

# Newsletter

## "OZB Highlights" 2011 - Volume 3 Number 1

### Summary :

#### **Efficient production of stably transfected Fibroblast expressing WRN gene with DreamFect™ reagent** 2

Ammazzalorso *et al.* ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery.  
The EMBO Journal 2010, 29: 3156 - 3169

#### **COX-2 gene silencing in MC3T3-E1 cells using SilenceMag transfection reagent** 3

F. Zhang *et al.* Interleukin-17A induces cathepsin K and MMP-9 expression in osteoclasts via celecoxib-blocked prostaglandin E2 in osteoblasts.  
Biochimie 2011, 93(2): 296-305

#### **Efficient delivery of anti-ricin antibody with Ab-DeliverIN™ in RAW264.7 cells** 4

F. Wu *et al.* Protective effects of anti-ricin A-chain antibodies delivered intracellularly against ricin-induced cytotoxicity.  
World J Biol Chem 2010, 1: 188-195



# Efficient production of stably transfected Fibroblast expressing WRN gene with DreamFect™ reagent.

## **Ammazzalorso et al. ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. The EMBO Journal (2010) 29, 3156 - 3169**

Improper response to fork replication arrest could give chromosomal rearrangements accumulation leading to chromosome fragility syndromes. The Werner syndrome is a disorder characterized by the appearance of premature aging caused by a mutation in the WRN gene which codes for a DNA helicase. The loss of WRN function engenders hypersensitivity to agent interfering with replication and fragility of chromosomal regions involved in replication fork stalling. Previous works already suggested that ATR/ATM phosphorylation of WRN protein is of importance for replication fork recovery during the response to perturbed replication, but such modifications are not fully appreciated.

In this paper Ammazalorso et al. focused their work on the influence of ATR/ATM phosphorylation of WRN on the different steps of the replication fork recovery reaction.

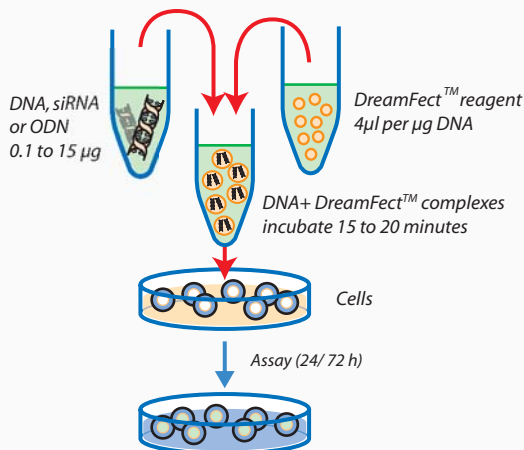
As ATR, but not ATM, is the primary kinase involved in the response to replication stress, the authors first showed that WRN is a substrate to ATR without overlapping ATM phosphorylation sites. By using specific cell model (Hela cells in which ATR is depleted) they identified 3 out of 6 Ser/Thr-Gln (S/TQ) sites of WRN only phosphorylated by ATR under replication arrest condition.

They then proved that WRN phosphorylation by ATR prevents double strand break (DSB) formation at stalled fork site allowing replication recovery. To reach this conclusion they employed WS fibroblast stably expressing wt WRN (WSWRN) or mutated for WRN at 3 (WSWRN3A) or 6 (WSWRN6A) S/TQ sites (unphosphorylatable position). These cell line models were generated by stably transfecting the corresponding plasmid into the WS cell line using **DreamFect™** (see scheme 1), a universal nucleic acid transfection reagent. After plasmid transfection and stress replication conditions the authors observed that phosphorylation of WRN by ATR is essential for:

- i) WRN re-localisation at sites of replication fork stalling
- ii) avoiding degeneration of forks into DSBs
- iii) recovering from perturbed replication.

Authors have also used **DreamFect™** to transiently transfect HeLa and HEK293 cells for immunocomplex kinase assay with Flag-ATM and Flag-ATR constructs.

Altogether the generation of stable transfectants using **DreamFect™** allowed the authors to decipher the vital role of WRN phosphorylation in the response to replication fork arrest.



