Evaluation of Metafectene-Pro mediated transfection of siRNA and/or plasmids into human and murine cells

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Introduction:

DNA double strand breaks (DSBs) can lead to chromosomal fragmentation and genomic rearrangements if they are not repaired in an accurate and timely manner. In mammalian cells, DSBs trigger a signalling response from the Ataxia Telangiectasia Mutated (ATM) pathway while DNA non-homologous end joining (NHEJ) and Homologous Recombination (HR) operate as the primary mechanisms of DSB repair[1]. Whilst the majority of DSBs are repaired by NHEJ in an ATM-independent manner, loss of ATM or those mediator proteins required for its activation or signalling (such as the Mre11/Rad50/NBS1 (MRN) complex, MDC1, 53BP1, BRCA1 or histone H2AX) results in a subtle defect characterized by ~10-25% of initially incurred DSBs that remain un-repaired[2]. Interestingly, mutation of the NHEJ processing nuclease, known as Artemis, also results in the same DSB repair defect and we have demonstrated that it in fact functions within the same pathway as ATM to repair breaks by NHEJ in G1-phase[2] or HR in G2-phase (manuscript in preparation).

These DSBs preclude 'normal' re-joining and are associated with a greater complexity that, after much work, we now know equates with increased heterochromatinization[3]. Central to this process is the transcriptional repressor and heterochromatin-building factor known as KAP-1, which has shown to be a substrate of ATM signalling[4]. Knockdown of KAP-1, to transiently perturb heterochromatin, can restore wildtype DSB repair in an ATM-signalling deficient cell. This work has been positively reviewed at *Molecular Cell* and we are currently addressing the reviewer's comments before resubmission in the beginning of March, 2008[3]. One important revision that we are currently trying to achieve is the re-introduction of KAP-1 expression to cells that have been treated with KAP-1 siRNA, in order to prove the specificity of the effect we have observed and introduce certain point-mutants of KAP-1 into KAP-1 depleted human cells (no KAP-1 deficient cells have been reported to date, as it is an essential gene). Furthermore, although significant progress has been made in the characterization of the ATM/Artemis DSB repair pathway, we still need to establish the role that the Artemis nuclease plays within heterochromatic DSB repair.

Experimental motivation for this study:

The transfection of less-transformed cells such as primary fibroblasts is notoriously challenging. When coupled with normally difficult-to-express proteins

such as the Artemis nuclease, it becomes a prohibitive experiment. We have, for some time, desired to transfect Artemis into primary human fibroblasts for DSB repair complementation analysis of Artemis-deficient patients and functional studies of the Artemis protein. We are also interested in improving the existing methods we have established for transfecting Artemis into more transformed cells (such as hTERT-immortalized fibroblasts or spontaneously transformed mouse-embryonic fibroblasts (MEFs)). To date, the more "traditional" methods of lipid-based transfection have not been useful for Artemis expression and only the (relatively expensive and time-consuming) AMAXA nucleofector technology (AMAXA biosystems) has resulted in a reasonable transfection efficiency (although still only 20%)[2]. Here, we examine the relative ability of Metafectene-Pro to transfect Artemis into MEFs compared to the commercially available Genejuice® (Merck Biosciences).

The simultaneous transfection of siRNA for knockdown and plasmids for expression can be technically challenging due to the different reagents and methods required to introduce each compound to cells. Indeed, we have previously used siPORT™NeoFX™ (Ambion) or HiPerFect Transfection Reagent (Qiagen) to transfect KAP-1 siRNA with much success, but neither of these reagents is useful for efficient plasmid transfection. Genejuice®, which can efficiently transfect plasmids into transformed cells, is not (in our experience) useful for siRNA studies and does not work very well with less-transformed cells such as primary human fibroblasts. Here, we show that Metafectene-Pro can be used to successfully introduce KAP-1 siRNA and either GFP- or HA-tagged KAP-1 expression plasmids (individually) into both transformed and primary human cells.

In the near future, we aim to examine whether the simultaneous introduction of KAP-1 siRNA and expression plasmids can be achieved using a single round of Metafectene-Pro mediated transfection. If successful, this reagent would become highly useful for these kinds of protein replacement studies, which are in increasing demand by reviewers at high impact journals and are of particular relevance to the mutational analysis of essential genes.

