Transfection of human endothelial cells with Metafectene Pro

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Introduction

Angiogenesis is a hallmark of diverse malignancies and inhibition of angiogenesis is considered a promising treatment strategy. A critical step in targeting tumor vasculature is the identification of markers on tumor endothelial cells that can serve as docking molecules for bioactive molecules for destruction or visualization of the vasculature.
We have previously performed extensive gene expression profiling analyses of endothelial cells of tumor and normal tissues [1] and identified different genes that are specifically overexpressed in tumor endothelial cells. To validate the contribution of the gene (product) to the process of tumor angiogenesis, it is crucial to have tools that allow modification of the expression level of the gene of interest. Constructs expressing the open reading frame or an shRNA to upregulate or downregulate the mRNA of the gene product in question serve this purpose. However, endothelial cells are generally very difficult to transfect, hampering proper target validation.
Here we have tested Metafectene Pro reagent for the transfection of different endothelial cells and optimized a protocol that will allow functional target validation studies to be performed. These will help elucidate the function and localization of potential targets for angiogenesis interfering therapies.

Materials and methods

Cell culture
Three different endothelial cell lines were used: HMEC, RF24 and EVLC2. The first has a microvascular origin, the second and third have a macrovascular origin. In addition, HUVEC were used that were isolated from umbilical cords by perfusion with trypsin. Cells were routinely cultured in gelatin-coated culture flasks, in RPMI-1640 supplemented with 10% FCS and 10% human serum, 1% glutamine and penicillin/streptomycin.

Transfection with Metafectene Pro
Cells ($5*10^4$ or as otherwise indicated) were seeded in gelatin-coated 48-wells plates one day prior to transfection, to ensure a true confluency of about 50%. pEGFP was added to 15µl RPMI and this solution was combined with 10µl RPMI containing Metafectene Pro. Complex formation was allowed to proceed for 20 minutes at room temperature. Complex, 25µl, was added dropwise to the cells in the 48-wells plates under continuous gentle swirling of the plates. Transfection complex was incubated with the cells for 12-16hrs after which transfection medium was removed and fresh medium added. After 24 hrs and 48 hrs cells were visually inspected for GFP expression under a fluorescence microscope, and for viability and morphology using an inverted microscope. Different amounts of Metafectene Pro and pEGFP were tested to determine the optimal transfection efficiency with minimal toxicity.

Flowcytometry
Forty-eight hours post-transfection, cells were harvested with trypsin and fixed with 1% paraformaldehyde. Cells were subject to flowcytometry analysis to measure fluorescence intensity and transfection efficiency.

Results

Optimization of Metafectene Pro transfection of endothelial cells

For our experimental setup in 48-wells plates containing 250ul medium, we tested 0.5µl, 1µl, 2µl and 4µl Metafectene Pro. Invariably, cells incubated with 2µl Metafectene Pro or more had a very poor morphology already a few hours posttransfection, presenting many floating dead cells with a granular appearance (data not shown). However, cells that did survive showed equal fluorescence intensity as cells transfected with a small amount of transfection reagent (data not shown). For further optimization, we used 0.5µl Metafectene Pro per well. We subsequently optimized the ratio Metafectene Pro:pDNA. Both the mean fluorescence intensity and the transfection efficiency generally increased to a ratio of 5 and declined somewhat with higher ratios (Figure 1). For subsequent experiments, we used a ratio of 5.

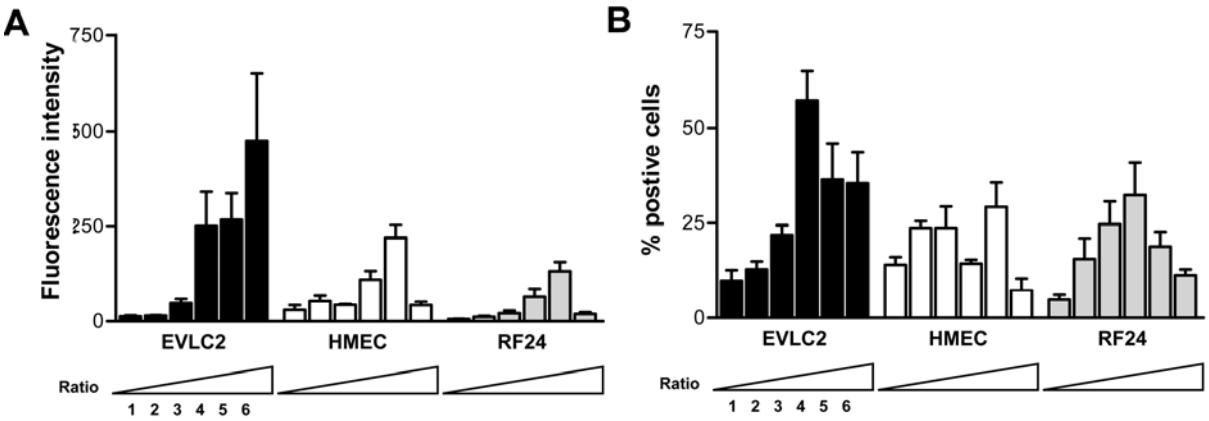

Figure 1: Optimization of Metafectene Pro:pDNA ratio for the transfection of different types of endothelial cells. **A)** Mea fluorescence intensity of cells analysed by flowcytometry. **B)** Percentage of cells positive for GFP assesed by flowcytometry.

