

Intracellular Cell Selection

Introduction

This disclosure relates to the selection and isolation of mammalian cells from a heterogeneous population of mammalian cells by affinity binding of intracellular antigens by
5 ligands, typically antibody fragments, and the use of magnetic particles to select said cells.

Background

Selective cell separation is an essential method in experimental biology and medicine. It is an increasingly important process that is driven by the ever increasing demands for sensitivity, selectivity, yield, time and economy of the process. The most popular aim for cell separation
10 is to achieve complete removal of the target population from a cell mixture. However, for some applications, a secondary aim may be to recover the selected cells for study for additional manipulation. Additionally, the purity of separation may be more important than the yield of the selected cells, or vice versa. Thus, the aims for cell separation determine the method that is chosen to achieve the separation. Other factors to take into account when
15 selecting the separation technique are the processing time, viability of residual cells, and exposure to non-medically approved reagents.

There are a number of cell separation techniques which can be broadly classified into two categories: techniques based on size and density, and techniques based on affinity (chemical, electrical, or magnetic) (Radisic et al, 2006).

20 Magnetic separation techniques are a common method in many laboratories and have been broadly used for targeted drug delivery, imaging and bioseparation such as in selective cell separations, antibody purification, protein affinity purification, immunoprecipitations, protein fractionations, organelle isolations, total nucleic acid purification and polyadenylated mRNA purification (Saiyed et al., 2003; Nord et al., 2001; Stark et al., 1988; Suzuki et al., 1996;
25 Weissleder et al., 1997).

The magnetic cell separation technique consists of magnetic beads coated with antibodies specific for cell surface antigens on the desired cells. When the antibody-magnetic beads are exposed to a mixed cell population, they attach to the surface of the desired cells via antibody-antigen interaction. The desired cell subpopulation can then be separated in the
30 presence of a strong magnetic field. The antibodies used in these applications are typically

monoclonal antibodies specific for cell surface antigens. However, antibody fragments such as single chain antibodies (scFv) have also been successfully coupled to magnetic beads and used in a variety of applications such as phage display libraries of scFv and in imaging of tumours (McConnell et al., 1999; Nord et al., 2001; Han et al., 2006).

5 Single VH and VL domains are the smallest functional modules of antibodies required for antigen binding. ScFv are a fusion of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of 10 to 25 amino acids. Their small size conveys them with distinct advantages over whole antibodies, in particular they have rapid pharmacokinetics, greater tumour penetration and lower immunogenicity
10 than intact IgG, F(ab')₂, or Fab (Yokota et al., 1992). They have also shown to be particularly useful in clinical applications involving the selective delivery of radionucleotides to tumours (Huston et al., 1996; Reisfeld et al., 1996; Laske et al., 1997). The scFv may be assembled from the variable regions of particular monoclonal antibodies (Huston et al., 1988; Brinkmann et al., 1991) or made de novo from phage display libraries (Winter et al., 1994;
15 Chester et al., 1994). They retain the specificity of the original immunoglobulin, and in many applications outperform whole IgG in construction speed, production yield, and engineering flexibility, as they can be easily expressed in bacterial expression systems. Indeed, many scFv have been produced in bacteria with good yields, either as insoluble inclusion bodies in *Escherichia coli* (Huston et al., 1988) or by secretion into the periplasm (Skerra et al.,
20 1993) or into the culture supernatant (Takkinen et al., 1991).

A major caveat of magnetic cell separation is that it is only applicable to cells that can be separated by their specific surface antigens. This can be particularly limiting when the cell specific markers are mainly found intracellularly and are not readily available on the cell surface. Indeed, in most cancers, the cancer markers of interest are predominantly expressed
25 inside the cells and therefore cannot be used for cell separations. The inherent difficulties surrounding cell internalisation of antibodies have made it difficult so far to use intracellularly expressed antigens in cell separations. Whole monoclonal antibodies cannot be internalised or be expressed in cells due to the reducing environment of the cell.