**Scheme 1:** The WS cell line was cultured in DMEM, supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Stably transfected WS cells were generated by transfection of various constructs using the transfection reagent **DreamFect™** followed by selection of stable transfectants using G418. Protein expression levels were analyzed by western blotting 48 h post-transfection.

### Green Fluorescent Protein expression in different cell lines transfected with DreamFect™



Cells ( $2 \times 10^5$ ) were transfected with 1 µg/well of pEGFP plasmid and 4 µl of **DreamFect™** reagent in 12-well plates. EGFP expression was monitored 48 h after transfection by fluorescence microscopy.

#### **Other successful DreamFect™ Gold & DreamFect™ transfections:**

- MDCK & BHK cells: Theard D. *et al.* The EMBO journal. 2010, 29:1499 - 1509
- HeLa cells: Miller EW. *et al.* PNAS. 2010, 107:15681-15686
- PC-12 & HEK-293 cells: Grohman M *et al.* PLoS ONE. 2010, 5(1): e8956
- C2C12 H. Bernardi *et al.* Am J Physiol Cell Physiol., doi:10.1152/ajpcell.00214.2010
- U87 & U118 & C272 & WI38 & SHSY5Y: M. Shao *et al.* Nucleic Acids Research 2011, doi:10.1093/nar/gkq1305
- MEF: Chen *et al.* Science Signaling. 2011, 4 (161), ra10

## F. Zhang et al. Interleukin-17A induces cathepsin K and MMP-9 expression in osteoclasts via celecoxib-blocked prostaglandin E<sub>2</sub> in osteoblasts.

*Biochimie (2011), 93(2):296-305*

Interleukin-17 (IL-17) is a pro-inflammatory cytokine secreted by activated T cells or neutrophils, that can stimulate the development of osteoclasts in the presence of osteoblasts.

Acting through osteoblasts, IL-17 has been shown to promote osteoclasts differentiation indirectly by the regulation of bone resorption-related enzymes mainly cathepsin K and the Matrix-Metalloproteinase (MMP)-9.

In a previous work, Zhang et al. used RAW264.7 as osteoclast precursors to study the direct effect of IL-17A on their differentiation. They showed that this interleukin suppressed the hydrolysis of matrix bone proteins during bone resorption by decreasing the production of cathepsin K and MMP-9.

In this paper, Zhang et al. deciphered the indirect effect of IL-17A using MC3T3-E1 cells (mouse calvarial cell lines) and RAW264.7 cells as osteoblasts and osteoclast precursors. Osteoblasts were cultured with IL-17A and/or celecoxib (a cyclooxygenase (COX)-2 inhibitor). The medium was then conditioned in presence of soluble RANKL, a member of TNF cytokine family important in bone metabolism. When celecoxib was added, the effects of IL-17A (number of nucleated cells and the expression of cathepsin K and MMP-9 increase) were blocked.

The authors confirmed the effect of COX-2 silencing on cathepsin K and MMP-9 protein expression in RAW264.7 cells by transfecting siRNAs with **SilenceMag** transfection reagent in MC3T3-E1 cells cultivated in  $\alpha$ -minimal essential medium containing 10% FBS.

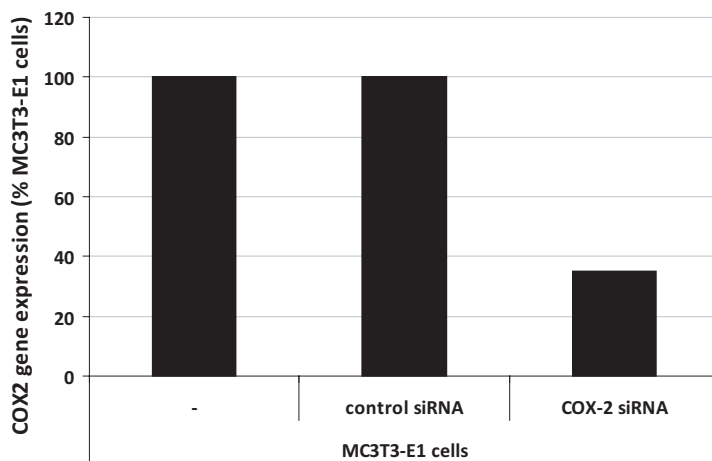
After reaching 80% confluence, MC3T3-E1 cells were transfected with a pool of 4 siRNAs (5 nM) directed against COX-2 using **SilenceMag**.

