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A Flexible Approach to Studying Post-Transcriptional Gene Regulation in Stably Transfected Mammalian Cells

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Abstract

The study of post-transcriptional regulation is constrained by the technical limitations associated with both transient and stable transfection of chimeric reporter plasmids examining the activity of 3'-UTR *cis*-acting elements. We report the adaptation of a commercially available system that enables consistent stable integration of chimeric reporter cDNA into a single genomic site in which transcription is induced by tetracycline. Using this system, we demonstrate the tight control afforded by this system and its suitability in mapping the regulatory function of defined *cis*-acting elements in the human TNF 3'-UTR, as well as the distinct effects of serum starvation on transiently transfected and stably integrated chimeric reporter genes.

Keywords

ARE; TNF; mRNA turnover; Translation; 3'-UTR

Introduction

Post-transcriptional events regulate gene expression at multiple steps, including splicing, polyadenylation, nuclear export, mRNA decay, and translation. Elucidating the contributions of *cis*-acting elements in the 5' and 3'-untranslated regions (UTR) of mRNA to these activities is largely dependent on transient transfection of reporter gene plasmids that lack or contain the sequence in question. Perhaps the most studied mediator of these pathways is the tumor necrosis factor- (TNF) 3'-UTR, that regulates nuclear export, cytoplasmic mRNA turnover and translation [1–12]. Transient transfection is a cumbersome, repetitive, labor-intensive process with many technical concerns (reproducibility, toxicity from the transfection process, gene expression from non-integrated plasmid DNA). Unfortunately, the generation of stable cell lines is equally problematic. In stable transfections, the inability to regulate the copy number or location of genomic integration results in significant differences due to variations in the transcriptional competence of integration sites. Moreover, sites of stable integration might independently result in changes in cellular phenotype due to effects on endogenous gene expression. Finally, transcriptional inactivation of the reporter gene by DNA methylation can vary considerably over the genome, and may change over time [13].

In this report, we describe a robust alternative for 3'-UTR sequence analysis through the adaptation of a commercially available system. Using this approach, we are able to generate many different 293 cell lines stably expressing single copy reporter gene integrants that demonstrate the suitability of this system for the study of the post-transcriptional regulation. This analysis examines the contributions of adenosine–uridine-rich (ARE)-dependent and -independent pathways, and demonstrates its suitability for the GFP reporter based system. These studies establish the strong potential of this system for increasing understanding of the regulatory function of the 3'-UTR.

Materials and Methods

Reagents

Chemicals were purchased from the following suppliers: blasticidin, hygromycin B and TRIzol were from Invitrogen (Carlsbad, CA); doxycycline was from Clontech (Palo Alto, CA); ionomycin and SB203580 were from Calbiochem (San Diego, CA); phorbol myristate acetate (PMA), rapamycin, SP600125, and wortmannin were purchased from Sigma (St. Louis, MO). Anti-GFP antibodies were from Invitrogen. Anti-tubulin antibodies were from Sigma.

Plasmid Constructs

FRT-GFP vectors—The globin-GFP-globin reporter gene contains the coding region from GFP with the 5'-UTR and 3'-UTR from the *X. laevis* b-globin gene [11, 14]. The globin-GFP-TNF reporter also contains 573 bases (nt 2781–3385, Accession # AY066019) of the human TNF 3'-UTR, introduced into the 3'-UTR of the GFP gene (Fig. 1a). The globin-GFP-TNF- ARE has the 34-nt ARE deleted from globin-GFP-TNF [11]. The globin-GFP-globin, globin-GFP-TNF, globin-GFP-TNF- ARE template sequences were PCR-cloned into pcDNA5/FRT-TO-Topo to create the FRT-GFP-globin, FRT-GFP-TNF, and FRT-GFP-ARE plasmids. Plasmid integrity was confirmed by DNA sequencing. The TNF Intron 3 and TNF No Intron 3 vectors were generated from genomic DNA and mRNA, respectively, and cloned into the luciferase reporter plasmid pGL3-Control (Promega, Madison, WI). The

same primer pairs were used to generate constructs from genomic and mRNA sources. TNF Intron 3 contains nucleotides 1833–3381 (Accession # X02910). TNF No Intron 3 contains nucleotides 396–1643 (Accession # X01394). Construct TNF Intron 3 ARE contains nucleotides 1833–2909 and 3131–3381.