However, scFv are routinely stably expressed in cells and have been reportedly been able to
30 internalise into cells with the help of carrier mediated systems such as transduction peptides (Futaki et al., 2001; Avignolo et al., 2008; Niesner et al., 2002; Ma et al., 2011). In addition, recent studies using magnetic particles have shown that it is possible to internalise these

particles into cells and control their location by means of an external magnet (Tseng et al., 2009). Moreover, they have been used in cancer therapeutics as a means to direct the cells containing anti-cancer agents to a desired location on the cancer cells by means of an external magnetic field, thus, promising to reduce the cytotoxic effects of drugs by increasing the bioavailability of therapeutic compounds at the site of action and improving pharmacokinetics (Cinti et al., 2011). Magnetic beads have also been used as a means to internalise nucleic acids (magnetofection) (Scherer et al., 2002) and small peptides into cells (Han et al., 2006) and have been previously coupled to cationic peptides (Smith et al., 2002) to enhance their uptake in vivo and allow their use for MRI scanning (Stark et al., 1988; Suzuki et al., 1996; Weissleder et al., 1997). Additionally they have been coupled to drugs for magnetic drug targeting in patients with solid tumours (Lubbe et al., 1996; Alexiou et al., 2000). Furthermore, these magnetic particles do not rely on receptors or other cell membrane bound proteins for their cellular uptake, it is possible to transfect cells that are normally non-permissive (Scherer et al., 2002). This is also true in primary cells which are known to be difficult to transfect (Krotz et al., 2003).

Statements of Invention

The present disclosure is directed to overcoming the deficiencies in magnetic cell separation by providing a method to separate a target population from a cell mixture on the basis of the expression of their intracellular markers and to then allow the recovery of these cells for additional downstream studies and to achieve therapeutic benefits.

This disclosure relates to a method for isolating specific cells from a mixed population of different cell types using intracellular markers rather than cell surface markers. This method involves the coupling of antibody fragments to a magnetic particle, where the antibody fragment is specific to the marker of interest expressed inside the cell.

According to an aspect of the invention there is provided a magnetic particle comprising: a ligand that specifically binds an intracellular antigen, a cell membrane penetrating cationic peptide and a peptide comprising an amino acid sequence that is adapted to interact with the secretory pathway in a cell.

In a preferred embodiment of the invention said intracellular antigen is substantially a nuclear antigen.

In a preferred embodiment of the invention said nuclear antigen is selected from the group consisting of: FoxO family members, TARP, p53, Rb, E2F, hK4, BRCA1, Cdk2, SATB1, TP53INP1, Myc, Fos, Jun, CREB, Ets, SRF, FAK, Pax6, Calpain, Elk1, Stats 1–3, Akt, p21

5 In an alternative preferred embodiment of the invention said intracellular antigen is substantially a cytoplasmic antigen.

In a preferred embodiment of the invention said cytoplasmic antigen is selected from the group consisting of: Jak1, Jak2, Tyk2, Jak3, GATA 1–4, Stats 1–6, CBP, NFkB, IKK, PIK3CA, B-raf, EBI3, eIF2 α , Akt, PI3K, IAP, Hsp, FAK, Raf, Ras, TNF, Src, Abl, Caspases 1–12.

10 In a preferred embodiment of the invention said cationic cell membrane penetrating peptide is a natural cell penetrating peptide.

In an alternative preferred embodiment of the invention said cell membrane penetrating peptide is a synthetic sequence.

15 In a preferred embodiment of the invention said cell membrane penetrating peptide is adapted to penetrate a mammalian cell.

In a preferred embodiment of the invention said cell membrane penetrating peptide is selected from the group consisting of: YARKKRRQRRR, YARKARRQARR, YARAAARQARA, YARAARRAARR, YARAARRAARA, YARRRRRRRRR, YAAARRRRRRR, PLSSIFSRIGDP, RQIKIWFQNRRMKWKK, WEIEDEDER,
20 GRKKRRQRRRPQ, RRRRRRRRRRRR.

In a preferred embodiment of the invention said cell membrane penetrating peptide consists essentially of the amino acid sequence: GRKKRRQRRRPQ.

Preferably said cell membrane penetrating peptide consists of the amino acid sequence: GRKKRRQRRRPQ.

25 In a preferred embodiment of the invention said cell membrane penetrating peptide consists essentially of the amino acid sequence: RRRRRRRRRRRR.

Preferably said cell membrane penetrating peptide consists of the amino acid sequence: RRRRRRRRRRRR.

The cell penetrating peptide and the secretory peptide can be engrafted into the CDR3 regions of the VL and VH domains.

