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Differential susceptibility of human primary aortic and coronary artery vascular cells to RNA interference

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ABSTRACT

Background: RNAi technology is a promising tool for gene therapy of vascular disease. However, the biological heterogeneity between endothelial (EC) and vascular smooth muscle cells (SMC) and within different vascular beds make them differentially susceptible to siRNA. This is further complicated by the task of choosing the right transfection reagent that leads to consistent gene silencing across all cell types with minimal toxicity. The goal of this study was to investigate the intrinsic RNAi susceptibility of primary human aortic and coronary artery endothelial and vascular smooth muscle cells (AoEC, CoEC, AoSMC and CoSMC) using adherent cell cytometry.

Methods: Cells were seeded at a density of 5000 cells/well of a 96well plate. Twenty four hours later cells were transfected with either non-targeting unlabeled control siRNA (50 nM), or non-targeting red fluorescence labeled siRNA (siGLO Red, 5 or 50 nM) using no transfection reagent, HiPerFect or Lipofectamine RNAiMAX. Hoechst nuclei stain was used to label cells for counting. For data analysis an adherent cell cytometer, Celigo was used.

Results: Red fluorescence counts were normalized to the cell count. EC displayed a higher susceptibility towards siRNA delivery than SMC from the corresponding artery. CoSMC were more susceptible than AoSMC. In all cell types RNAiMAX was more potent compared to HiPerFect or no transfection reagent. However, after 24 h, RNAiMAX led to a significant cell loss in both AoEC and CoEC. None of the other transfection conditions led to a significant cell loss.

Conclusion: This study confirms our prior observation that EC are more susceptible to siRNA than SMC based on intracellular siRNA delivery. RNAiMax treatment led to significant cell loss in AoEC and CoEC, but not in the SMC populations. Additionally, this study is the first to demonstrate that coronary SMC are more susceptible to siRNA than aortic SMC.

1. Background

Intimal hyperplasia (IH) remains the leading cause of arterial bypass graft failure. In recent years gene therapy approaches such as RNA interference (RNAi) gained interest as a possible treatment modality for IH. Several studies have applied RNAi technology to modulate the response to vascular injury *in vitro* and *in vivo* [1–3]. To facilitate sufficient siRNA delivery it is important to define optimal transfection reagent as well as route of delivery [4]. In addition, it is known that, EC and SMC from different segments of the vasculature have inherently different biological properties [5,6]. We have previously shown that EC derived from human coronary arteries are more susceptible to siRNA delivery than the corresponding SMC [7].

Interestingly, in our own observations, target gene knockdown achieved in primary human coronary SMC was significantly higher compared to AoSMC under identical transfection conditions (data not shown). This observation suggested a different intrinsic susceptibility of these subsets of SMC towards siRNA. Particularly, if less susceptible cells such as primary human AoSMC are to be targeted, the choice of transfection modality can be pivotal [4]. We recently showed that fluorescently labeled transfection indicator siRNAs (siGLO Red, Dharmacon Inc., Lafayette, CO) and adherent cell cytometry can be used to quickly assess rates of transfection of live adherent AoSMC [4]. Furthermore, cellular fluorescence inversely correlated with levels of gene knockdown measured by Q-RT-PCR [4].

After having previously examined the susceptibility of AoSMC's towards siRNA, it is logical to investigate the response of AoEC, CoEC and CoSMC towards RNAi as these cell populations represent some of the most relevant cell types for vascular gene therapy [4].

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This study focuses on siRNA delivery into these cell populations and cross comparisons between them.

2. Methods

In order to specifically assess and cross compare the susceptibility of AoEC, CoEC, AoSMC and CoSMC towards RNAi and commercial transfection reagents, the established adherent cell cytometry protocol were applied again [4]. This protocol allowed rapid analysis of different transfection conditions with a reduced dependence on Q-RT-PCR.

2.1. Cell culture

Human aortic and coronary endothelial and smooth muscle cells (Lonza, Walkersville, MD) were cultured in basal medium (LifeLine, Walkersville, MD) enriched with the supplied EC and SMC growth supplements. The media was maintained in a humidified incubator at 37 °C with 5% CO_2. Cells from passages 6–9 were used in the experiments.