FRT-GFP Cell Lines

Single copy, single-site integrant cell lines were created by recombination of the FRT-GFP plasmids into the genome of 293 cells at a single chromosomal site. The Flp-In™ System (Invitrogen) utilizes a Flp Recombinase Target site (FRT), engineered into a transcriptionally active chromosomal location (Fig. 1b). The FRT site includes two 13 base pair inverted repeats located 5' and 3' to an 8 base pair core sequence. Recombination between the reporter gene plasmid and the Flp site is mediated by the Flp recombinase, derived from *S. cerevisiae*, acting at the FRT site.

The FRT-GFP-globin, FRT-GFP-TNF, and FRT-GFP- ARE plasmids were separately co-transfected with pOG44 (encoding the Flp recombinase) into Parent FRT cells (Flp-In-T-REx-293 cells, Invitrogen). Recombination activates the plasmid hygromycin resistance gene, and cell lines were selected and maintained with hygromycin. The pcDNA5-FRT-TO-Topo plasmid utilizes a BGH polyadenylation signal, and contains a “TET-On” promoter for doxycycline-inducible gene expression. Blasticidin selects for stable expression of the Tet repressor in Flp-In-T-REx-293 cells. To screen for positive cell line clones, gene expression was induced with doxycycline and GFP protein expression was measured by flow cytometry and GFP mRNA was measured by RT-PCR.

The FRT-GFP cell lines were maintained at 37°C in DMEM (Mediatech, Manassas, VA) supplemented with 10% tetracycline-free fetal bovine serum (Clontech), blasticidin (15 µg/ml), and hygromycin B (200 µg/ml). On day 0, sufficient trypsinized cells were added to 24-well (15 mm) or 6-well (35 mm) plates to produce 50% confluence on day 1. Doxycycline (1.5 µg/ml) was added on day 1 for 18 h to induce expression of the GFP reporter genes.

For studying the effects of cell signaling on GFP expression, FRT-GFP 293 cells were cultured for 18 h induction with doxycycline, then treated with concentrations of PMA (20 ng/ml) and ionomycin (1 µM) for 4 h. PMA and ionomycin concentrations were used that provided optimal GFP induction and mRNA stabilization (data not shown). Solvent controls were used for each kinase inhibitor. For signaling pathway inhibition studies, cells were pretreated for 15 min with SB203580 (10 µM), SP600125 (10 µM), rapamycin (10 nM), wortmannin (100 nM), or solvent control (DMSO, methanol, or ethanol) prior to addition of PMA and ionomycin.

Serum Starvation Studies

Stable 293 cell lines expressing GFP-globin, GFP-TNF or GFP-TNF- ARE were treated for 18 h with doxycycline (1.5 µg/ml) to induce expression. Medium was changed to DMEM plus FBS (control), or DMEM without serum, and incubated for 24 h. Cells were trypsinized and levels of GFP fluorescence measured by flow cytometry. For transient transfection experiments, parent FRT cells were plated in 15 mm wells to achieve ~50% confluence on the day of transfection. Human TNF 3'-UTR plasmids (containing or lacking the Intron 3) were complexed with Lipofectamine 2000, following manufacturer's directions (Invitrogen), and added to cells in serum-free DMEM medium. After 4 h, medium with FBS (Control) or without FBS (starvation) was added to cells (final concentration of FBS was 10%). After 24 h, cells were lysed in 100 µl of CCLR lysis buffer (Promega) and the luciferase activity in 20 µl of cell lysates was measured in triplicate on a Berthold luminometer.

Flow Cytometry

Levels of GFP fluorescence were measured in trypsinized cells that were washed twice with cold phosphate-buffered saline (PBS), and resuspended in cold PBS. An aliquot of 5 μ l (~20,000 cells) was removed and diluted to 200 μ l total volume for flow cytometry. The remaining cells from each sample were used for RT-PCR analysis. To eliminate irrelevant signal, cells were gated to exclude fluorescence from dead cells and cellular debris. GFP protein levels were measured by FACScan (Becton–Dickinson, Franklin Lakes, NJ) set on the FITC channel, and data was processed using CellQuest (Becton–Dickinson) measuring 4000 gated events. For the purposes of these studies, background was considered to be the level of gated fluorescence from cells not induced with doxycycline. Background fluorescence from 293 cells without FRT-GFP genes was the same as background from FRT-GFP cells not treated with doxycycline. To confirm that GFP protein was expressed by FRT-GFP cells, proteins from cell lysates were separated on SDS-PAGE gels, transferred to nitro-cellulose, and probed with antibodies to GFP and tubulin (loading control).