Results/methods:

Comparison of Metafectene-Pro versus Genejuice mediated transfection of mouse embryonic fibroblasts with human Artemis expression constructs.

Logarithmically growing wildtype MEFs were trypsinized and diluted with fresh medium (MEM+10%FCS) to a concentration of 1 x 10^5 cells/mL.

Metafectene transfection: 1 ug of the plasmid Artemis^{WT}-PCI-neo-myc or an equivalent volume of water (=mock transfection) was combined with 50 uL of OPTI-MEM® (Gibco-Invitrogen). 5 uL of Metafectene-Pro was combined with 50 uL of OPTI-MEM® and immediately added to the diluted plasmid. Diluted Metafectene-Pro and plasmid were incubated for 15

minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

Genejuice® transfection: The recommended manufacturer's instructions were followed. 3 uL of Genejuice® was combined with 100 uL of OPTI-MEM® and incubated for 5 minutes at room temperature. Diluted Genejuice® was then added drop-wise to 1 ug of the plasmid Artemis^{WT}-PCI-neo-myc or an equivalent volume of water (=mock transfection), was incubated for 10 minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

2 mL of the wildtype MEFs (at 1 x 10^5 cells/mL) were gently pipetted on top of each of the transfection reactions. 24 or 48 hours later, cells were fixed, permeablized, immunostained for Myc-tag and counterstained for DAPI. Transfection efficiency was assessed by immunofluorescence. The number of MEFs successfully expressing Myc-tagged Artemis was two-fold greater in Metafectene-Pro transfected cells versus Genejuice® transfected cells, for both the 24 and 48 hour time points (Figure 1).

Comparison of Metafectene-Pro versus Genejuice mediated transfection of transformed human fibroblasts with KAP-1 expression constructs.

Logarithmically growing 1BRneo cells were trypsinized and diluted with fresh medium (MEM+10%FCS) to a concentration of 1 x 10^5 cells/mL. Plasmids are described in [4].

Metafectene transfection: 1 ug of the plasmids pEGFPC1-KAP$^{WT,\ S824A\ or\ S824D}$, pCMVsport6-HA-KAP$^{WT,\ S824A\ or\ S824D}$, pCLXSN-HA-KAP$^{WT,\ S824A\ or\ S824D}$ or an equivalent volume of water (=mock transfection) was combined with 50 uL of OPTI-MEM® (Gibco-Invitrogen). 5 uL of Metafectene-Pro was combined with 50 uL of OPTI-MEM® and immediately added to the diluted plasmid. Diluted Metafectene-Pro and plasmid were incubated for 15 minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

Genejuice® transfection: The recommended manufacturer's instructions were followed. 3 uL of Genejuice® was combined with 100 uL of OPTI-MEM® and incubated for 5 minutes at room temperature. Diluted Genejuice® was then added drop-wise to 1 ug of the plasmids (as above) or an equivalent volume of water (=mock transfection), was incubated for 10 minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

2 mL of the 1BRneo cells (at 1 x 10^5 cells/mL) were gently pipetted on top of each of the transfection reactions. 48 hours later, cells were fixed, permeablized and stained for anti-HA and DAPI. Transfection efficiency was assessed by GFP or HA-tag immunofluorescence. The number of cells successfully expressing KAP-1 was two-fold greater in Metafectene-Pro transfected cells versus Genejuice® transfected cells, for all plasmids examined (Figure 2).

Comparison of Metafectene-Pro versus HiPerFect or siPORT™NeoFX™ mediated transfection of human primary fibroblasts with KAP-1 siRNA.

Near-confluent 1BR3 human primary fibroblasts were trypsinized and diluted with fresh medium (MEM+15%FCS) to a concentration of 1 x 10^5 cells/mL. The KAP-1 siRNA was a custom designed STEALTH™ siRNA synthesised by Invitrogen (CAGUGCUGCACUAGCUGUGAGGAUA; cDNA nt 450-475).