2.2. siRNA/siGLO Red transfection

Cells were seeded at a density of 5000 cells/well in a BD Falcon 96-well black-bottom plate (Fisher, Pittsburg, PA). Twenty four hours later, cells were transfected with either non-targeting unlabeled siRNA (50 nM) (no fluorescence control, CAT#ID D-001206-13-20, Dharmacon, Lafayette, CO), siGLO Red® (5 or 50 nM) (Dharmacon, Lafayette, CO) using no transfection reagent, HiPerFect® (Qiagen, Valencia, CA), or Lipofectamine RNAiMAX™ (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. A master mix was created for each individual condition in order to eliminate pipetting errors and to increase consistency between each well. A total of 576 sample wells were analyzed. The experimental set-up of the transfection conditions for each cell type is outlined in Fig. 1.

2.3. Plate analysis with the adherent cell cytometry system Celigo™

In brief, cells were stained with fluorescence nuclear stain (Hoechst nuclei stain, 2.6 μg/mL, Invitrogen) to obtain absolute cell counts and red fluorescence tagged siRNA, siGLO Red was used to quantify cellular siRNA uptake. The Celigo system allowed rapid quantification of cellular fluorescence expression as previously described [4]. Plates were analyzed using the adherent cell cytometer equipped with brightfield and fluorescent channels: a blue (DAPI) filter for the Hoechst nuclear stain and a red filter (PE) for the siGLO Red.

Gating parameters were adjusted for each fluorescence channel to exclude background and other non-specific signals. The Celigo system provided a gross quantitative analysis for each fluorescence channel and individual well, including total count and average integrated red fluorescence intensity (AIRFI) of gated events.

2.4. Fluorescence expression analysis

Identical gating and exposure settings were used for each cell type and experiment to allow for cross-comparison between the groups. In all treatment groups only minimal background fluorescence was detected (Supplementary Fig. 1). Fluorescence expression analysis was performed, in two different ways, fluorescent pixel count or AIRFI.

