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Proliferation and Migration of Tumor Cells in Tapered Channels

Yuan Wan, MD, PhD^{1,2,3,#}, Deepika Tamuly, MS², Peter B. Allen, PhD⁴, Young-tae Kim, PhD^{2,3}, Robert Bachoo, MD^{5,6,7}, Andrew D. Ellington, PhD⁴, and Samir M. Iqbal, PhD^{1,2,3,8,9,*}

¹Nano-Bio Lab, University of Texas at Arlington, Arlington, TX 76019, USA

²Department of Bioengineering, University of Texas at Arlington, Arlington, TX 76019, USA

³Nanotechnology Research and Education Center, University of Texas at Arlington, Arlington, TX 76019, USA

⁴Institute for Cell and Molecular Biology, University of Texas at Austin, Austin, TX, USA

⁵Annette G. Strauss Center for Neuro-Oncology

⁶Department of Neurology, University of Texas Southwestern Medical Center, Dallas, TX, USA

⁷Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA

⁸Department of Electrical Engineering, University of Texas at Arlington and University of Texas Southwestern Medical Center at Dallas, University of Texas at Arlington, Arlington, TX 76019, USA

⁹Joint Graduate Committee of Bioengineering Program, University of Texas at Arlington and University of Texas Southwestern Medical Center at Dallas, University of Texas at Arlington, Arlington, TX 76019, USA

Abstract

Tumor cells depict two deviant tendencies; over-proliferation and vigorous migration. A tapered channel device is designed and fabricated for *in vitro* studying inhibited proliferation and migration of human glioblastoma (hGBM) cells when exposed to a novel aptamer targeting epidermal growth factor receptors (EGFR). The device is integrated with controlled ambient and microscope for providing real-time and quantitative characterization of the tumor cell behavior *in vitro*. The results show that hGBM cells loose proliferation and motility when exposed to the anti-EGFR aptamers. The aptamer directly inhibits and blocks EGF-induced EGFR phosphorylation. This also reduces the ability of cells to remodel their internal structure for invasion through narrow constrictions. This provides a framework for possible studies on efficacy of other inhibiting molecules.

Introduction

Glioblastoma multiforme (GBM) is the most aggressive of the gliomas, which is also the most common in humans (Holland 2000). The median survival of GBM patients is less than a year (Krex et al. 2007). Treatment of GBM is not effective due to the invasion of malignant cells into surrounding normal brain and induction of local tumor recurrence following initial treatment (Demuth and Berens 2004). Therefore, inhibition of GBM cell

*To whom correspondence should be addressed: Samir M. Iqbal, Ph.D., 500 S. Cooper St, M.S. 19072, Room #217, University of Texas at Arlington, Arlington, TX 76019, smiqbal@uta.edu.

#Now at Mawson Institute, University of South Australia, Mawson Lakes, Adelaide, SA 5095, Australia

migration and proliferation is considered important towards effective treatment (Liang et al. 2009).

2D and 3D *in vitro* assays are mainly used for cell migration studies. A number of proteins such as laminin, myelin, fibronectin and collagen are immobilized on substrate surfaces for studying their roles in cell migration (Aubert et al. 2008; Enam et al. 1998; Giese et al. 1996; Koshikawa et al. 2000). These assays have heavy emphasis on molecular mechanism of cell migration influenced by various proteins. However, these devices discount the fact that cancer cells can change their morphology by cytoskeletal rearrangement and squeeze into and through confined extracellular spaces (Demuth and Berens 2004). 3D assays including gel invasion, microfluidic device, organotypic tissue culture and Boyden chamber do mimic the *in vivo* microenvironment, but real-time quantification of cell migration and proliferation is hard to achieve, especially for studies of single cells. Other issues such as 3D morphogenesis within an ECM, control of microenvironment, precise flow regulation, real-time imaging, etc. are also challenging (Chung et al. 2010). It is thus essential to design new assays for real-time quantification of cell migration *in vitro*.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) oncogene, frequently overexpressed in many human malignancies. When various ligands, such as epidermal growth factor (EGF), or transforming growth factor- α (TGF- α) selectively bind to the EGFR extracellular domain, the receptor forms a dimer, and further triggers receptor autophosphorylation through tyrosine kinase activity (Citri and Yarden 2006). Autophosphorylation initiates the recruitment and phosphorylation of several further intracellular substrates that promote cell proliferation, angiogenesis, apoptosis resistance and migration (Mendelsohn 2004). The expression level of EGFR in cancer cells can be 10 to 100 times higher than that in normal cells (Carpenter 1983; Carpenter and Cohen 1979; Wan, et al. 2011). The frequent overexpression of EGFR in tumors therefore supports receptor-mediated targeted therapeutic approaches (Lorimer et al. 1996). Efficiently blocking the interactions between EGF and EGFR to inhibit cell proliferation, tumor growth, and migration can be a novel therapeutic approach. It has been reported that an anti-EGFR aptamer can block EGF binding to A431 cells (epidermoid carcinoma cell line). Western blot analyses show that anti-EGFR aptamer can block EGF-induced EGFR phosphorylation (Li et al. 2011). The anti-EGFR aptamer may provide a tangible impact on human glioblastoma (hGBM) cell proliferation and migration *in vitro*.