The extent of GFP expression and the transfection efficiency were not dramatically influenced by reducing the cell number (Figure 2). Furthermore, GFP expression was quickly induced and sustained for up to 72 hrs in a transient transfection protocol (Figure 3). Maximum GFP expression was observed around 48hrs posttransfection.

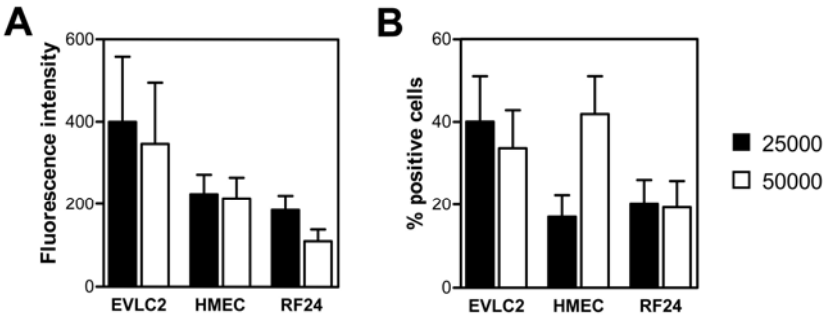

Figure 2: Optimization of cell number for Metafectene Pro transfection. The mean fluorescence intensity **(A)** and percentage successfully transfected cells **(B)** were evaluated with 25000 (black bars) or 50000 cells (white bars) seeded per well the day prior to transfection. Except for HMEC, no significant differences were observed between the different cell densities.

In summary, endothelial cells are best transfected using small amounts of Metafectene Pro (0.2% v/v), in combination with 0.2µg pDNA/ul Metafectene Pro. Furthermore, cells should be seeded relatively dense ($5*10^4$ cells/cm^2), and (the effect of) transgene expression can already be monitored from 18hrs posttransfection.

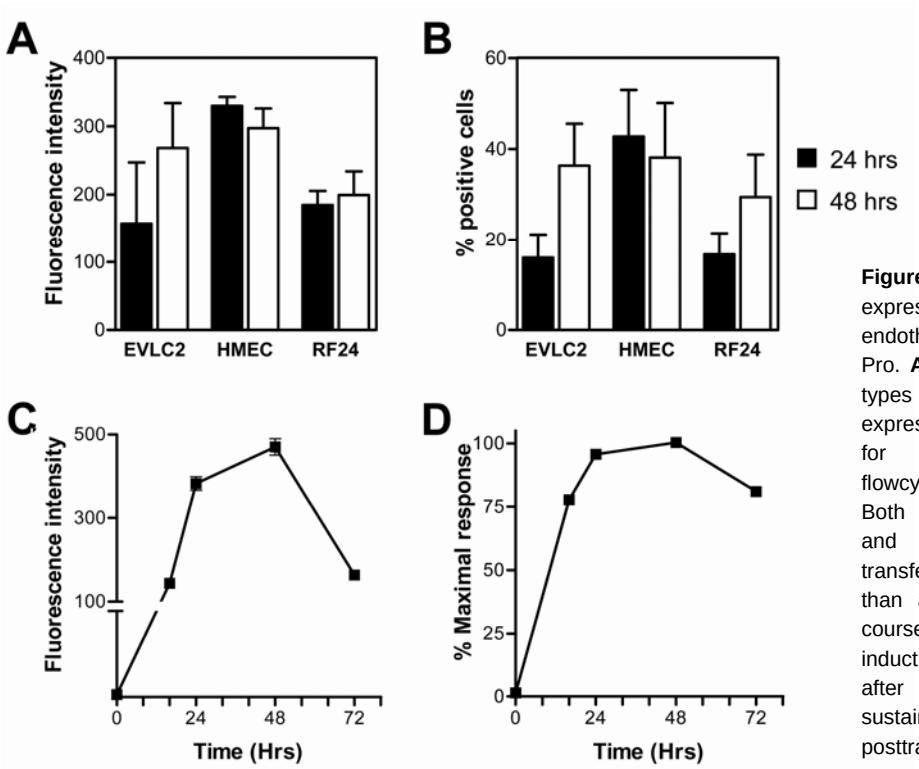

Figure 3: Time course of GFP expression after transfection of endothelial cells with Metafectene Pro. **A, B)** Different endothelial cell types were transfected with GFP expression constructs and analyzed for GFP expression by flowcytometry after 24 and 48hrs. Both mean fluorescence intensity and percentage successfully transfected cells was higher after 24 than after 48 hrs. **C, D)** A time course analysis showed rapid induction of GFP expression already after 16hrs, and expression was sustained to at least 72 hrs posttransfection.