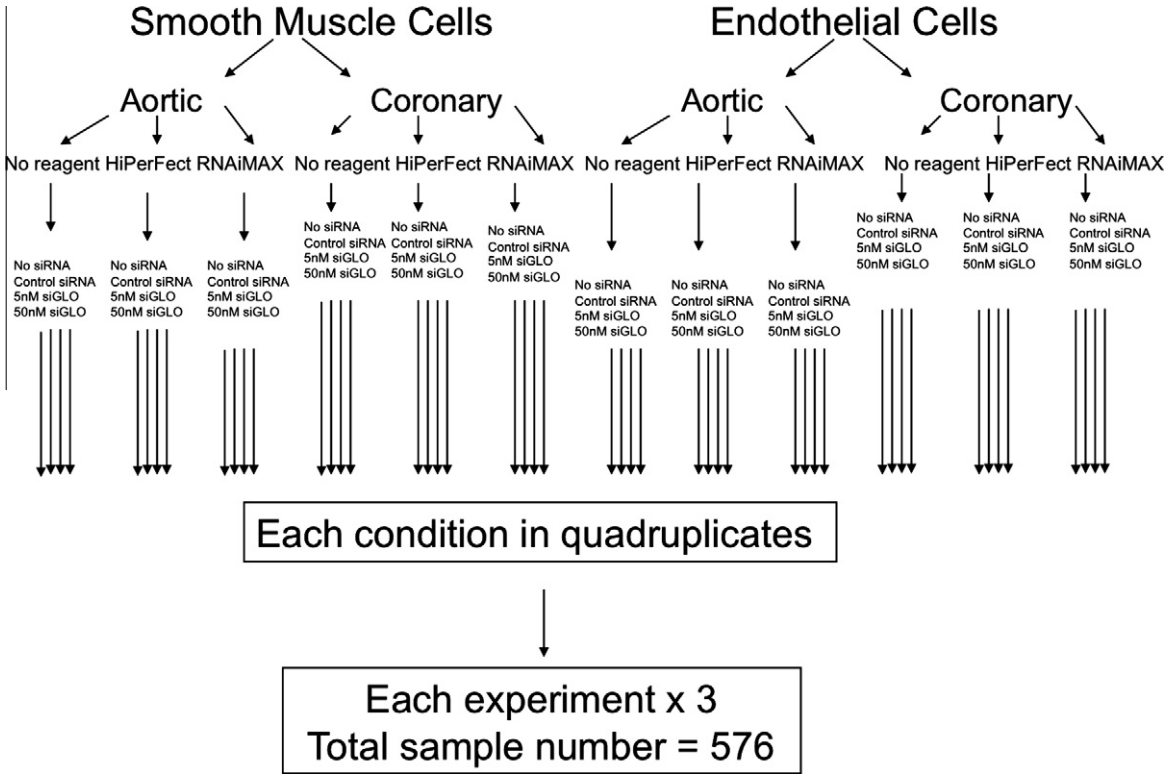

Fig. 1. Experimental conditions and total number of samples. Human aortic and coronary SMC and EC were transfected in the presence or absence of a transfection reagent (HiPerFect or RNAiMAX; 0.375 μl/100 μl each). Each group was further divided into four sub-groups: no siRNA, 50 nM unlabeled control siRNA, and 5 nM and 50 nM of siGLO Red transfection indicator. Each treatment was carried out in quadruplicates and each experiment was repeated three times for a total sample size of 576.

2.5. Statistical Analysis

At least three independent experiments were performed and results were analyzed using Graph Pad Prism Version 5.0 software (Graph Pad Software Inc., La Jolla, CA). For cell viability and fluorescence expression analysis, two-way ANOVA with Bonferroni post hoc analysis was used to assess statistical significance. A '*p*' value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Cell count and cytotoxicity of transfection reagents

In order to quantify cytotoxicity of our treatments, cell counts were assessed 16 h post-transfection. Cell counts (i.e. nuclear counts) were obtained using the fluorescence expression analysis based on Hoechst nuclear stain. RNAiMAX treatment led to a significant cell loss in both AoEC and CoEC ($p < 0.05$) transfected with unlabeled control siRNA or siGLO Red irrespective of concentration. Interestingly, no other transfection condition caused any significant cell loss in any cell type investigated ($p > 0.05$) (Fig. 2).

3.2. AoSMC and CoSMC analysis (fluorescent pixel count/per cell)

Similar to a previous study, there were significant differences in siRNA delivery in AoSMC and CoSMC [4]. Under identical transfection conditions more siGLO Red could be delivered into CoSMC compared to AoSMC (Fig. 3, Supplementary Tables 1 and 2). The transfection differences depended on siRNA concentration and choice of transfection reagent. RNAiMAX proved to be superior to HiPerFect or no reagent (Fig. 4A, Supplementary Tables 1 and 2). These differences reached statistical significance when 50 nM siGLO Red were complexed with either HiPerFect or RNAiMAX (Supplementary Table 1). Extracellular background fluorescence was negligible in either case (Supplementary Fig. 1, Supplementary Tables 1 and 2).

For a general cross-comparison with EC the AIRFI instead of fluorescence pixel count per cell was used (Fig. 4B). AIRFI is a function of size (pixel count per gated event) and fluorescence intensity of the individual pixel within a gated event (value between 0 and 255). AIRFI is a standardized analytical algorithm of the Celigo system and given the technical setting, was better suited to quantify the blotchy fluorescence signals observed in AoEC and CoEC and permit individual signal cross-comparison between EC and SMC.