RNA Extraction and Quantitative RT-PCR

Total cellular RNA was isolated by TRIzol (Invitrogen), dissolved in RNase free water, and analyzed by spectrophotometry. Ten micrograms of RNA was DNase I treated (Turbo DNA-free, Ambion, Austin, TX), and RNA was analyzed again by spectrophotometry. One microgram of RNA was reverse transcribed with oligo (dT) and Superscript II RT (Invitrogen). Reverse transcribed RNA was analyzed for GFP mRNA transcripts using 5'-GCTCGC CGACCACTACCAGCAGAACAC-3' (upper primer) and 5'-GCAGGACCATGTGATCGCGCTTCTC-3' (lower primer) with SYBRTM Green Supermix (Bio-Rad, Hercules, CA) by real-time PCR on a Bio-Rad I-Cycler. Each RT was simultaneously examined for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 5'-CAAGGCTGTGGGCA AGGTCA-3' (upper primer) and 5'-ACCACCTTCTTGAT GTCATC-3' (lower primer) as an internal control. GFP mRNA levels were normalized to GAPDH mRNA levels from the same sample. For studies of mRNA turnover, doxycycline was removed from culture and actinomycin D (5 μ g/ml final) added with RNA extraction at the specified times.

Statistical Analysis

Mean and standard deviation (SD) were determined for experiments with triplicate samples. Mean and range are shown for experiments using duplicate samples. Statistical significance was determined by Student's *t*-test, using GraphPad Prism and a value of $P < 0.05$ was considered to show significant differences.

Results

For the potential purpose of screening using intact viable cells, we utilized a GFP reporter to examine the effect of the human TNF 3'-UTR (Fig. 1b). Cell lines containing stable integrants of the FRT-GFP-globin, FRT-GFP-TNF, FRT-GFP-ARE plasmids were generated by co-transfection with pOG44 (encoding the FLP recombinase) into parent FRT cells (FLP-In-T-REx-293 cells, Invitrogen) followed by hygromycin selection as described above. These data result in integration of the GFP-TNF fusion gene at the single, transcriptionally active tetracycline-inducible FRT site engineered into this cell line. In multiple (>30) transfections with different vectors, single copy, doxycycline-inducible transfectants are obtained on the first attempt over 50% of the time (data not shown). Typically 50% of hygromycin-resistant cells are doxycycline inducible. GFP expression is controlled by a tetracycline-inducible promoter, and the signal to noise ratio is 2 orders of magnitude (see "Globin," Fig. 2a). Moreover, we found the level of expression with these

lines to be stable over months in prolonged culture as well as between thaws of different vials 3 years apart (data not shown).

The FRT-GFP system was used to evaluate TNF ARE-dependent and ARE-independent regulation of GFP reporter expression. GFP fluorescence exhibits linear expression relative to mRNA levels over several logs [15, 16]. Following overnight induction with doxycycline, marked increases in GFP expression were seen by flow cytometry (Fig. 2a). Expression of the GFP-globin control yielded far more fluorescence than either the GFP-TNF or GFP-

ARE cells. In four experiments, GFP expression decreased by 98% in GFP-TNF cells and by 93% in GFP- ARE cells relative to GFP-globin cells (Fig. 2b). Slightly less inhibition of GFP mRNA accumulation was seen relative to mean fluorescent intensity (MFI) with the GFP-TNF and GFP- ARE mRNA levels reduced to 19% and 27% of GFP-globin, respectively (Fig. 2b). These results demonstrate the effects of ARE- and non-ARE-pathways in the TNF 3'-UTR in regulating mRNA accumulation and translation [1, 2, 8]. Immunoblotting of cell lysates confirmed these results, showing the high levels of GFP protein expression by GFP-globin cells following doxycycline treatment (Fig. 2d). Consistent with the flow cytometry data, GFP protein from GFP- ARE cells treated with doxycycline was markedly inhibited with little or no signal seen under these exposure conditions (Fig. 2d).

