR3 was not increased. These results suggest that translation of FGFR3 was stimulated in hypoxia or protein stability was increased. On the contrary, mRNA of FGFR3 was markedly increased under serum-starved conditions. These results suggest that the enhanced expression of FGFR3 is induced through transcriptional and translational regulation that is dependent on the environmental conditions.

In normal cells, the expression of alternatively spliced isoforms of FGFRs is regulated in a tissue-specific manner. Usually, IIIb type of FGFRs, FGFR2IIIb and FGFR3IIIb are expressed in normal epithelial cells and IIIc type of FGFRs,

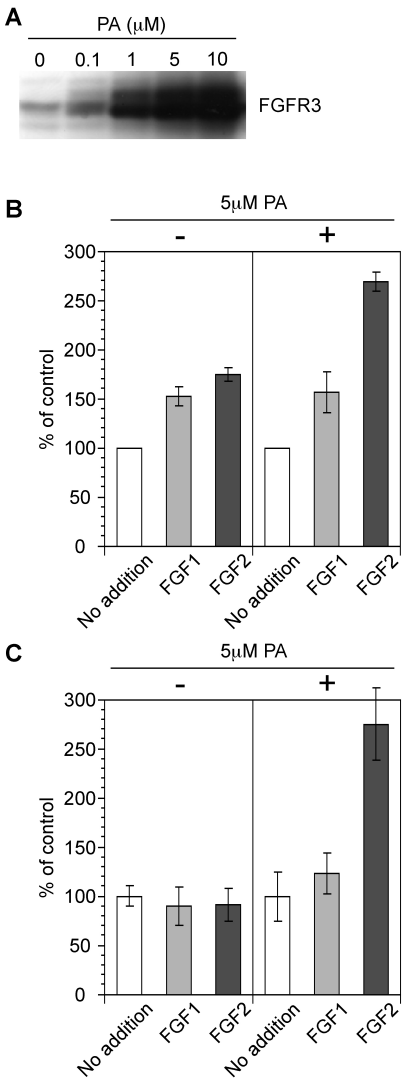

Fig. (3). Expression of FGFR3IIIc stimulates anchorage-dependent and -independent cell growth in response to FGF2. (**A**) PA induces the expression of FGFR3IIIc in DJM1-IIIc cells. Treatment of DJM1-IIIc cells with the indicated concentrations of PA for 24h induces FGFR3IIIc protein. The cellular proteins were extracted and analyzed by Western blot using an anti-FGFR3 antibody. (**B**) FGF2 stimulates anchorage-dependent growth in DJM1-IIIc cells expressing FGFR3IIIc. The expression of FGFR3IIIc in DJM1-IIIc cells was induced by the addition of 5μM PA (+) or not induced by no addition (-). Then, the cells were stimulated by FGF1 or FGF2 (10 ng/ml), or not stimulated (no addition) and incubated for 4 days. The cell numbers were counted and mean cell number (± S.D.) of triplicate determinations are expressed as % of control wells. (**C**) FGF2 stimulates anchorage-independent growth in DJM1-IIIc cells expressing FGFR3IIIc. DJM1-IIIc cells were seeded in 10%FBS-DMEM containing 0.36% agar. FGFR3IIIc was induced by 5μM PA (+) or not induced (-). Then, the cells were treated with 10 ng/ml of FGF1, FGF2, or without (no addition) and incubated for 25 days. Colonies were stained with MTT to detect living cells. The number of colonies was counted and mean colony number (± S.D.) of triplicate determinations are expressed as % of control wells.

FGFR2IIIc and FGFR3IIIc are expressed in mesenchymal cells [17, 19]. They differ in ligand-specificity. FGFR3IIIc

has higher affinity to FGF2 than FGF1, and FGFR3IIIb does not bind to FGF2 [15, 16]. These results suggest that the dysregulation of expression and alternative splicing of FGFR3 mRNA may occur in the tumor environment thus influencing tumor growth and survival.

In our study, serum starvation in DJM1 cells induced growth arrest and markedly increased expression of FGFR3IIIb and IIIc. However, it did not increase FGFR2IIIb expression, suggesting that expression of FGFR2 and FGFR3 is differently regulated and that FGFR2 and FGFR3 play significantly different functional roles in epithelial cancer cells (Fig. **2**).