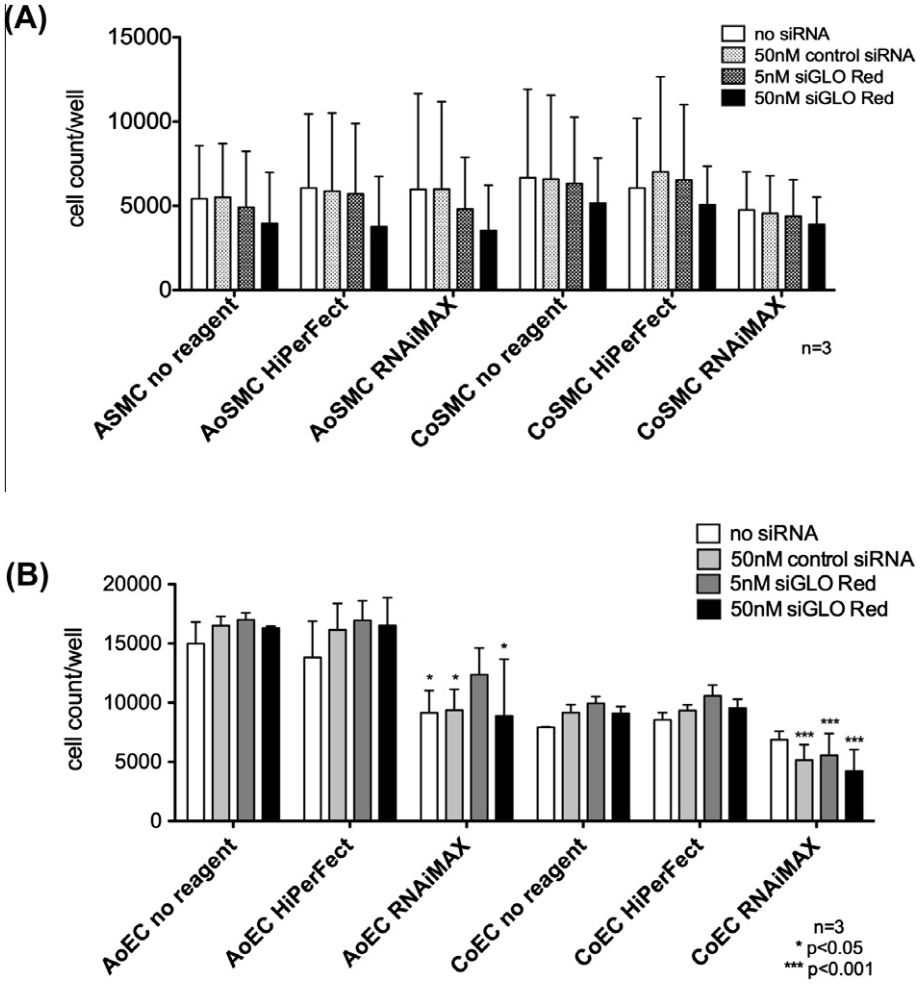

Fig. 2. Effect of transfection reagents on cell counts. (A) In neither AoSMC nor CoSMC significant cell losses was observed after incubation with either transfection reagent. No significant differences in overall cell counts between AoSMC and CoSMC were seen. A slight trend in cell loss was observed in the AoSMC groups that appeared to be siRNA (siGLO Red) concentration dependent yet independent of the transfection reagent. (B) Cell loss was observed in human AoEC after transfection with RNAiMAX transfection reagent irrespective of the presence of siRNA. In human CoEC, RNAiMAX alone did not lead to a significant cell loss, however the combination of RNAiMAX and siRNA or siGLO Red did lead to cell loss. Cell loss was overall less compared to that seen in the AoEC. Interestingly, AoEC appeared to proliferate faster in culture during the same time interval compared to CoEC.