In a preferred embodiment of the invention said peptide adapted to interact with the mammalian secretory pathway is selected from the group consisting of:
5 MCPARSLLLVATLVLLDHLSLA, MDAMKRGLCCVLLLCGAVFVSPS, MATGSRTSLL
LAFGLLCLPWLQEGSA, MYRMQLLSICIALSLALVTNS or MGVKVLFALICIAVAE.

In a preferred embodiment of the invention said peptide is consists essentially of the amino acid sequence MGVKVLFALICIAVAE.

In a preferred embodiment of the invention said peptide is consists of the amino acid
10 sequence: MGVKVLFALICIAVAE.

In a preferred embodiment of the invention said peptide is consists essentially of the amino acid sequence: MYRMQLLSICIALSLALVTNS.

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15 In a preferred embodiment of the invention said ligand that binds said intracellular antigen is an antibody fragment.

Various fragments of antibodies are known in the art, e.g. Fab, Fab₂, F(ab')₂, Fv, Fc, Fd, scFvs, etc. A Fab fragment is a multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light
20 chain variable region, covalently coupled together and capable of specifically binding to an antigen. Fab fragments are generated via proteolytic cleavage (with, for example, papain) of an intact immunoglobulin molecule. A Fab₂ fragment comprises two joined Fab fragments. When these two fragments are joined by the immunoglobulin hinge region, a F(ab')₂ fragment results. An Fv fragment is multimeric protein consisting of the immunologically
25 active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically binding to an antigen. A fragment could also be a single chain polypeptide containing only one light chain variable region, or a fragment thereof that contains the three CDRs of the light chain variable region, without an associated heavy chain variable region, or a fragment thereof
30 containing the three CDRs of the heavy chain variable region, without an associated light

chain moiety; and multi specific antibodies formed from antibody fragments, this has for example been described in US patent No 6,248,516. Fv fragments or single region (domain) fragments are typically generated by expression in host cell lines of the relevant identified regions. These and other immunoglobulin or antibody fragments are within the scope of the invention and are described in standard immunology textbooks such as William E. Paul, Fundamental Immunology or Janeway et al. Immunobiology (cited above). Molecular biology now allows direct synthesis (via expression in cells or chemically) of these fragments, as well as synthesis of combinations thereof. A fragment of an antibody or immunoglobulin can also have bispecific function as described above.

10 Methods to deliver antibody fragments to cells intracellularly are known in the art; for example see WO2007/064727; WO2004/030610; WO03/095641; WO02/07671; WO01/43778; WO96/40248; and WO94/01131 each of which is incorporated by reference in their entirety.

15 In a preferred embodiment of the invention said antibody fragment is an ScFv or a single VH or VL domain.

In an alternative preferred embodiment of the invention said ligand that binds said intracellular antigen is a peptide or a peptide aptamer.

Peptides that have binding affinity to a target antigen are within the scope of the invention. For example peptides that mimic the binding affinity of antibodies and antibody fragments; 20 see Chattopadhyay, A, Tate, S., Beswick, R., Wagner, S.D. and Ko Ferrigno, P. A peptide aptamer to antagonise BCL-6 function. Oncogene, 25 2223-2233 (2006).

In a preferred embodiment of the invention said cell is a mammalian cell.

In an alternative preferred embodiment of the invention said magnetic particle further comprises a nuclear export peptide.

25 In a preferred embodiment of the invention said nuclear export peptide comprises or consists essentially of the amino acid sequence: NELALKLAGLDINKTEGEEDAQ

According to a further aspect of the invention there is provided a method for the selection and isolation of a mammalian cell from a heterogeneous population of cells comprising:

- i) forming a first preparation comprising a heterogeneous population of mammalian cells;
- ii) contacting said population with a second preparation comprising a magnetic particle according to the invention to provide a combined preparation and providing conditions that allow penetration and binding of said magnetic particle to an intracellular antigen;
- iii) incubating said combined preparation to remove unbound magnetic particles by the secretory pathway of said mammalian cell, optionally by application of a magnetic field; and
- iv) applying a magnetic field to said combined preparation to select and isolate mammalian cells comprising magnetic particles bound to an intracellular antigen.

In a preferred method of the invention said mammalian cell is a stem cell.

Preferably said stem cell is a cancer stem cell.