Most transformed cells of human origin have lost the dependence on adhesion for survival and have acquired the ability to proliferate in an anchorage-independent manner [27, 28]. For example, constitutive levels of FGFR1 or FGFR3 have been shown to be essential for FGF-stimulated anchorage-independent growth of human adrenal adenocarcinoma cells [29]. In the present study, to assess the biological function of FGFR3IIIc in skin metastatic cancer cells, we used anchorage-dependent and -independent growth assays. As shown in Fig. (**3**), ecdysone-induced expression of FGFR3IIIc in skin cancer cells enhanced cell proliferation and anchorage-independent growth in response to FGF2. In the present study, we showed that PA-induced expression of FGFR3IIIc in DJM1-IIIc cells markedly promoted anchorage-dependent and -independent growth in response to FGF2. Most transformed cells of human origin have lost the dependence on adhesion for survival and have acquired the ability to proliferate in an anchorage-independent manner [28]. Because anchorage-independent growth correlates with tumor growth *in vivo* as well as metastatic potential, our results suggest that the up-regulation of FGFR3IIIc in human epithelial cancers may play an important role in cancer metastasis. In the present study, exogenously added FGF1 or FGF2 did not increase the anchorage-independent growth of the DJM1-IIIc cells, which are not expressing FGFR3IIIc (Fig. **3C**). This suggests either that endogenously expressed FGFR2IIIb and FGFR3IIIb are not capable of stimulating the anchorage-independent growth or that these receptors are already stimulated with endogenously expressed FGF1 or FGF2. However, the induced expression of FGFR3IIIc in DJM1-IIIc cells treated with PA greatly increased the anchorage-independent growth of the cells. Together with our results, the enhanced expression of FGFR3IIIc contributes to tumor growth and survival in the tumor environment *in vivo*. These findings indicate that FGFR3IIIc stimulated by FGF2 is capable of inducing growth and survival in the skin squamous carcinoma cells.

FGF1 and FGF2 have potent biological activities implicated in malignant tumor development [30]. It has been reported that high levels of FGF2 are expressed in metastatic human esophageal tumor cells [31] and a poor prognosis in human pancreatic cancer patients who express FGF2 [32]. The main source of FGF2 in the DJM1 tumor and other malignant tumor cells *in vivo* may be the tumor associated stromal cells. We have shown that DJM1 tumors also increased the transcription factor HIF-1α expression *in vivo* (Fig. **1D**). Hypoxia changes cellular gene expression through stabilization of HIF-1α, including vascular endothelial growth factor (VEGF) [5, 33]. This may help to explain that the xenografts of DJM1 cells developed blood vessels and stromal cells infiltrated into the tumor tissue (Fig. **1B, C**). Cancer cells can alter their adjacent stroma to the activated tumor stroma by producing a range of stroma-modulating growth factors such as VEGF and proteases [34]. The activated stroma can then release a myriad of angiogenic factors including VEGF and FGF2.

In conclusion, we showed that the expression of FGFR3 is up-regulated in response to the tumor environment *in vivo* and that FGF signaling through the up-regulated FGFR3 plays an important role in tumor growth and survival which may lead to metastasis. This study highlights its importance as a target for antitumor therapy.

ACKNOWLEDGEMENTS

We greatly thank Drs. Reiko Sasada and Tsutomu Kurokawa (Takeda Chemical Industries, Ltd.) for human recombinant FGF1 and FGF2 and Dr. Hiroshi Katayama for DJM1 cells. The work was supported by the Foundation for the Scientific Research Center in Kyoto Sangyo University from the Ministry of Education, Science, and Culture of Japan.

ABBREVIATIONS:

FGFR3 = fibroblast growth factor receptor 3

FGF2 = fibroblast growth factor-2

HIF-1 = hypoxia inducible factor-1

PA = Ponasterone A

REFERENCES

[1] Dang, C.V.; Semenza, G.L. Oncogenic alterations of metabolism. *Trends Biochem. Sci.*, **1999**, *24*, 68-72.

[2] Folkman, J. Anti-angiogenesis: new concept for therapy of solid tumors. *Ann. Surg.*, **1972**, *175*, 409-416.

[3] Semenza, G.L.; Roth, P.H.; Fang, H.M.; Wang, G.L. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.*, **1994**, *269*, 23757-23763.