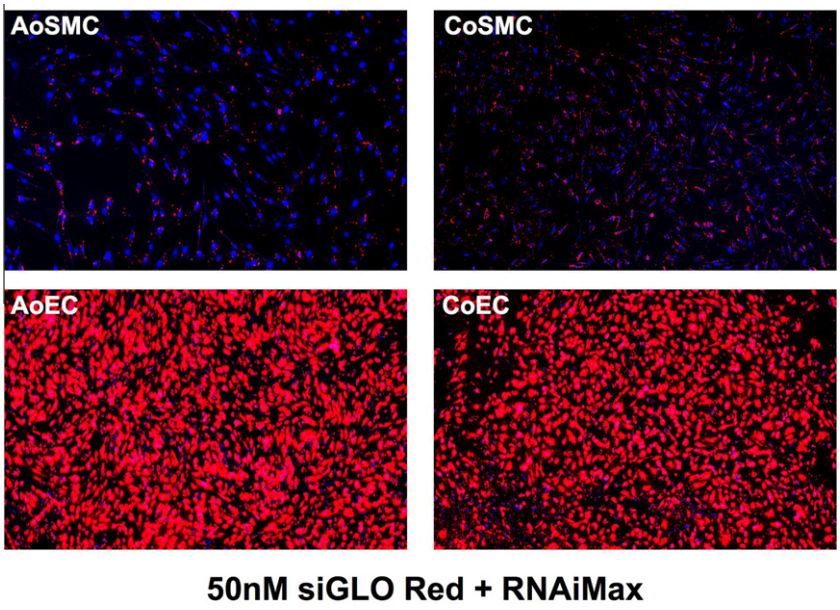

Fig. 3. RNAi susceptibility of AoSMC, CoSMCm AoEC and CoEC. All cells were transfected with 50 nM siGLO Red complexed with RNAiMAX. Note the significant differences between the SMC and EC populations. More subtle differences in siGLO Red uptake were seen between AoSMC and CoSMC with CoSMC showing more siGLO Red than AoSMC under identical transfection condition. In this experimental setting no apparent differences in siGLO Red uptake were seen between AoEC and CoEC. Blue represents Hoechst stained nuclei and red represents siGLO Red. Identical microscope exposure settings were used to obtain the images.

However, given the homogenous, punctuated siGLO Red distribution pattern observed in SMC treatment groups, AIRFI is not perfectly suited to discriminate transfection results amongst the SMC population.

3.3. AoEC and CoEC analysis (average integrated red fluorescence intensity)

Size of fluorescence events observed in EC was magnitudes larger than in SMC so that individual fluorescence pixel merged into large confluent blotches. In this setting the use of fluorescence red pixel count/nuclei resulted in falsely low values and made an accurate analysis impossible (Fig. 3). AIRFI is a function of size (pixel count per gated event) and fluorescence intensity of the individual pixel within a gated event (value between 0 and 255) [4]. AIRFI is a standardized analytical algorithm of the Celigo system and given the technical setting, was best suited to quantify the blotchy fluorescence pattern observed in AoEC and CoEC.

Strong differences in siGLO Red uptake were observed in AoEC and CoEC depending on the choice of transfection reagent and concentration of siGLO Red (Supplementary Table 2). Similar to SMC, RNAiMAX was more efficient in delivering siGLO Red into EC than HiPerFect or no transfection reagent. Unlike in SMC, there were no significant differences in siGLO Red uptake between AoEC and CoEC (Fig. 3 and Supplementary Table 2).

3.4. Comparison of transfection results in human primary AoEC, CoEC, AoSMC, CoSMC

Similar background fluorescence values for all four investigated cell types were observed (Supplementary Fig. 1, Supplementary Tables 1 and 2). SMC were less susceptible to siRNA than EC under identical transfection conditions (Fig. 3). 50 nM siGLO Red complexed with RNAiMAX led to the highest siRNA uptake in all cell types (Fig. 4C, Supplementary Tables 1 and 2).

4. Discussion

The vascular milieu poses various biological challenges for gene therapy. The present study analyzes some of these challenges in an automated and objective manner. The biology of EC and SMC is remarkably different. Additionally, significant differences have been described within EC and SMC populations from different vascular beds [5,6]. Previously our lab demonstrated that adherent cell cytometry is useful to assess uptake of fluorescently labeled siRNA into AoSMC. Importantly, the level of cellular siRNA fluorescence signal inversely correlated with the level of gene silencing [4].

The present study expanded on our previous finding depicting higher susceptibility towards RNAi of primary human EC vs. SMC within the same vascular bed [7].

Our current study provides further evidence for this observation. Interestingly, on average, siRNA uptake was higher in CoSMC than in AoSMC under identical transfection conditions. These differences reached significance when SMC were transfected with 50 nM siGLO Red complexed with RNAiMAX. This data matched Q-RT-PCR results from our lab, which indicated that under identical transfection conditions gene knockdown in primary human CoSMC on average is higher than in AoSMC (data not shown).

A direct side-by-side comparison of AoEC and CoEC did not reveal significant differences.

The findings of this study are relevant for the development of vascular RNAi therapies. A successful therapy must not only factor in the difference in susceptibility of EC and SMC towards RNAi but also the differential toxicity that the transfection reagents might exert on the cells. This is particularly important when transitioning from *in vitro* experiments to *in vivo* models or when targeting different segments of the vasculature since a 'one size fits all' approach cannot be employed. For example, siRNA delivery modality for treating aortic aneurysms will be different than treating intimal hyperplasia, even if the target cells could be SMC in both cases.