- The concept of a cancer stem cell within a more differentiated tumour mass, as an aberrant form of normal differentiation, is now gaining acceptance over the current stochastic model of oncogenesis, in which all tumour cells are equivalent both in growth and tumour-initiating capacity (Hamburger et al., 1977; Pardal et al., 2003). For example, in leukaemia, the ability to initiate new tumour growth resides in a rare phenotypically distinct subset of tumour cells (Bonnet et al., 1997) which are defined by the expression of CD34+CD38 surface antigens and have been termed leukemic stem cells (LSC). Similar tumour-initiating cells have also been found in 'solid' cancers such as prostate (Collins et al., 2005), breast (AlHajj et al., 2003), brain (Singh et al., 2004), lung (Kim et al., 2005), colon (O'Brien et al., 2007; Ricci Vitiani et al., 2007) and gastric cancers (Houghton et al., 2004). A list of intracellular cancer stem cell markers is available in Table 1.

According to an aspect of the invention there is provided a kit for customized modification of the magnetic particles containing a selection of cell membrane penetrating cationic peptides selected from the group of YARKKRRQRRR, YARKARRQARR, YARAAARQARA, YARAARRAARR, YARAARRAARA, YARRRRRRRRR, YAAARRRRRRR, PLSSIFSRIGDP, RQIKIWFQNRRMKWKK, WEIEDEDER, GRKKRRQRRRPQ, RRRRRRRRRRRR, a selection of secretory proteins selected from the group MCPARSLLLVATLVLLDHLSLA, MDAMKRGLCCVLLLCGAVFVSPS,

MATGSRTSLLLAFLGLCLPWLQEGSA, MYRMQLLSIALSLALVTNS or
MGVKVLFALICIAVAE and an activation, wash and blocking/storage buffer.

5 In a preferred embodiment of the invention said kit further comprises a magnet for the use of
said customized magnetic particles.

The magnetic particles may be coupled to an antibody fragment (scFv, single domain,
dibody), a cell penetrating peptide and a secretory peptide, or to an antibody fragment and a
secretory peptide.

10 The cell penetrating peptide and the secretory peptide may be either directly coupled to the
magnetic particle or to the antibody fragment via expression as a fusion protein or via
chemical coupling between the antibody and the peptide, or they may be engrafted within the
CDR3 regions of the VH or VL domains as previously described by (Jeong et al. 2011).

The magnetic antibody fragments are incubated with the cells and allowed to internalize into
15 the cells using the cationic peptide or the positive charge of the magnetic particle as the
delivery vehicle. After achieving cell internalisation of the antibody magnetic particles, the
cells are allowed a period of incubation in cell media to allow the magnetic antibody
fragments bind to their targets. Any unbound magnetic antibody fragments will then be
secreted from the cells by the secretory peptide with or without the help of an external
20 magnet. The cells containing just the bound antibody fragments to their respective targets can
be selected with the use of an external magnet where any unbound cells are washed away.
The selected cells can then be further incubated for other downstream applications (Figure 1).

Throughout the description and claims of this specification, the words “comprise” and
“contain” and variations of the words, for example “comprising” and “comprises”, means
25 “including but not limited to”, and is not intended to (and does not) exclude other moieties,
additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the
plural unless the context otherwise requires. In particular, where the indefinite article is used,
30 the specification is to be understood as contemplating plurality as well as singularity, unless
the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

5 **Table 1.** Intracellular Cancer stem cell markers

TP53INP1	tumor protein p53 inducible nuclear protein 1	ENSG00000164938
Axl	AXL receptor tyrosine kinase	ENSG00000167601
GATA-3	GATA binding protein 3	ENSG00000107485
USP22	ubiquitin specific peptidase 22	ENSG00000124422
BIRC6	baculoviral IAP repeat containing 6	ENSG00000115760
Hh	sonic hedgehog	ENSG00000164690

Figure 1. Diagram depicting the sequence of events in the magnetic intracellular cell separation technique (MICELS). A magnetic particle (1) is coupled to a cell penetrating positively charged peptide (2), a cell export peptide (3) and an scFv antibody fragment (4).

- 10 Step 1. The magnetic-protein complex is incubated with the mixed cell population for a length of time until the complex has fully internalised into the cells. Step 2. The scFv antibody fragments specifically bind to their antigen targets (Ag) available in the cells and are thereby anchored inside these cells. Step 3. Any magnetic-protein complexes that are not bound to the specific target antigens are secreted to the extracellular milieu via the export peptide through the cell secretory pathway. This step avoids the selection of cells that do not
15 express the specific target antigens. Step 4. An external magnetic field is applied to the cells allowing only the cells containing the magnetic complexes to bind to the magnet. The remainder of the cell population can be washed away. The selected cells can be now used for further downstream applications.