Here, a simple biomimetic cell proliferation and migration assay is developed on a chip that provides temporal observation of cell behavior in constricted space *in vitro*, meanwhile providing quantitative means to measure effects of anti-EGFR aptamers on blocking EGFR phosphorylation. The changes in migration and proliferation of GBM cells can be directly observed in this device and quantification of temporal behavior of cells provide insights into EGF signaling pathways. The data shows that anti-EGFR aptamer significantly inhibits hGBM cell proliferation and suggest that it may be possible to prevent their migration *in vivo* with strong possibility to eliminate metastasis.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Aptamer Preparation

The isolation of anti-EGFR RNA aptamer ($K_d = 2.4$ nM) has been reported before. (Osborne et al. 1997; Wan et al. 2010). Briefly, it was isolated by performing iterative selection of binding species against purified human EGFR (R&D systems, Minneapolis, MN). The

sequence for anti-EGFR aptamer was (5'-GGC GCU CCG ACC UUA GUC UCU GUG CCG CUA UAA UGC ACG GAU UUA AUC GCC GUA GAA AAG CAU GUC AAA GCC GGA ACC GUG UAG CAC AGC AGA-3') and that for mutant aptamer was (5'-GGC GCU CCG ACC UUA GUC UCU GUU CCC ACA UCA UGC ACA AGG ACA AUU CUG UGC AUC CAA GGA GGA GUU CUC GGA ACC GUG UAG CAC AGC AGA-3'). We used 2'-fluoro modified CTP and UTP for aptamer synthesis with Epicentre Durascribe *in vitro* transcription kits (Illumina, Madison, WI), so all aptamers used in the experiments were nuclease resistant.

hGBM Cell Culture with EGF and Aptamers

hGBM cells were obtained from consenting patients at the University of Texas Southwestern Medical Center at Dallas, Texas, with the approval of IRB. These were stably transduced with a lentivirus expressing *m-cherry* fluorescent protein. The cells were suspended in serum-free Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, consisting of 20 ng/ml mouse EGF (Peprotech, Rocky Hill, NJ), 1× B27 supplement (Invitrogen, Carlsbad, CA), 1× Insulin-Transferrin-Selenium-× (Invitrogen), and gentamycin (Invitrogen). The cells were plated at a density of 30,000 cells per well in 8 mm diameter 24 well plate. Cells were divided into four groups for culture with combinations of EGF and the two aptamers. The four groups were: only EGF (EGF+ve), no EGF (EGF-ve), with EGF and anti-EGFR aptamer (EGF+Anti-Apt), and with EGF and mutant aptamer (EGF+Mut-Apt). Cells were cultured at 37 °C in 5% CO₂ for 72 h, and the culture media were changed after every 24 h (in vitro half-life of aptamer is about 5 to 15 hours). After 72 hrs of culture, 20 images were randomly taken from each well. The images were analyzed with *Image-Pro Plus* software. The total number of cells was counted automatically, and the cell densities (number of cells per mm²) were calculated. In the cell migration study, cell culture followed the same protocol; except that the cell seeding density was higher (60,000 cells per well in the 24 well plate).