72 hours later, total RNA was extracted and supernatants were collected, conditioned and used for RAW264.7 cell culture. Real time PCR analysis showed that 5 nM of siRNA-COX-2 transfected with **SilenceMag** induced a significant decrease in COX-2 gene expression of 65% compared to control siRNA (figure 1).

Moreover, the indirect induction of cathepsin K and MMP-9 protein expression in RAW264.7 cells by IL-17A was dramatically inhibited when COX-2 expression in MC3T3-E1 cells was inhibited by RNA silencing.

Taken together the results highlighted that IL-17A stimulates not only the differentiation of RAW264.7 cells into osteoclast-like cells but also the expression of cathepsin K and MMP-9 in osteoclast-like cells.

In addition, PGE<sub>2</sub> (Prostaglandine E<sub>2</sub>) production from MC3T3-E1 cells increased in the presence of IL-17A indicating that IL-17A indirectly induces the differentiation and function of osteoclasts via a COX-2-dependant prostaglandin in osteoblasts.



**Figure 1: COX-2 gene silencing in MC3T3-E1 cells using SilenceMag transfection reagent.**

The gene expression level was determined using real-time PCR and expressed as a percentage of untransfected cells. Supernatants of COX-2 KO cells were further used to stimulate RAW264.7 cells.

### Other successful SilenceMag applications:

- HUVEC & HMEC1: S.Simoncini *et al.* Circ Res. 2009, 104(8):925-7
- Neurons: G. de Lartigue *et al.* Endocrinology. 2010, 151(8):3589-3599
- C2C12: Y.Tajika *et al.* Acta Histochem. Cytochem. 2010, 43 (4): 107-114
- Primary Gastric Gland: S. Kenny *et al.* Am J Physiol Gastrointest Liver Physiol. 2008, 295:431-441
- A7r5: M Li *et al.* Pharmacological Research, 2008, 58(5-6):308-315

## F. Wu et al. Protective effects of anti-ricin A-chain antibodies delivered intracellularly against ricin-induced cytotoxicity. *World J Biol Chem* 2010, 1, 188-195.

Ricin, extracted from castor bean (*Ricinus communis*) is the most toxic substance in the plant kingdom.

It consists of a heterodimeric protein of two polypeptidic chains, the A-chain (RTA) responsible for the poisoning effect of ricin, and the B-chain (RTB), a galactose-binding protein that facilitates cell entry of the toxin. The RTA unit is an active enzyme that cleaves ribosomal RNA causing inhibition of protein synthesis.

Because of its stability, ease of production and its multiple ways of administration, ricin is considered as a potential powerful biological weapon, for which no antidote is available to date. There is therefore a tremendous demand for antitoxins that can be administered to counteract ricin poisoning lethal effect.

It has already been demonstrated that passive antibody administration following ricin exposure was efficient in protecting mouse against ricin-induced lethality, although the protective effect was highly dependent upon the delay between exposure and antibody treatment.

It was hypothesized that at later time point, antibodies failed to enter the cytosol and where therefore unable to neutralize the internalized ricin toxin. Intracellular delivery of anti-ricin antibodies, as opposed to previous passive administration, was expected to circumvent this limitation and thus prolong the therapeutic window after ricin exposure.

In this paper, Wu et al. investigated the ability of anti-RTA antibodies delivered intracellularly to protect against ricin-induced cytotoxicity in murine macrophages RAW264.7 cells.

In a first set of experiments, the protective effects of two anti-ricin antibodies (Anti-deglycosylated ricin-A chain antibody dgA Ab and RAC18 anti ricin-A monoclonal antibody RAC18 mAb) was assessed.