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Figure 2. MICELS selection of CHO and A549 cells with scFv magnetic-protein complexes. (A) MICELS selection of A549 cells in a 24 well plate with a serial dilution of the scFv magnetic-protein complexes (40µg-2.5µg). The blue arrows indicate the location of the selected cell pellets. Decreasing amounts of selected cells were obtained with decreasing
25 concentrations of magnetic-protein complexes. The best results for cell selection were obtained with 40µg of the magnetic-protein complexes. (B) Confocal images of CHO (A, B)

and A549 (C, D) cells after 4.30h incubation with scFv magnetic-protein complexes. The cells were washed vigorously with PBS before imaging. The green magnetic-protein complexes localised at the cell membrane, the perinuclear membrane and in the cytoplasm consistent with the sub-cellular localisation of activated Ras in the A549 lung cancer cell line.

- 5 No magnetic-protein complexes were observed in CHO cells following 4.30h incubation.

Figure 3. MICELS selection of A549 and CHO cells with 40µg scFv magnetic-protein. Images of A549 cells (A) and CHO cells (B) selected using MICELS were imaged using a 10x objective. The images were taken after an extensive wash with PBS followed by
10 trypsinisation and cell selection with the magnet. Minimal background of CHO cells was observed following selection. (C,D) Fluorescent and bright field confocal image of the A549 cell pellet obtained after selection. (E,F) Merged confocal images of A549 cells that had been placed back into culture following MICELS selection. The images were taken at 24h and no significant cytotoxicity was observed.

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Materials and Methods:

Production of H-RasG12V scFv

The sequence of an scFv (scFv#6) specific for the mutant H-RasG12V (Tanaka et al., EMBO Journal 2007) served as a template for the synthesis of scFv#6 by GeneArt® (Life
20 Technologies) with flanking BamHI and EcoRI sites at the 5' and 3' ends, respectively. The gene was subcloned into BamHI and EcoRI sites of the pRSETa bacterial expression vector (Life Technologies) to generate in-frame fusion scFv protein with a 6x histidine tag. The plasmid was transformed into BL21 (DE3) bacterial cells (Merck-Millipore) and expression of the scFv#6 fragment was induced in 500ml culture via the addition of 1 mM IPTG at
25 OD₆₀₀ of 0.55 followed by incubation for 3 h at 37°C. The cells were harvested by centrifugation at 5000 × g at 4°C and the final cell pellets were stored at -80°C prior to scFv purification.

scFv protein was extracted from bacterial pellets in 25ml of B-PER bacterial lysis buffer (Thermo Scientific) in the presence of a cocktail of protease inhibitors (containing 1.5 µg/ml
30 Chymotrypsin, 0.8 µg/ml Thermolysin, 1 mg/ml Papain, 1.5 µg/ml Pronase, 1.5 µg/ml Pancreatic extract, 0.002 µg/ml Trypsin) (Roche) at RT for 30min. The lysate was

centrifuged at $15000 \times g$ at 4°C and the supernatant was diluted in 25ml lysis buffer (50 mM Na phosphate, pH 8.5, 300 mM NaCl, 10 mM imidazole). The His-tagged scFv#6 proteins were purified by gravity flow through 1ml of Ni-NTA agarose (Qiagen), followed by a wash with 20ml of wash buffer (50 mM Na phosphate, pH 8.5, 300 mM NaCl, 20 mM imidazole) and eluted in 5ml of elution buffer (50 mM Na phosphate, pH 8.5, 300 mM NaCl, 250 mM imidazole). The eluted protein was subsequently dialysed against 0.1M MES pH6.0, snap frozen and stored at -80°C at a concentration of 1.4mg/ml

Magnetic Particles

Biodegradable magnetic nanoparticles with an average size of 100 nm (by dynamic light scattering) were purchased from Chemicell, Berlin, Germany. These particles are referred to as nano-screenMAG particles with an extension to their name -ARA, referring to the surface coating of the iron oxide core with Glucuronic acid used for the binding to biomolecules, and are covered by a lipophilic fluorescence dye emitting light in the range of green (Exc.= 476 nm / Em_{max}. = 490 nm).