The FRT-GFP cells were next used to study signaling pathways that regulate TNF 3'-UTR-dependent gene expression. Following doxycycline induction, PMA/Ionomycin treatment (4 h) increased the fold-change in MFI and GFP mRNA accumulation of both GFP-TNF and GFP- ARE cells to a greater degree than that seen in GFP-globin cells (Fig. 2c). This increase in expression in the GFP-TNF and GFP- ARE cell lines was insensitive to inhibitors of JNK II (SP600125), mTOR (rapamycin), or PI3K (wortmannin) (data not shown). In contrast, p38 inhibition by SB203580 (10 mM) treatment induced a 50% decrease in GFP expression by the GFP-TNF cell line (data not shown). SB203580 treatment decreased GFP-TNF mRNA levels to a lesser degree than the reduction in GFP expression, consistent with reduced translation efficiency [2, 11, 17–19]. No effect was found on GFP expression in GFP- ARE cells. We conclude that the FRT-GFP cell lines accurately reflect the effect of p38 activation on ARE-dependent gene expression, as previously shown [2, 17–22].

Transient transfection of 293 cells demonstrated that the TNF 3'-UTR ARE increased mRNA translation in the context of fetal bovine serum (FBS) starvation [8]. We compared the effects of serum starvation on transiently transfected and stably integrated reporter constructs. The ARE-dependent regulatory pathway in GFP-globin, GFP-TNF, or GFP- ARE lines was compared by doxycycline induction followed by serum starvation for 24 h. Although the total MFI was slightly reduced (25%) by serum starvation (data not shown), the fold effect (No FBS treatment/ FBS treatment) was the same in the three cell lines (Fig. 3a). In contrast, transient transfection with luciferase reporter plasmids confirmed the observation that the ARE enhanced translation under conditions of serum starvation (Fig. 3b). Luciferase activity of the reporter vector containing the TNF 3'-UTR ("No intron") increased 3-fold with serum starvation. Inclusion of the TNF 3'-UTR and the intron (Intron 3) resulted in a smaller, but significant increase (65%) as a function of serum starvation. Removing the ARE (Intron 3-ARE) eliminated the effect of serum starvation. Thus, serum starvation increases expression of transiently transfected reporter genes through the TNF 3'-UTR ARE [8]. Identical ARE-dependent effects of serum starvation were seen with transient transfection with four additional sets of chimeric reporters (data not shown). These results show that the serum starvation effect was specific to transiently transfected reporter plasmids, but not seen with stably integrated reporter genes.

The finding of ARE-independent effects on gene expression in the human TNF 3'-UTR were noteworthy in terms of their potency. A codominant, equipotent second *cis*-acting element (constitutive decay element, CDE) was identified in the murine TNF 3'-UTR using transient transfection [23]. Our studies with the GFP-ARE cell line suggested a similar activity in the human TNF 3' UTR. Deletion of the ARE had no effect on mRNA turnover (Fig. 4; Table 1), consistent with the model that at least one separate codominant instability element exists 3' to the ARE [23]. In a series of experiments, GFP mRNA was always very stable in the GFP-globin cell line (no decline in mRNA levels up to 6 h, $n = 9$). The presence of the TNF 3'-UTR in the GFP-TNF ($n = 6$) or GFP-ARE ($n = 4$) cell lines markedly reduced GFP mRNA stability with nearly identical half-lives ~45 min. In the mouse, the ARE-independent effect on mRNA turnover of the murine TNF 3'-UTR was mapped to 99 nucleotides, the CDE [23], that occurred immediately 3' to the ARE (Fig. 1a). The murine CDE sequence is nearly completely conserved in the human 3'-UTR.