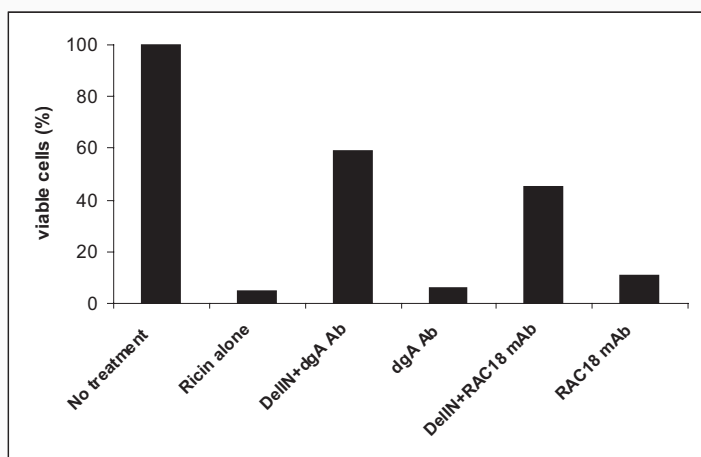
To this end, the antibodies were delivered intracellularly in RAW264.7 with **Ab-DeliverIN™**, a **specific reagent for antibody delivery within cells**. After 8h, cells were washed to remove residual extracellular antibodies and then exposed to ricin. Only cells treated with antibody / DeliverIN were protected against ricin toxicity (fig. 1).

In contrast, no protection was observed when cells were treated with dgA Ab or RAC18 mAb without **Ab-DeliverIN™** reagent, thereby indicating that intracellular localization of the antibody, mediated by **Ab-DeliverIN™** was necessary to exert its protective effect. Fluorescence observations further confirmed successful delivery of antibodies with **Ab-DeliverIN™** and the intracellular colocalization of Alexa-labeled ricin and RAC18 mAb.

In order to mimic a more realistic situation where antibodies would function as an antidote after ricin exposure, cells were first exposed to ricin, and peptide-conjugated antibodies were then administered at various time points. Up to 4h after ricin exposure, antibody delivery was able to increase cell survival.

In summary, the authors showed that intracellular delivery of neutralizing antibodies before or after ricin exposure confers protection against the toxin cytotoxic effects.

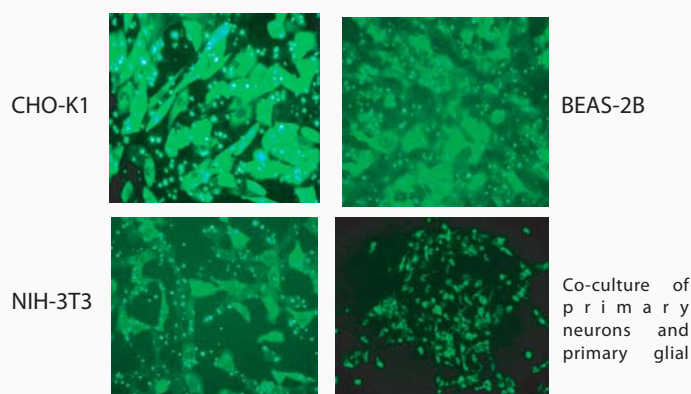
Cell-permeable antibodies might therefore represent an innovative approach to extend the therapeutic window after ricin intoxication.



**Figure 1: Protective effect of Ab-DeliverIN (DelIN) mediated delivery of antibodies against ricin-induced toxicity.**

RAW264.7 cells treated with dgA Ab or RAC18 mAb showed increased survival after ricin exposure when the antibodies were delivered with Ab-DeliverIN™ as compared to antibodies alone.

**Ab-DeliverIN™ is suitable for numerous cell types. This reagent has been successfully tested on a variety of immortalized cell lines as well as some primary cells.**



Fluorescently-labeled IgG delivery

### **Ab-DeliverIN™ is the first dedicated intracellular antibody delivery reagent**

- Functionally active antibodies delivery
- Highly efficient in many primary cells and cell lines
- Serum compatible
- Biodegradable and no cytotoxicity
- Easy: straightforward protocol and ready-to-use