Coupling of fluorescent magnetic particles to proteins

FITC labelled Tat cell penetrating peptide 5(6)-Carboxyfluorescein-GRKKRRQRRRPQ and Gaussia Luciferase secretory peptide 5(6)-Carboxyfluorescein-MGVKVLFAICIAVAEA were synthesised by JPT Peptide Technologies GmbH. The lyophilised peptides were resuspended in 100µl of DMSO and subsequently diluted down to a concentration of 1mg/ml with 10mM PBS pH7.4, snap frozen and stored at -80°C in aliquots.

The magnetic particles were activated by 2 washes with 1ml 0.1M MES buffer pH5.0 using the MagnetoPure (Chemicell, Berlin, Germany) magnetic separator. After the second wash the particles were resuspended in 0.25ml 0.1M MES buffer pH5.0. The carboxyl groups on the green fluorescent magnetic beads nano-screenMAG/G-ARA were activated with freshly prepared EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) (Sigma) by dissolving 10mg EDC in 0.25ml 0.1M MES buffer. The EDC buffer was added to the particles and gently mixed at RT for 10min. Following this incubation, the magnetic particles were washed 2 x with 1ml 0.1M MES buffer pH5.0 and resuspended in 0.25ml of 0.1M MES buffer, pH5.0.

200µg of scFv#6 purified protein were mixed with 150µg of Tat cell penetrating peptide and 200µg of Gaussia luciferase export peptide in 0.25ml of 0.1M MES buffer, pH 5.0. The

protein mixture was added to 10mg of activated particles and gently mixed for two hours at room temperature to generate magnetic-protein complexes. The particles were washed 3 x with 1 ml PBS and resuspended in 500µl Blocking/Storage buffer (10mM PBS pH7.4, 0.1 % BSA, 0.05 % sodium azide).

5 Cell Culture

Chinese Hamster Ovary (CHO) cells and the A549 human lung carcinoma cell line (which constitutively express mutant K-RasG12V antigen) were maintained in selective Gibco® Ham's F12 Nutrient Mixture Media (Life Technologies) supplemented with 10% heat-inactivated foetal bovine serum, 100 µg/mL streptomycin, 0.29 mg of L-glutamine at 37°C in a humidified atmosphere of 5% CO₂.

Magnetic intracellular cell separation technique (MICELS)

The CHO and A549 cell lines were seeded the day before the magnetic selection, at 2×10^5 cells/well in a 24 well glass bottom plate (In Vitro Scientific) in 1ml of growth media to allow the cells reach 90-95% confluency at the time of the magnetic cell separation. The following day the media was replaced with fresh growth media pre-warmed at 37°C. A serial dilution ranging between 40µg and 2.4µg of the magnetic-protein mix was gently mixed with 100µl of serum free Ham's F12 media and added drop wise to the cells. The cells were incubated with the magnetic-protein complexes for 1.30h at 37°C in a humidified atmosphere of 5% CO₂ to allow these to transduce into the cells. The cells were then vigorously washed five times with PBS and incubated further for 3h. Following the 3 hour incubation, any unbound magnetic-protein complexes are secreted from the cells through the cell secretory pathway. The cells were then vigorously washed five times with PBS and trypsinised in the 24 well plate with 0.05% trypsin-EDTA (Invitrogen). 0.5ml of fresh F12 growth media was added to each well to inactivate the Trypsin and the 24 well plate was placed on top of a MagnetoPURE (Chemicell, Berlin, Germany) magnetic separator for cell selection. At this point any cells that did not contain magnetic-protein complexes were gently washed away 3 times with 500µl PBS on the magnetic separator. The magnetic separator was then removed and the remaining cells containing the magnetic-scFv complexes bound to the activated form of Ras were further incubated in 500µl of growth media at 37°C in a humidified atmosphere of 5% CO₂ for 48h.

Image acquisition Laser scanning confocal microscopy.