Target validation using transfected endothelial cells

EVLC2 cells were transiently transfected with Metafectene Pro using the optimized protocol described above. GFP-tagged expression constructs for HMGB1 were used to determine the cellular localization of the protein. We observed a predominantly nuclear staining in comparison to endothelial cells transfected with GFP only (Figure 4). However, to evaluate gene function in an *in vitro* model system, functional assays need to be performed. Different angiogenesis assays are used to evaluate the pro-angiogenic or anti-angiogenic function of compounds or genes. One such assay uses endothelial cells grown in spheroids that are subsequently embedded in collagen gel to allow sprouting of the endothelium. After transfection with Metafectene Pro, the cells were not compromised in their ability to form spheroids and to sprout; however, the relatively long time span of this assay did somewhat compromise the transgene expression (Figure 4). This may well be overcome by stable transfection of the cells.

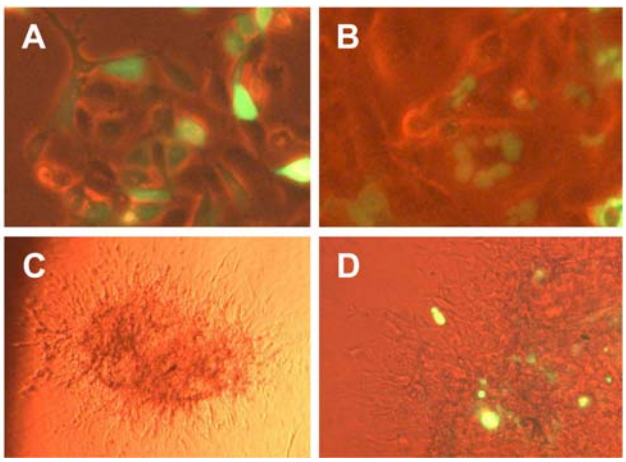

Figure 4: Application of endothelial cell transfections for functional target validation. **A,B)** Expression of GFP-tagged proteins can be used to determine the subcellular localization of the protein of interest. Native GFP is expressed throughout the cell (A), whereas HMGB1-GFP is expressed predominantly in the nucleus (B). **C,D)** Metafectene Pro transfection does not compromise the capacity of endothelial cells to function in angiogenesis assays. Endothelial cells were grown to spheroids, and allowed to sprout in collagen gel (C). Trangene expression is still detectable in the spheroids, approximately 7 days posttransfection (D).

Finally, we tested the optimized protocol on primary endothelial cells, HUVEC. We observed only a minimal percentage of successfully transfected cells as determined by flowcytometry on GFP expression, though these few cells expressed very high amounts of GFP (Figure 5). However, a subpopulation of the cells showed a small shift in overall fluorescence, indicating a considerable amount of cells was successfully transfected (Figure 5), but expressed only low levels of GFP. Hence, additional optimization might be required for primary cells.

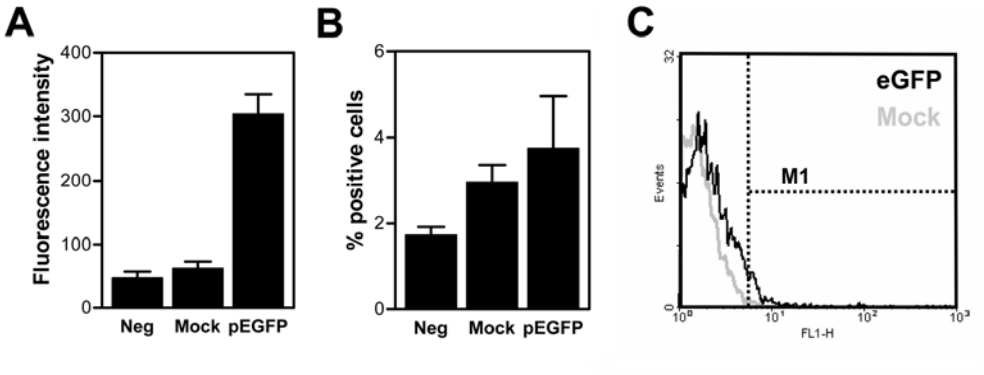

Figure 5: Metafectene Pro transfection of primary HUVECs. **A)** Clear increase in mean fluorescence intensity of HUVEC after Metafectene Pro transfection with eGFP. **B)** Only a very small percentage of cells expressed more GFP than non-transfected or mock transfected cells. **C)** A minor shift in overall fluorescence is observed of GFP expressing HUVEC.

Conclusions

Though endothelial cells are generally hard to transfect, Metafectene Pro is capable of transfecting these cells with a high efficiency and low toxicity. Functional validation of putative target genes for interfering with angiogenesis using expression constructs greatly benefits from efficient transfection protocols.

References

1. van Beijnum, J.R., et al., *Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature.* Blood, 2006. **108**(7): p. 2339-48.