BrdU Immunostaining for Examining Cell Proliferation

A solution of 1 mM Bromodeoxyuridine (BrdU) was added into each ml of cell culture media, and was incubated with cells at 37 °C for 1 h, then culture media were removed and cells were fixed with 4% paraformaldehyde in 1× PBS at 4 °C for 1 h (Zink et al. 1998). After removing paraformaldehyde, the samples were washed with 1× PBS twice. For BrdU immunostaining, the samples were incubated with washing solution (0.5% triton in 1× PBS) at room temperature (RT) for 30 min. The samples were then treated with ice-cold 1N HCl for 10 min and 2N HCl for 30 min at 37°C respectively. After removing the acidic solution, the samples were washed with 1× PBS three times. The samples were treated with blocking solution (4% goat serum in wash solution) for 1 h at RT. Pre-cold primary BrdU antibody (1:500 mIgG1) was incubated with samples at 4 °C overnight. The samples were again washed with wash solution thrice, and incubated with the secondary antibody, goat anti-mIgG1 Dylight 488 (Jackson ImmunoResearch Laboratories, West Grove, PA) at RT for 1 h. After removing the secondary antibody, the samples were again washed thrice with wash solution. Finally, 4',6-diamidino-2-phenylindole (DAPI) was dissolved in PBS to 1 µg/ml concentration and incubated with cells at RT for 20 min (Hamada and Fujita 1983). Samples were washed with 1× PBS three times and stored in fresh 1× PBS at 4 °C for imaging. For analysis, 200 representative images were randomly taken without any overlaps in the imaged regions (50 images for each group × 4 groups). The DAPI stained cell nuclei were counted with *ImageJ* software, and the cell densities (number of cells per mm²) were calculated.

Monitoring of hGBM Cell Migration

The tapered-channel polydimethylsiloxane (PDMS) devices had microchannels with inlets of 20 µm on proximal side. The microchannels gradually tapered down to 5 µm on distal

side. The channels were 15 μm in height and were 580 μm long. The devices were fabricated using soft lithography and the transparency of PDMS provided a direct modality to study and record cell migration and growth behavior in situ. The PDMS devices were punched, sterilized, treated with oxygen plasma for 30 min and then bonded to sterilized glass coverslips. The glass surfaces were coated with 10 $\mu\text{g}/\text{ml}$ laminin which would increase hGBM cells' radial migration (Demuth and Berens, 2004). Thirty thousand live cells were seeded in the proximal side reservoir and were allowed to migrate through the microchannels. Once hGBM cells entered into microchannels, these vigorously migrated towards the shorter ends and exited to the distal side reservoir. Images of migrating hGBM cells were taken every 12 hours for 5 days. Differential Interference Contrast (DIC) and fluorescence micrographs were overlapped for quantification. The number of microchannels that contained cells and those through which cells transited were counted and the transit times of cells were calculated.

Results and Discussion

Cell Proliferation and Division

Cells were evenly seeded in the proximal side reservoirs of devices and treated for 72 h at 37 °C. The treatments were: EGF+ve, EGF-ve, EGF+Anti-Apt and EGF+Mut-Apt. After BrdU and DAPI staining, cell proliferation and division was monitored and imaged (Fig. 1). Figures 1(A)–(D) show DAPI stained nuclei in each group; green fluorescent signals from nuclei show dividing cells. The results show 828.9 (S.D: 111.3), 167.8 (S.D: 40.2), 381.2 (S.D: 77.3), and 766.3 (S.D: 100.1) hGBM cells per mm^2 in each group respectively (Fig. 1(E)). Cell density of EGF+Anti-Apt group was less than that of EGF+ve or EGF+Mut-Apt groups, and the later two groups did not show difference in cell density. With EGF+Anti-Apt group as reference, T-tests were run for both EGF+ve and EGF+Mut-Apt groups. The P-value was less than 0.01. The cell density of EGF-ve group was the lowest. The data indicated cell division rates varied in the four groups. BrdU staining images were used to further characterize cell division status. There were 37.3% (S.D: 3.4), 23.2% (S.D: 5.5), 28.3% (S.D: 3.8), and 37.8% (S.D: 4.5) cells undergoing division in respective groups. Even if we consider varying proliferation rates for different groups, the cells in EGF+Mut-Apt group could keep normal division; while cells in the EGF+Anti-Apt group showed inhibited cell division, and the division rate was close to that of the group with EGF-ve, but less than the other two groups ($P < 0.01$).