Laser scanning confocal microscopy was carried out using an Inverted Zeiss LSM 510 META Axiovert 200M confocal microscope using a 10x, 20x and a 40x/1.3NA oil immersion objective. FITC was imaged using an Argon 488-nm laser light and a 505–530-nm BP emission filter

EXAMPLES

A method to separate whole live cells using intracellular proteins rather than cell surface proteins (MICELS-magnetic intracellular cell separation) was developed by coupling magnetic particles to scFv antibody fragments and to cell penetrating and cell export peptides (Figure 1). In this study the proto-oncogene mutant RasG12V was used as a model system to test the MICELS technology. The Ras protein belongs to a class of proteins known as small GTPases, and plays key roles in signal transduction as molecular switches. These proteins are mediated through two switch regions displaying conformational differences between active (GTP bound) and inactive (GDP bound) states (Vetter and Wittinghofer, 2001). Occasionally, mutations occur in these Ras proteins (K, H or NRAS) resulting in constitutively activated GTP-bound forms that promote cell transformation in a signal-independent manner (Adjei, 2001), thereby, promoting cell proliferation and protection from apoptosis. Approximately 30% of human cancers contain oncogenic Ras mutants, with higher frequencies in pancreas, colon and lung adenocarcinoma (Grewal et al., 2011). The Ras isoforms (H-Ras, N-Ras and K-Ras) are mainly localised to the inner cell membrane, however, in recent years they have been shown to associate, as well, with intracellular organelles such as the Golgi, the ER, endosomes and the mitochondria (Grewal et al., 2011).

An scFv antibody fragment (scFv#6) previously shown to bind specifically to the activated form of Ras, rather than to particular RAS mutants (Tanaka et al., EMBO Journal 2007), was employed for this study. The gene was synthesised by GeneArt (Life Technologies) and subsequently subcloned into the bacterial expression system pRSET to generate His-Tagged scFv. The protein was expressed in BL21 (DE3) bacterial cells and coupled to green fluorescent magnetic beads together with FITC labeled Tat penetrating peptide to facilitate the transport of the complexes into cells. A FITC labeled Gaussia luciferase export peptide

was coupled, as well, to generate the MICELS complexes. The export peptide would direct the magnetic complexes to the extracellular milieu by either following the classical or an unconventional cell secretory pathway (Nickel, 2005). Any complexes that had not anchored to the activated Ras proteins, would be secreted.

- 5 A serial dilution of the magnetic-protein complexes ranging from 40 μ g and 2.4 μ g were incubated with the CHO and A549 cells in a 24 well plate for 1.30h to allow them to transduce into the cells. The cells were then extensively washed with PBS to remove any complexes that had not transduced into the cells. These were further incubated for another 3h to allow for the export peptide to re-localise the complexes in the cells thereby allowing them to exit via the cell export system. This was to ensure the removal of any complexes that had not recognised the Ras antigen. The cells were then vigorously washed again before trypsinisation and selection with the magnet (Figure 2).

- The cells containing the magnetic-protein complexes slowly migrated towards the magnet. These were gently washed with PBS by magnetic separation to ensure the removal of cells that did not contain the magnetic-protein complexes. The supernatant was removed and whole live cells containing activated Ras were selected using the MICELS method and placed back into culture (Figure 3-A,B,C,D). 24h later the cells had divided diluting down the cells in culture that still contained the magnetic-protein complex (Figure 3-E,F). The lipophilic fluorescent dye and the polysaccharid Glucuronic acid coating of the magnetic-protein complexes eventually degrade inside the cell leaving just the iron oxide core of the magnetic bead, which is non-toxic to the cells. It is thought that the iron oxide nanoparticles reside inside lysosomes (Becker et al., 2007) and are eventually degraded into iron ions via enzyme hydrolysis (Shuayev et al., 2009).

The magnetic-protein complexes localise to subcellular compartments.

- 25 Depending on the cell signaling events undergoing in the cell, the H-Ras, N-Ras and K-Ras isoforms have all been shown to localise to the cell membrane as well as to a number of other subcellular compartments such as the ER, the Golgi, endosomes and to the perinuclear membrane (Grewal et al., 2011). In this study the scFv magnetic-protein complexes localised to the cell membrane, to the perinuclear membrane and to distinct subcellular vesicles in the cytoplasm consistent with the localisation pattern of activated Ras in the A549 lung cancer cell line. Optimal results for cell selection were obtained by using 40 μ g of complexes. The

number of cells selected decreased concomitantly with the decrease in the concentration of complexes (Figure 2-A).

A simple method is described here for selection of cells using intracellular proteins by coupling antigen-specific antibody fragments to magnetic beads and by using cell penetrating peptides to facilitate the cellular uptake of these complexes, and cell export peptides to allow the secretion of any unbound complexes via the cell secretory pathway.

The method is fast and simple, and requires minimal work. Furthermore, no cellular cytotoxicity was observed allowing the cells to be used for other downstream applications.