Metafectene transfection: 25 pmol of KAP-1 siRNA or an equivalent volume of water (=mock transfection) was combined with 30 uL of OPTI-MEM® (Gibco-Invitrogen). 5 uL of Metafectene-Pro was combined with 50 uL of OPTI-MEM® and immediately added to the diluted siRNA. Diluted Metafectene-Pro and siRNA were incubated for 15 minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

HiPerFect transfection: The recommended manufacturer's instructions were followed. 25 pmol of KAP-1 siRNA or an equivalent volume of water (=mock transfection) diluted to 100 uL with OPTI-MEM® (Gibco-Invitrogen). 3 uL of HiPerFect Transfection Reagent was added to the diluted siRNA, mixed and incubated for 10 minutes at room temperature before being pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

siPORT™NeoFX™ transfection: The recommended manufacturer's instructions were followed. 25 pmol of KAP-1 siRNA or an equivalent volume of water (=mock transfection) diluted to 100 uL with OPTI-MEM® (Gibco-Invitrogen). 5 uL of siPORT™NeoFX™ was combined with 95 uL of OPTI-MEM® and incubated for 10 minutes at room temperature. Diluted siPORT™NeoFX™ and diluted siRNA were incubated for 10 minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish.

2 mL of the 1BR3 cells (at 1 x 10^5 cells/mL) were gently pipetted on top of each of the transfection reactions. 48 hours later, cells were fixed, permeablized, immunostained for KAP-1 and counterstained for DAPI. Transfection efficiency

was assessed by immunofluorescence (Figure 3A,B). Compared to the mock-transfection reaction, the number of 1BR3 human primary fibroblast successfully knocked down for KAP-1 expression was negligible for the siPORT™NeoFX™ transfection. Both the HiPerFect Transfection Reagent and Metafectene-Pro transfected cells showed substantial KAP-1 knock-down compared to the mock-transfections, indicating the equivalent ability of these reagents to introduce siRNA duplexes into primary human fibroblasts.

Use of Metafectene-Pro to express GFP in primary human fibroblasts

Near-confluent 1BR3 human primary fibroblasts were trypsinized and diluted with fresh medium (MEM+15%FCS) to a concentration of 1 x 10^5 cells/mL. 1 ug of the plasmid pEGFP-N1 or an equivalent volume of water (=mock transfection) was combined with 50 uL of OPTI-MEM® (Gibco-Invitrogen). 5 uL of Metafectene-Pro was combined with 50 uL of OPTI-MEM® and immediately added to the diluted plasmid. Diluted Metafectene-Pro and plasmid were incubated for 15 minutes at room temperature and then pipetted into the middle of a glass coverslip placed into the centre of a 6 cm tissue culture dish. 2 mL of the 1BR3 cells (at 1 x 10^5 cells/mL) were gently pipetted on top of the transfection reaction. 48 hours later, cells were fixed, permeablized and counterstained for DAPI. Transfection efficiency was assessed by GFP immunofluorescence (Figure 3C). Remarkably, a number of 1BR3 human primary fibroblasts were clearly observed to be successfully expressing GFP. Although the transfection efficiency was low (<15%), this is a substantial improvement over prior attempts. Previously, we have been unable to achieve any degree of expression in 1BR3 primary human fibroblasts from transfected expression constructs (such as GFP) using standard reagents such as Genejuice® (data not shown).

Summary:

In summary, we find that Metafectene-Pro demonstrates a two-fold improvement over Genejuice® in the transfection efficiency of a wide variety of expression constructs into either MEFs or transformed human cells. Importantly, Metafectene-Pro achieves modest transfection of GFP into primary human fibroblasts, a cell line we have hitherto been unable to transfect. The versatility of of Metafectene-Pro is evident in its ability to transfect siRNAs as well as plasmids. This advantage is further highlighted by the ability of this reagent to transfect primary human cells with siRNA to a similar degree as HiPerFect Transfection Reagent from Qiagen (which is not useful for plasmid expression). We are presently examining whether Metafectene-Pro may be used for simultaneous KAP-1 knockdown by siRNA and KAP-1 mutant expression from siRNA-resistant plasmid constructs using a single round of transfection. If

successful, such a technique would become highly valuable for carrying out now routinely asked for, but technically challenging ‘protein replacement’ studies.