It has been demonstrated that hGBM cell lines with medium and low levels of EGFR grow slowly without EGF. In EGF-ve group, after 72 h, not only the cell number was far less than the other three groups, the cell division rate was also the lowest. However, when suitable concentration of EGF was present, it stimulated their growth. On the contrary, cell division is known to be slowed down or to cease altogether in serum-free conditions (Engelbraaten et al. 1993; Lund-Johansen et al. 1990; Westphal et al. 1988). As we mentioned before, EGF binding to EGFR triggers a series of intracellular pathways that can stimulate cell proliferation. EGF also has an anti-apoptotic effect (Woodburn 1999), and it can induce cyclin D₁ for cell cycle progression from G₁ (Perry et al. 1998). As expected, in EGF+ve groups, cells number increased significantly during the culture period, and the division rate was higher than that for EGF-ve group. Interference in EGF activity by the anti-EGFR aptamer was then the only reason for inhibited cell growth and division. High-concentration (100 μM) of anti-EGFR aptamer showed inhibition of autophosphorylation of EGFR as well. In EGF+Anti-Apt group, the cell density and division rate were lower than those for EGF+ve culture and EGF+Mut-Apt control groups. In EGF+Mut-Apt group, the mutant aptamer without the correct structure could not recognize and bind to EGFR, and therefore it could not inhibit cell proliferation. No significant difference in cell density was thus found between EGF+ve and EGF+Mut-Apt groups.

It is known that hGBM cells frequently express EGFRvIII, which can phosphorylate without EGF binding. Figure 1B shows that the cells in EGF-ve group still had some division activities. The same autophosphorylation activities also existed in other three groups. It is possible that the EGFRvIII was responsible for the remaining proliferation under EGF-ve conditions or conditions containing the anti-EGFR aptamer. If we consider the 23.2% cell division rate in EGF negative group as reference and normalize the data, anti-EGFR aptamer inhibition effect shows only 5.1% of cells undergoing division compared to 14.1% and 14.6% for those in EGF+ve and EGF+Mut-Apt groups, respectively.

Morphology of Cancer Cells

Cell morphology has close relationship to its proliferation and division rate. Although cells were cultured on non-natural surfaces, the morphology of cell in different groups still showed significant differences. The morphology of aptamer treated cells closely resembled the EGF-ve control cells. This was expected, as cell spreading, migration and morphology on the surface was mediated by its surrounding extracellular matrix (ECM) (Fukushima et al. 1998). It has been found that laminin, fibronectin and type IV collagen can promote cell attachment and migration (Friedlander et al. 1996), and hGBM cells also express these ECM proteins. So the content of these ECM proteins on cell surface might affect cell attachment and migration on laminin coated surface. In EGF+ve and EGF+Mut-Apt groups, cell proliferation rates were higher than those for the other two groups. With sufficient ECM proteins, cells could easily attach on the surface and migrate. In EGF-ve and EGF+Anti-Apt groups, cell proliferation and division were inhibited or interfered, that resulted in a discernable change in their respective morphology.

Cell morphology images were taken after the first 12 h culture and at the end of culture (Fig. 2). Images were taken randomly, so the images provide an unbiased survey of the population. Figs. 2(A), 2(C), 2(E) and 2(G) show cells incubated in EGF+ve, EGF-ve, EGF +Anti-Apt and EGF+Mut-Apt respectively, at 37 °C for 12 h. Figs. 2(B), 2(D), 2(F) and 2(H) show cell morphology after 72 h culture in respective groups. The cells were fixed before imaging. In EGF+ve and EGF+Mut-Apt groups, hGBM cells showed astrocyte-like morphology with multiple long thin processes extending from the cell body. Most of the cells did spread well on the laminin coated surface (Figs. 2(A) and 2(G)). On the contrary, in EGF-ve and EGF+Anti-Apt groups, although long thin processes formed, cells spread less on the substrates, and cells maintained rounded shapes (Fig. 2(C) and 2(E)). After 72 h culture, in EGF+ve and EGF+Mut-Apt groups, cells further spread on the surface; in certain regions, few cells even overlapped each other (Fig. 2(B) and 2(H)). However, in EGF-ve group, cell bodies further shrank and formed humps (Fig. 2(D)). Most of the cells showed bipolar morphology, while long thin processes were still observable. In EGF+Anti-Apt group, cell morphology was very much the same in shape to the first two groups but their spreading was limited compared to the two groups (Fig. 2(F)). This can be explained in terms of a few EGF that did make it EGF-EGFR complex, even in the presence of Anti-Apt. However, the internalization of lesser number of EGF indeed showed in similar shape but limited spreading. Understandably, in EGF-ve group, cells did not show any proliferation and thus showed behavior as observed.