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Claims

1. A magnetic particle comprising: a ligand that specifically binds an intracellular antigen, a cell membrane penetrating cationic peptide and a cell export peptide comprising an amino acid sequence that is adapted to interact with the secretory pathway in a cell.
2. The magnetic particle according to claim 1, wherein said intracellular antigen is substantially a nuclear antigen.
3. The magnetic particle according to claim 2, wherein said nuclear antigen is selected from the group consisting of: FoxO family members, TARP, p53, Rb, E2F, hK4, BRCA1, Cdk2, SATB1, TP53INP1, Myc, Fos or Jun.
4. The magnetic particle according to claim 1, wherein said intracellular antigen is substantially a cytoplasmic antigen.
5. The magnetic particle according to claim 4, wherein said cytoplasmic antigen is selected from the group consisting of: Jak3, GATA 1-4, Stats 1-6, CBP, NFkB, IKK, PIK3CA, BRAF, PIK3CA, EML4-ALK, VEGF, EBI3, eIF2 α , Akt, PI3K, IAP, Hs, Hsp, FAK, Raf, Ras, TNF, Src, Wnt or Abl.
6. The magnetic particle according to any one of claims 1-5, wherein said cationic cell membrane penetrating peptide is a natural cell penetrating peptide.
7. The magnetic particle according to any of claims 1-5, wherein said cationic cell membrane penetrating peptide is a synthetic sequence.
8. The magnetic particle according to claim 6 or 7 wherein said cationic cell membrane penetrating peptide is adapted to penetrate a mammalian cell.
9. The magnetic particle according to any one of claims 6 to 8 wherein said cationic cell membrane penetrating peptide is selected from the group consisting of:

YARKKRRQRRR, YARKARRQARR, YARAAARQARA, YARAARRAARR,
 YARAARRAARA, YARRRRRRRRR, YAAARRRRRRR, PLSSIFSRIGDP,
 RQIKIWFQNRRMKWKK, WEIEDEDER, GRKKRRQRRRPQ or,
 RRRRRRRRRRRR.

10. The magnetic particle according to any one of claims 1 to 13, wherein said cell export peptide is selected from the group consisting of: MCPARSLLLVATLVLLDHLSLA, MDAMKRGLCCVLLLCGAVFVSPS, MATGSRTSLLLAFGLLCLPWLQEGSA, MYRMQLLSICIALSLALVTNS or MGVKVLFIKICIAVAE.

11. The magnetic particle according to any one of claims 1 to 10 wherein said ligand that binds said intracellular antigen is an antibody fragment or an aptamer.

12. The magnetic particle according to any one of claims 1-11 wherein said magnetic particle further comprises a nuclear export peptide.

13. The magnetic particle according to claim 12 wherein said nuclear export peptide comprises or consists essentially of the amino acid sequence: NELALKLAGLDINKTEGEEDAQ.

14. A method for the selection and isolation of a mammalian cell from a heterogeneous population of cells comprising:

- i) forming a first preparation comprising a heterogeneous population of mammalian cells;
- ii) contacting said population with a second preparation comprising a magnetic particle according to any one of claims 1-13 to provide a combined preparation and providing conditions that allow penetration and binding of said magnetic particle to an intracellular antigen;
- iii) incubating said combined preparation to remove unbound magnetic particles by the secretory pathway of said mammalian cell, optionally by application of a magnetic field; and

iv) applying a magnetic field to said combined preparation to select and isolate mammalian cells comprising magnetic particles bound to an intracellular antigen.

15. A kit for customized modification of magnetic particles containing a selection of cell membrane penetrating cationic peptides selected from the group of YARKKRRQRRR, YARKARRQARR, YARAAARQARA, YARAARRAARR, YARAARRAARA, YARRRRRRRRR, YAAARRRRRRR, PLSSIFSRIGDP, RQIKIWFQNRRMKWKK, WEIEDEDER, GRKKRRQRRRPQ, RRRRRRRRRRRR, a selection of cell export peptides selected from the group MCPARSLLLVATLVLLDHLSLA, MDAMKRGLCCVLLLCGAVFVSPS, MATGSRTSLLAFGLLCLPWLQEGSA, MYRMQLLSICIALSLALVTNS or MGVKVLFALICIAVAE, activation, wash and blocking/storage buffer and optionally a nuclear export peptide and a magnet.