References:

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3. Goodarzi AA, Noon AT, Deckbar D, Ziv, Y, Shiloh, Y, Löbrich M, Jeggo PA. (2008) ATM signalling facilitates repair of DNA double strand breaks associated with heterochromatin. *Currently completing revisions for Mol Cell.*

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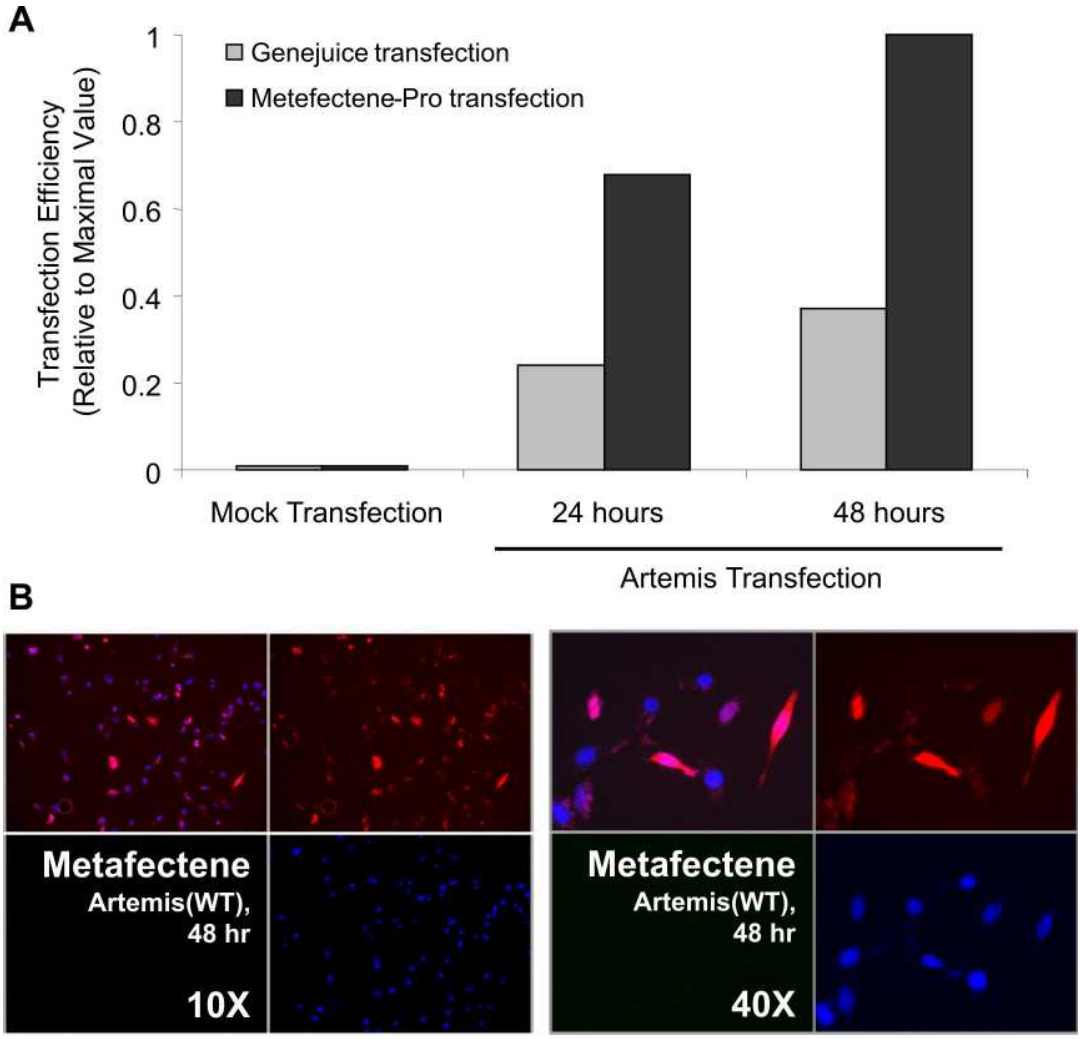

Figure 1: Relative Artemis Expression in MEFs transfected by Metafectene-Pro versus Genejuice. Panel A: Relative transfection efficiency of Genejuice (light grey bars) versus Metafectene-Pro (dark grey bars) mediated Artemis-Myc expression in wildtype MEFs. Panel B: Sample images of Myc-signal (red) and DAPI (blue) at 10X magnification and 40X magnification.