Cell Migration through Microchannels

Cancer cells remarkably deform their cell shape to squeeze through narrow spaces during migration. Considering this factor as a cell behavior criterion, we designed tapered channels. The channel size gradually decreased over fixed distance (from 20 micrometers width to 15, 10, 8 and 5 micrometers). This simulated the migration of cancer cells in space constricted environment. Cells are seeded in the proximal end reservoir and morphology was studied during the course of migration towards the distal end reservoir. The taper design allowed

monitoring of a single cell morphology change while travelling towards the distal end reservoir, forcing the cells to use intracellular mechanisms to overcome space constrictions during migration. The *in vitro* platform presented here can be used for variety of single cell migration studies to understand cellular mechanisms against other therapeutic agents.

Cells were seeded on the proximal side of the microchannels and were cultured with 20 ng/ml mouse EGF until cells migrated into at least 10 channels which took around 48 hours. At this point, the original culture medium containing EGF was removed and media containing EGF+ve, EGF-ve, EGF+Anti-Apt and EGF+Mut-Apt were added into respective devices. After 5 days of tracking, the number of channels just containing cells and the number of channels where cell passed through to the distal end were counted. We found number of tapered channels that showed complete passage of cells significantly varied between the four groups (Fig. 3). There were 96.4% such channels for EGF+ve case (54 of 56), 14.1% of channels showed passage for EGF-ve group (9 of 64), while 35.2% (25 of 71) and 95.1% (58 of 61) of the channels showed passage for EGF+Anti-Apt and EGF+Mut-Apt groups. It is important to note that the number of cells that passed through also varied between the four groups. The EGF+ve or EGF+Mut-Apt channels were full of cells all the way from proximal to distal ends just after only 24–36 hours of migration (average: 12.7, S.D.: 2.1, and average: 12.5, S.D.: 2.7, respectively). This indicated vigorous proliferation and migration of cells (Fig. 3(A) and 3(D)), and also revealed that mutant aptamer had no significant effect on hGBM cell migration. On the contrary, in the channels with EGF-ve or EGF+Anti-Apt there were very few cells in the channel (average: 2.8, S.D.: 1.1, and average: 4.3, S.D.: 1.8, respectively). Even after 100 h, in these two groups of channels few cells aggregated at the 20 μ m wide segment, and very few cells adapted shape to fit the 5 μ m distal end (Fig. 3(B) and 3(C)). We also found that when cells arrived at 15 and 10 μ m wide sections, these lacked sufficient morphological flexibility to move forward and to enter into narrower 5 μ m wide part. Some cells even moved back to the wider region of the channels (Fig. 4(B) and 4(C)). This indicated that cells without EGF or inhibited with anti-EGFR aptamer lost their normal morphological flexibility. These two kinds of cells could easily adapt to 20 μ m channel but very few cells adapted to the smaller size channel (less than 15 μ m). In addition, it also took them longer to transit. On the contrary, cells in EGF+ve or EGF+Mut-Apt groups could pass through the whole channel quickly. This showed these hGBM cells maintained enough deformability to adapt to surrounding constrictions.

The channel transit time of cells in each group was also different. Here, the total time was recorded from when the cell first entered the channel all the way to when it passed out of the distal end. In EGF+ve and EGF+Mut-Apt groups, cells could traverse the whole channel within 24 to 36 h (Fig. 4(A) and 4(D)). There were 148 and 140 cells respectively on the distal side, for these two groups. However, cells in distal side reservoir were composed of immigrant cells and their newly divided passage cells that were not discernable. So the exact number of cells which passed through the channel in these two groups was difficult to enumerate. On the other hand, in EGF-ve and EGF+Anti-Apt groups, only 10 and 28 cells, respectively, passed through the channels in 96 hours or even more. Thus our results are conservative: cell migration rate might have been slower if we had treated cells with EGF-ve or EGF+Anti-Apt through the whole experiment. We compared the cell number in the channels, the morphological flexibility, and the transit time among each group. We found cells treated with EGF+Anti-Apt were inhibited from entering, slowed down in transit and their morphological flexibility reduced due to aptamer-mediated inhibition of cellular pathways clearly disrupting proliferation and mobility.