[4] Towle, H.C. Metabolic regulation of gene transcription in mammals. *J. Biol. Chem.*, **1995**, *270*, 23235-23238.

[5] Semenza, G.L. Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer*, **2003**, *3*, 721-732.

[6] Acevedo, V.D.; Ittmann, M.; Spencer, D.M. Paths of FGFR-driven tumorigenesis. *Cell Cycle*, **2009**, *8*, 580-588.

[7] Feng, S.; Wang, F.; Matsubara, A.; Kan, M.; McKeehan, W.L. Fibroblast growth factor receptor 2 limits and receptor 1 accelerates tumorigenicity of prostate epithelial cells. *Cancer Res.*, **1997**, *57*, 5369-5378.

[8] Kornmann, M.; Ishiwata, T.; Matsuda, K.; Lopez, M.E.; Fukahi, K.; Asano, G.; Beger, H.G.; Korc, M. IIIc isoform of fibroblast growth factor receptor 1 is overexpressed in human pancreatic cancer and enhances tumorigenicity of hamster ductal cells. *Gastroenterology*, **2002**, *123*, 301-313.

[9] Plowright, E.E.; Li, Z.; Bergsagel, P.L.; Chesi, M.; Barber, D.L.; Branch, D.R.; Hawley, R.G.; Stewart, A.K. Ectopic expression of fibroblast growth factor receptor 3 promotes myeloma cell proliferation and prevents apoptosis. *Blood*, **2000**, *95*, 992-998.

[10] Richelda, R.; Ronchetti, D.; Baldini, L.; Cro, L.; Viggiano, L.; Marzella, R.; Rocchi, M.; Otsuki, T.; Lombardi, L.; Maiolo, A.T.; Neri, A. A novel chromosomal translocation t(4; 14)(p16.3; q32) in multiple myeloma involves the fibroblast growth-factor receptor 3 gene. *Blood*, **1997**, *90*, 4062-4070.

[11] Jang, J.H.; Shin, K.H.; Park, J.G. Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. *Cancer Res.*, **2001**, *61*, 3541-3543.

[12] Shotelersuk, V.; Ittiwut, C.; Shotelersuk, K.; Triratanachat, S.; Poovorawan, Y.; Mutirangura, A. Fibroblast growth factor receptor 3 S249C mutation in virus associated squamous cell carcinomas. *Oncol. Rep*., **2001**, *8*, 1301-1304.

[13] Cappellen, D.; De Oliveira, C.; Ricol, D.; de Medina, S.; Bourdin, J.; Sastre-Garau, X.; Chopin, D.; Thiery, J.P.; Radvanyi, F. Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. *Nat. Genet.*, **1999**, *23*, 18-20.

[14] Rieger-Christ, K.M.; Mourtzinos, A.; Lee, P.J.; Zagha, R.M.; Cain, J.; Silverman, M.; Libertino, J.A.; Summerhayes, I.C. Identification of fibroblast growth factor receptor 3 mutations in urine sediment DNA samples complements cytology in bladder tumor detection. *Cancer*, **2003**, *98*, 737-744.

[15] Chellaiah, A.T.; McEwen, D.G.; Werner, S.; Xu, J.; Ornitz, D.M. Fibroblast growth factor receptor (FGFR) 3. Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. *J. Biol. Chem*., **1994**, *269*, 11620-11627.

[16] Ornitz, D.M.; Xu, J.; Colvin, J.S.; McEwen, D.G.; MacArthur, C.A.; Coulier, F.; Gao, G.; Goldfarb, M. Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem*., **1996**, *271*, 15292-15297.

[17] Johnson, D.E.; Lu, J.; Chen, H.; Werner, S.; Williams, L.T. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol. Cell. Biol.*, **1991**, *11*, 4627-4634.

[18] Wuechner, C.; Nordqvist, A.C.; Winterpacht, A.; Zabel, B.; Schalling, M. Developmental expression of splicing variants of fibroblast growth factor receptor 3 (FGFR3) in mouse. *Int. J. Dev. Biol.*, **1996**, *40*, 1185-1188.

[19] Murgue, B.; Tsunekawa, S.; Rosenberg, I.; deBeaumont, M.; Podolsky, D.K. Identification of a novel variant form of fibroblast growth factor receptor 3 (FGFR3 IIIb) in human colonic epithelium. *Cancer Res.*, **1994**, *54*, 5206-5211.