In conclusion RNAi therapy although promising, requires considerable customization with respect to target cell delivery of

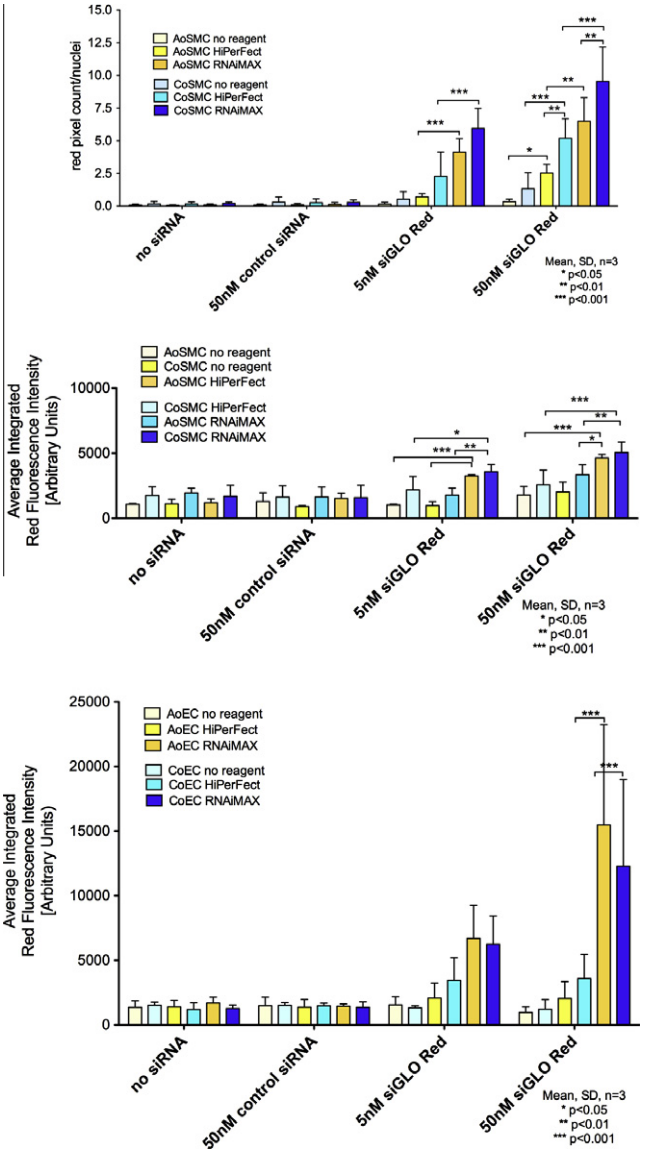

Fig. 4. Comparison of siGLO Red counts per cell between aortic and coronary SMC. (A) Comparison of siGLO Red pixel count/cell between different transfection reagents in AoSMCs that have either not been transfected (no siRNA) or were transfected with 50 nM control siRNA (not fluorescently labeled), 5nM siGLO Red or 50 nM siGLO Red. In both AoSMC and CoSMC, a siGLO Red dose dependent and transfection reagent dependent increase in red pixel/cell count was observed. RNAiMAX proved to be superior to HiPerFect or no transfection reagent used. (B) Instead of red pixel/nuclei the parameter 'average integrated red fluorescence intensity (AIRFI)' was used for cross comparison between SMC populations. AIRFI is a function of the amount of pixel per gated event and their individual signal intensity. This parameter is better suited to analyze the blotchy fluorescence signals obtained in EC. Given the homogenous, punctuated siGLO Red distribution pattern observed in SMC treatment groups, AIRFI is not well suited to discriminate transfection results amongst SMC. However, the general trends seen in the prior SMC analysis (red pixel/nuclei) are also seen using AIRFI. (C) Cross comparison of AIRFI values in AoEC and CoEC. In particular in the RNAiMAX group the differences in AIRFI values become apparent between the EC and SMC (B).

siRNA and transfection modality especially if subsets of vascular cells from different beds are targeted.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CSN – Experimental design, data acquisition and analysis, editing of the manuscript.
MC – Data acquisition, editing of the manuscript.
LPN – Experimental design, manuscript review.
SY – Editing of the manuscript.
FWL – Experimental design, manuscript review.
All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.078.

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