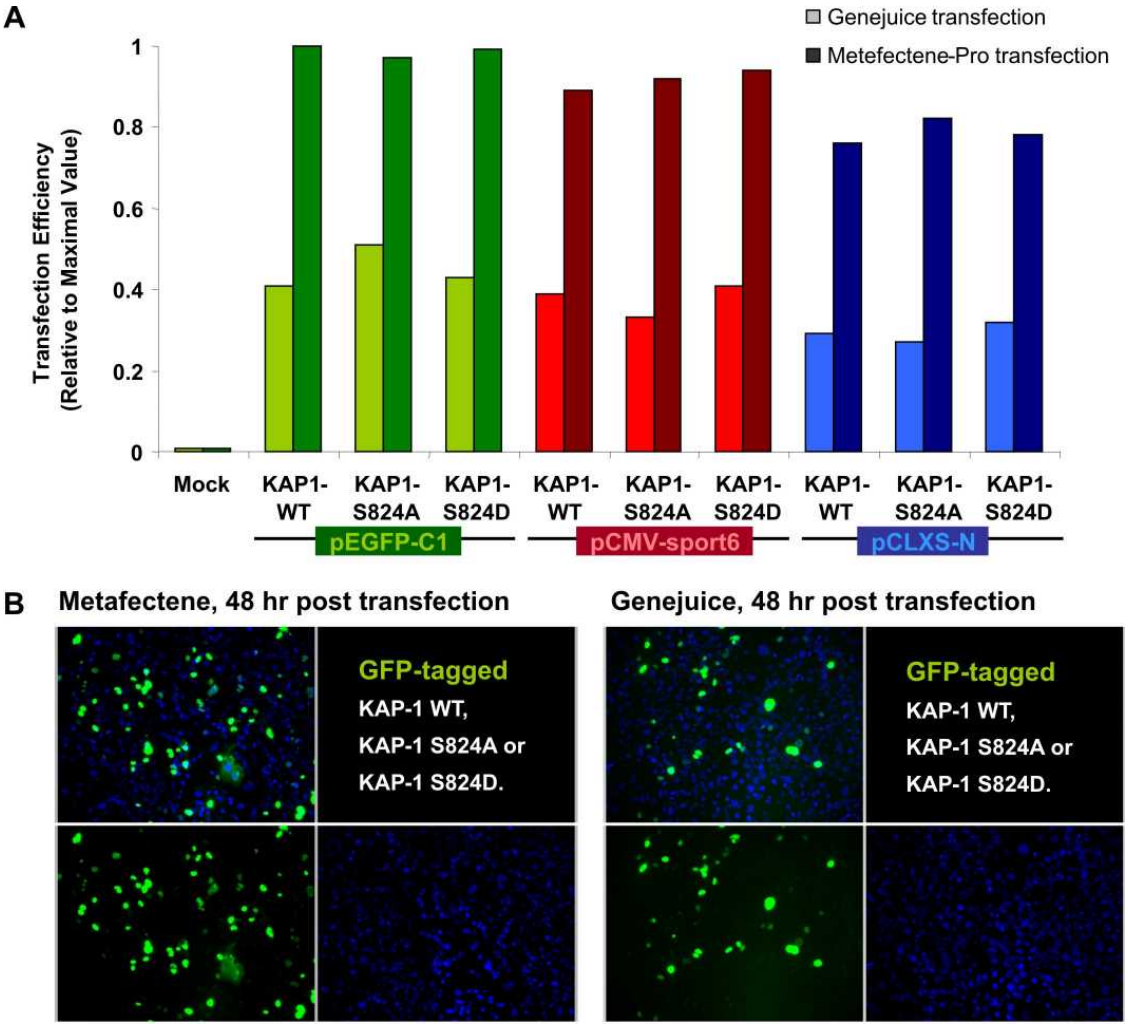

Figure 2: Relative Expression of KAP-1 expression constructs in 1BRneo human cells transfected by Metafectene-Pro versus Genejuice. Panel A: Relative transfection efficiency of Genejuice (light colour bars) versus Metafectene-Pro (dark colour bars) mediated expression of pEGFP-C1 (green), pCMV-sport6 (red) and pCLXS-N vectors encoding either WT, S824A or S824D KAP-1 into 1BRneo cells. Panel B: Sample images of GFP-signal (green) and DAPI (blue) at 10X magnification.