ECM proteins and cell adhesion molecules play a key role in cell migration. Laminin, collagen type IV, integrins, etc. have been reported to stimulate hGBM cell migration (Demuth and Berens 2004). A great variety of methods aiming to inhibit cell migration on

different levels have been studied (Hauck et al. 2001; Lakka et al. 2004; Loftus et al. 2009; Tamura et al. 1999; Tysnes et al. 1996). It is known that activation of tyrosine kinase (e.g. by EGF) can trigger a series of downstream pathways, including phospholipase C- γ (PLC- γ), mitogen-activated protein kinase (MAPK), and factor receptor tyrosine (FAK). These stimulate cell migration via reorganizing actin cytoskeleton, initiating the asymmetric motile phenotype and modulating integrin adhesive function (Jorissen et al. 2003). Attenuated EGFR signaling can inhibit EGF-dependent migration. The anti-EGFR aptamer inhibits EGF induced autophosphorylation, disturbs EGFR signaling pathways, and inhibits cell migration. In this work, we did not add TGF- α , platelet derived growth factor, or fibroblast growth factor to stimulate cell growth and still observed clear differences in growth, proliferation and mobility between EGF+ve and EGF-ve cases. We may thus discount the effects of small amount of autocrine growth factors, and assume that hGBM cell proliferation and migration depended primarily on EGF in these experiments.

Conclusion

Anti-EGFR aptamer can intercept the RTK signal pathway by blocking EGF autophosphorylation effects upon binding to EGFR, and therefore inhibits hGBM cells proliferation to one third of their normal level. Meanwhile, anti-EGFR aptamer treated cells lose their normal transformation and migration ability. Compared to other groups with EGF treatment, these cells spend 2–3 times longer to completely pass through tapered microchannels in a PDMS device. The low cell number in the channel further demonstrates that these lack the typical EGF induced rates of proliferation and migration. The work clearly shows that the anti-EGFR has *in vitro* inhibition effect on hGBM cells proliferation and migration. We suggest that this assay to measure cancer cells ability to invade small spaces is a novel and direct approach that can help probe new cancer therapeutics.

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Figure 1.

The density and division proportion of hGBM cells in each group. Cells were evenly divided into four groups, and incubated with EGF+ve, EGF-ve, EGF+Anti-Apt and EGF+Mut-Apt at 37 °C for 72 h. Cells were treated with BrdU and DAPI staining. (A) to (D) show DAPI stained cell nuclei and green fluorescent nuclei (BrdU stained) show dividing cells. (E) shows the average cell density (per mm²) in each group. * P < 0.01 between EGF-ve and the others; ** P < 0.01 between EGF+Anti-Apt and the others; *** not significant between EGF+Mut-Apt and EGF+ve. (F) shows proportion of dividing cells in each group respectively. *

$P < 0.05$ between EGF-ve and the others; ** $P < 0.05$ between EGF+Anti-Apt and the others; *** not significant between EGF+Mut-Apt and EGF+ve.

Figure 2. Cell morphology after the four treatments. (A), (C), (E) and (G) show cells incubated in EGF+ve, EGF-ve, EGF+Anti-Apt and EGF+Mut-Apt at 37 °C for 12 h, respectively. (B), (D), (F) and (H) show cell morphology after 72 h culture at 37 °C of respective group.

Figure 3.

Cell migration through the microchannels. (A), (B), (C) and (D) show cell migration in the channels containing EGF+ve, with EGF-ve, EGF+Anti-Apt and EGF+Mut-Apt; cells in all groups were cultured at 37 °C for 96 h. (E) depicts the number of channels (as percentage of total number of channels) through which the cells passed to the distal ends.

Figure 4. Cell migration through the microchannels. Cells were cultured at 37 °C for 24 to 96 h. (A), (B), (C) and (D) show cells spent different times to pass through the microchannels with EGF+ve, with EGF-ve, EGF+Anti-Apt and EGF+Mut-Apt respectively.