[20] Kanai, M.; Goke, M.; Tsunekawa, S.; Podolsky, D.K. Signal transduction pathway of human fibroblast growth factor receptor 3. Identification of a novel 66-kDa phosphoprotein. *J. Biol. Chem.*, **1997**, *272*, 6621-6628.

[21] Choi, D.Y.; Toledo-Aral, J.J.; Lin, H.Y.; Ischenko, I.; Medina, L.; Safo, P.; Mandel, G.; Levinson, S.R.; Halegoua, S.; Hayman, M.J. Fibroblast growth factor receptor 3 induces gene expression primarily through Ras-independent signal transduction pathways. *J. Biol. Chem*., **2001**, *276*, 5116-5122.

[22] Wang, Q.; Green, R.P.; Zhao, G.; Ornitz, D.M. Differential regulation of endochondral bone growth and joint development by FGFR1 and FGFR3 tyrosine kinase domains. *Development*, **2001**, *128*, 3867-3876.

[23] Webster, M.K.; Donoghue, D.J. Enhanced signaling and morphological transformation by a membrane-localized derivative of the fibroblast growth factor receptor 3 kinase domain. *Mol. Cell. Biol*., **1997**, *17*, 5739-5747.

[24] Onose, H.; Emoto, N.; Sugihara, H.; Shimizu, K.; Wakabayashi, I. Overexpression of fibroblast growth factor receptor 3 in a human thyroid carcinoma cell line results in overgrowth of the confluent cultures. *Eur. J. Endocrinol*., **1999**, *140*, 169-173.

[25] Katayama, H.; Yamane, Y.; Furukawa, Y.; Kitajima, Y.; Yaoita, H.; Fujijyo, Y.; Sakai, S. Malignant trichilemmal cyst secreting a cell adhesion factor. A case report for lymphatic metastases. *Hifuka-no Rinsho*, **1989**, *31*, 1721-1724.

[26] Katayama, H.; Yamane, Y.; Furukawa, Y.; Kitagawa, S.; Nakamura, Y.; Yoshino, K. Activation of focal adhesion kinase in detached human epidermal cancer cells and their long-term survival might be associated with cell surface expression of laminin-5. *Acta Derm. Venereol.*, **2008**, *88*, 100-107.

[27] Schwartz, M.A. Integrins, oncogenes, and anchorage independence. *J. Cell Biol*., **1997**, *139*, 575-578.

[28] Hanahan, D.; Weinberg, R.A. The hallmarks of cancer. *Cell*, **2000**, *100*, 57-70.

[29] Estes, N.R., 2nd; Thottassery, J.V.; Kern, F.G. siRNA mediated knockdown of fibroblast growth factor receptors 1 or 3 inhibits FGF-induced anchorage-independent clonogenicity but does not affect MAPK activation. *Oncol. Rep*., **2006**, *15*, 1407-1416.

[30] Billottet, C.; Elkhatib, N.; Thiery, J.P.; Jouanneau, J. Targets of fibroblast growth factor 1 (FGF-1) and FGF-2 signaling involved in the invasive and tumorigenic behavior of carcinoma cells. *Mol. Biol. Cell*., **2004**, *15*, 4725-4734.

[31] Kitadai, Y.; Onogawa, S.; Kuwai, T.; Matsumura, S.; Hamada, H.; Ito, M.; Tanaka, S.; Yoshihara, M.; Chayama, K. Angiogenic switch occurs during the precancerous stage of human esophageal squamous cell carcinoma. *Oncol. Rep*., **2004**, *11*, 315-319.

[32] Yamanaka, Y.; Friess, H.; Buchler, M.; Beger, H.G.; Uchida, E.; Onda, M.; Kobrin, M.S.; Korc, M. Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage. *Cancer Res*., **1993**, *53*, 5289-5296.

[33] Carmeliet, P.; Jain, R.K. Angiogenesis in cancer and other diseases. *Nature*, **2000**, *407*, 249-257.

[34] Mueller, M.M.; Fusenig, N.E. Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat. Rev. Cancer*, **2004**, *4*, 839-849.

Received: February 05, 2009 Revised: April 09, 2009 Accepted: April 10, 2009