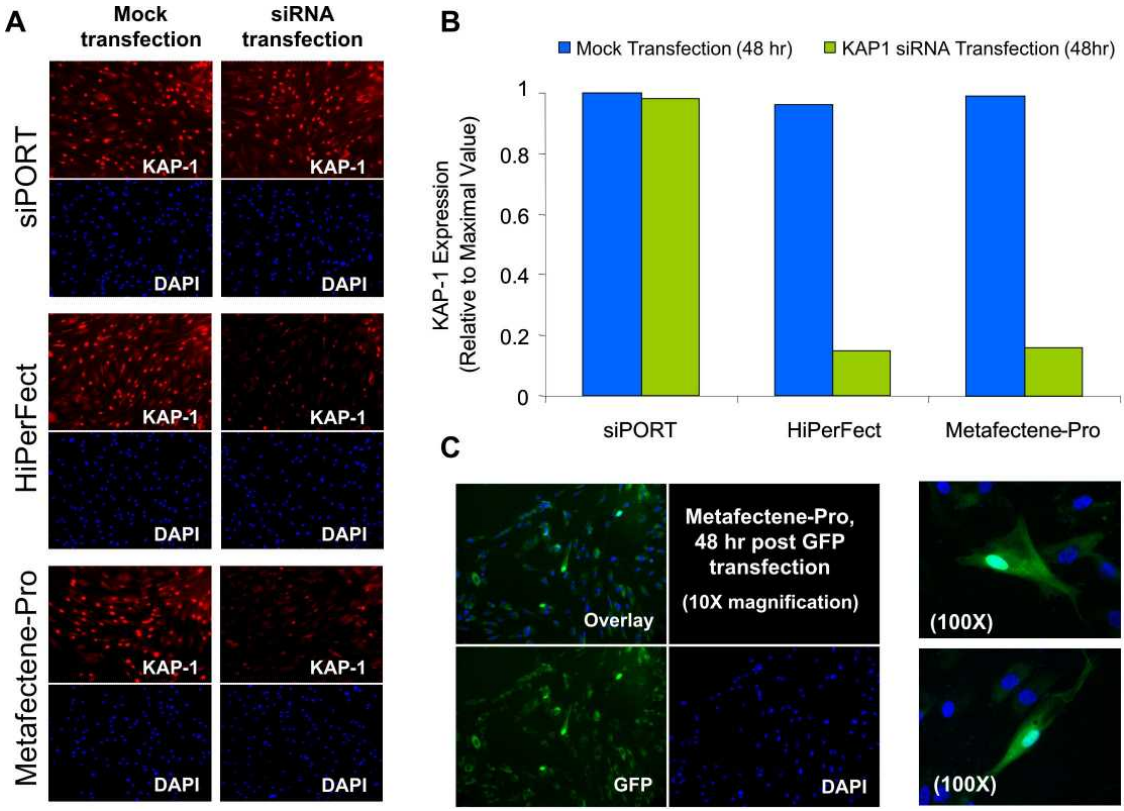

Figure 3: Transfecting Primary Human Fibroblasts with either siRNA or GFP expression constructs. Panel A: Sample images of endogenous KAP1 signal (red) and DAPI (blue) in Metafectene-Pro versus HiPerFect or siPORT™NeoFX™ mediated KAP-1 knockdown in 1BR3 primary human fibroblasts transfected with KAP-1 siRNA at 10X magnification. Panel B: Relative transfection efficiency of (A). Panel C: Sample images of GFP-signal (green) and DAPI (blue) at 10X magnification (right) or 100X magnification (left) for 1BR3 primary human fibroblasts transfected with pEGFP-N1 by Metafectene-Pro.