Discussion

In this report, we adapted a commercially available *in vivo* system for the study of post-transcriptional regulation by 3'-UTR *cis*-acting elements using the GFP reporter. This innovative application takes advantage of the ability to reproducibly integrate single copy reporter genes in a transcriptionally active genomic locus. The 3'-UTR of interest is cloned into a commercially available vector that includes a FRT site downstream of a reporter sequence. This plasmid is cotransfected with a Flp recombinase expression plasmid into Parent FRT cells containing a single FRT site, resulting in single copy integration of the plasmid DNA. The use of a GFP reporter enables screening of intact cells showing that ~50% of all hygromycin-resistant cells express the gene of interest with minimal differences in expression (data not shown). The GFP reporter signal is remarkably consistent and stable across cell lines, establishing the robust nature of this system. In addition, (a) the GFP protein is non-toxic compared to fluorophores such as fluorescein isothiocyanate (FITC), (b) allows real time observation by fluorescence microscopy without damaging cells, (c) has been used in live animals without adverse biological affects, and (d) permits quantitation of cells by convenient and sensitive flow cytometry [24, 25].

This methodology has advantages over transient or stable transfection approaches. Transient transfections are labor-intensive, technically challenging, and hard to control for the effects of transfection or expression from non-integrated circular DNA. Several studies have indicated an inter-relationship between chromatin structure and cotranscriptional events, including splicing and polyadenylation [26–29]. One mRNA binding protein, AUF1/hnRNPd, interacts with a chromatin remodeling complex [30], and subsequently regulates mRNA maturation and export to the cytoplasm. It is likely that the transcriptional environment of either an integrated gene or non-integrated plasmid DNA differs in the nature of the mRNA–protein complex that is exported to the cytoplasm, with subsequent consequences for mRNA stability and translation. In addition, the use of stably transfected cells avoids inconsistencies associated with plasmid preparations, transfection efficiency, and the effects of transfection or the transfection reagent itself. These advantages are not present with the conventional generation of stable cell lines in that integration is controlled, invariably occurring at a single chromosomal site that can be verified by its induction with tetracycline. Hence, the need for analysis by Southern blotting is not required. Second, the site of integration is transcriptionally active without any evidence of inactivation over prolonged (>3 months) cell culture. As stated above, the level of expression is consistent over years, indicating a truly stable phenotype.

The FRT approach confirmed the activity of the ARE in regulating both mRNA stability and translation efficiency as well as the role of the p38 pathway in regulating expression, as

previously described [2, 6]. In contrast, the effect of serum starvation on ARE-dependent gene expression in 293 cells was only seen with transiently transfected reporter plasmids rather than stably integrated GFP reporters [8]. These data suggest selectivity of the effects of serum starvation on transiently transfected reporters relative to mRNA transcribed from a chromosomal location. Sucrose starvation in *Arabidopsis* was associated with wide-spread mRNA translational repression coupled with increased histone H4 acetylation due to a decrease in histone deacetylase activity [31]. An obvious possibility is that the normal chromosomal environment of integrated genes is not replicated on non-integrated plasmid DNA. In addition, regulatory proteins that associate with chromosomal sites of transcription, and then modify and transit mRNA, may not localize with non-chromosomal plasmid DNA. As a result, the effects of serum starvation on translation of mRNA arising from chromosomal sites might be very different relative to that seen with transcription of transiently transfected circular plasmid DNA.

One striking finding of this model system was the clear evidence of ARE-independent effects of the human TNF 3'-UTR on mRNA turnover and translation. Moreover, this ARE-independent pathway was regulated differently, unaffected by either serum starvation or p38 inhibition. These data are identical to that reported with the mouse TNF 3'-UTR where transient transfection studies mapped this activity to a highly conserved 99 nucleotides immediately 3' to the TNF ARE [23].

In conclusion, we demonstrate the utility of FRT reporter system to analyze post-transcriptional gene regulation. This system can be easily adapted to any 3' UTR and serve as a highly tractable, reproducible system. The use of GFP reporters enables studies of live cells as well as calculation of the level of distribution of expression on a per cell basis. Second, the data with serum starvation indicates that the use of stably integrated and transient reporters can yield different results, emphasizing that chromosomal context may need to be considered when different patterns of post-transcriptional regulation are seen between the native mRNA and transient transfection of reporter plasmids containing putative *cis*-acting elements. Finally, the FRT GFP model system we have described should facilitate high-throughput screening of drugs that target the contribution of specific pathways regulating gene expression.

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Fig. 1.

a Schematic diagram of FRT-GFP-globin constructs that lack or contain the human TNF 3'-UTR. Specified portions (573 nt) of the human TNF 3'-UTR were cloned downstream of the Globin-GFP-Globin reporter. **b** Site-directed recombination of the GFP-TNF-3'-UTR fusion gene into a single locus. Plasmids were cotransfected with a Flp recombinase expression vector (pOG44) into the Zeocin-resistant parental Flp-In-293 cell line (Parent FRT, Invitrogen) that contains a single FRT (Flp recombination target) site. The integration site is transcriptionally competent, with the GFP-TNF-3'-UTR fusion conferring hygromycin resistance and tetracycline inducibility

Fig. 2.

a Detection of green fluorescent protein in FRT-TNF stable cell lines by flow cytometry. Cell lines with single-site integrants of GFP-globin, GFP-TNF or GFP- ARE were treated $\pm$ doxycycline (18 h) along with PMA/Ionomycin addition for the last 4 h. Mean fluorescent intensity (MFI) is shown. **b** Magnitude of the effects of the TNF 3'-UTR on MFI and GFP mRNA. MFI of GFP expression (*white bars*) and mRNA accumulation (*black bars*) relative to GFP-globin are shown as mean $\pm$ SD ($n=4$). The effect of the GFP-TNF and GFP- ARE on MFI and GFP mRNA was statistically significant ($P<0.005$ for all values). **c** Activation with PMA/ Ionomycin affects GFP protein and mRNA in FRT-GFP cells. GFP-FRT cells induced with doxycycline, and then activated for 4 h with PMA/Ionomycin. MFI and GFP mRNA levels were expressed as fold-change relative to un-activated cells ($n=3$). The changes in MFI and mRNA for GFP-TNF and GFP- ARE were greater than GFP-globin ($P<0.05$ for all values). **d** Doxycycline induction of GFP expression—immunoblotting. To confirm that FRT-GFP cells express GFP protein, cell lysates were immunoblotted with

anti-GFP. Wildtype (no FRT-GFP gene, “293”), Parent (no GFP gene), FRT-globin or FRT-ARE cells were untreated (*lanes* 1–4) or treated with doxycycline (*lanes* 5–8)

Fig. 3.

a Effects of serum starvation on GFP expression in FRT-TNF stable cell lines. GFP-globin, GFP-TNF and GFP- Δ ARE cell lines were induced (18 h) with doxycycline, and then cultured with and without 10% FBS (control versus starvation) for 24 h. The effects of serum starvation were calculated using the ratio of the MFI: GFP without FBS/GFP with FBS. Results are the mean $\pm$ SD ($n = 3$). **b** Effects of serum starvation on TNF expression using transient transfection. Parent FRT cells were transfected in triplicate with the empty pGL3 vector or the specified luciferase plasmids: 4 h after transfection, cells were brought to 10% FBS (control), or were not given FBS (starvation). After 24 h, luciferase activity was measured and normalized to luciferase from cells transfected with the pGL3 empty vector. The effects of starvation are shown as the ratio of mean luciferase activity (No FBS/FBS). Values were decreased with TNF Intron 3 Δ ARE compared to TNF Intron 3 ($P < 0.044$). Values were increased with TNF No Intron compared to TNF Intron 3 ($P < 0.0001$).

Fig. 4. ARE-independent effects on GFP mRNA stability in doxycycline treated FRT-GFP cells. Following doxycycline induction overnight, transcription was arrested with removal of doxycycline and addition of actinomycin D. Changes in GFP mRNA accumulation at the specified times are shown as a function of the amount of GFP mRNA present at the time of transcriptional inhibition. Half-life values from 5 to 8 experiments are also shown in Table 1

Table 1

Effect of TNF 3'-UTR on GFP-Globin mRNA stability

Cell line	$T_{1/2}$ (min)	# Experiments
GFP-Globin	>240	8
GFP-TNF	49 ± 5	7
GFP- ARE	55 ± 5	5

Effects of TNF 3'-UTR on reporter mRNA stability was measured in FRT-GFP cell lines. Cell lines with TNF 3'-UTR (GFP-TNF) or TNF 3'-UTR with the ARE deleted (GFP- ARE) were treated with doxycycline, and the half-life ($T_{1/2}$) of GFP mRNA was determined following treatment with actinomycin D. Results (minutes) are the average and SD from multiple experiments