Transcription regulation in time and space: Engineered cell systems to modulate the epigenetic chromatin structure: The role of Methyl-CpG-binding protein 2

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Chapter 2

Generating cell clones to measure transcription dynamics in an induced variable epigenetic chromatin state

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Abstract

To investigate the influence of the chromatin context on transcription regulation in single cells at the level of single genes we created a reporter gene cassette to be inserted into defined genomic sites of human cells. The cassette is equipped with a readout to measure real-time mRNA and protein level kinetics of a reporter gene upon modulating the epigenetic chromatin state of the reporter gene. The reporter gene is driven by a mammalian promoter that induces constitutive gene expression. The reporter gene cassette containing cells with a single integration site are designed to measure the mRNA and protein levels in real-time and the response time to alter the transcription output upon modulating the epigenetic chromatin state of the cassette. This approach should enable us to determine the gene-specific kinetic characteristics of transcription and the contribution of epigenetic regulation. Here we provide an overview of the set-up of our experimental approach and we discuss our design compared to the current state-of-the-art to visualize mRNA with high temporal and spatial resolution. Here we show that the reporter gene cassette is being expressed when transfected in human cells and that the target constructs bind to a tet operator repeat. In future studies we plan to integrate the cassette via homologous integration in the human genome and measure single gene, single cell real-time transcription upon modulating the chromatin structure.

Introduction

Epigenetic gene regulation is essential to orchestrate gene expression patterns and plays a major role in determining cellular identity. Epigenetic regulatory mechanisms, such as histone modifications and DNA methylation, have been directly linked with transcriptional regulation and changes in higher-order chromatin folding (Deng and Blobel, 2014; Rafalska-Metcalf et al., 2010; van Steensel, 2011; Wendt and Grosveld, 2014). Currently, we lack quantitative understanding of these complex interactions. Mechanistic interactions of individual epigenetic factors that determine single gene transcription dynamics, are largely unknown. Such regulatory networks are often simply represented as all-or-none, i.e. gene silencing or activation.

Gene regulatory networks are inherently noisy due to the low amount of reacting components. The noise caused by the transcription regulatory network is considered to represent a form of intrinsic noise. Noise can be propagated from one cellular compartment to another within the same cell (Maheshri and O'Shea, 2007). Such propagated noise is considered to represent an extrinsic noise source (Johnston et al., 2012; Maheshri and O'Shea, 2007). Noise in transcription kinetics is still largely unexplored, but an important factor when measuring at the single cell, single gene level. In a previous theoretical study the role of noise in transcription regulation is explored studying nonexponentially distributed lifetimes of gene states (Schwabe et al., 2012). Schwabe et al. represented a stochastic model entitled 'the molecular ratchet' that considers the concerted action of several proteins assuming an ordered, multistep and cyclic mechanism involving a sequence of transitions between distinct chromatin states. The molecular ratchet transitions are assumed to involve reversible protein complex formation on chromatin followed by irreversible posttranslational histone modifications. To experimentally test this theoretical model, single gene transcriptional measurements are inevitable. In the present study, we set-out to develop a reporter gene cassette containing cell line to quantitatively study the contribution of the epigenetic chromatin state in regulating the rate of transcription at single gene level in single cells. Using this cell line we are able to test the behavior of the theoretical molecular ratchet model. The integration of such theoretical transcription regulation models with transcription real-time single cell imaging will undoubtedly provide more insight into the complex regulation and stochastic nature of transcription.

We constructed a reporter gene cassette consisting of a reporter gene that consists of MS2 tagged RNA which can be detected by constitutively expressed fluorescently tagged MS2 protein and that contains DNA binding sequences allowing to target epigenetic regulatory proteins to modulate the chromatin structure of the cassette. We engineered the reporter gene cassette such that we can perform systematic quantitative measurements allowing to plot the transcription rate as function of (i) the integration of the reporter gene cassette in a defined genomic chromatin context, (ii) targeting and accumulation of epigenetic regulatory proteins to the reporter gene cassette and (iii) the

local chromatin structure of the reporter gene cassette. This set-up will enable us to interpret transcriptional cell-cell variability and the contribution of the epigenetic chromatin state to transcriptional noise. In the present study, we show the cloning and transient transfection of this reporter gene cassette and the creation of epigenetic targeting constructs to modulate the chromatin structure of the cassette.

Results

Design of the reporter gene cassette

To quantitatively study the contribution of the epigenetic chromatin state in regulating the rate of transcription, we designed a cell line consisting of a reporter gene cassette containing several features. These features represent (i) an array of tet operator (tetO) binding sequences allowing binding of tetracyclin-induced Cherry tet repressor (tetR)-tagged transactivator VP16 (tTA) fused to the ligand binding domain of the estrogen receptor (ER), i.e. Cherry-tTA-ER targeting, (ii) a mTurquoise-NES-PEST tagged reporter gene driven by a phosphoglycerate kinase (PGK) promoter and (iii) a bacteriophage MS2 hairpin repeat to detect RNA transcripts via YFP tagged MS2 protein binding (Figure 1A and B).

To measure the effect of both transcriptional repressors and activators at protein level, we used a reporter gene coding for a short-lived fluorescent protein. Short-lived proteins allow to measure changes in their protein level upon transcriptional repression in time. Many short-lived proteins have a life time shorter than 2 hours and they consist of a degradation signal, e.g. a PEST sequence, named after the amino acids they represent. The PEST sequence is present in short-lived proteins such as mouse ornithine decarboxylase (MODC), which has a life time 30 minutes. Li et al. noticed that fusion of the PEST part of the MODC protein to a GFP reporter gene drastically reduced the life time of the reporter gene (Li, 1998). We fused this PEST signal to the mTurquoise reporter gene to reduce the life time of the mTurquoise reporter gene. In addition, we added a nuclear export signal (NES) to the mTurquoise reporter gene to export the fluorescent

protein outside of the nucleus and measure its protein levels in the cytoplasm (Figure 1A and B).

We selected the mammalian PGK promoter to drive the exon-only sequence of mTurquoise. This cyan fluorescent protein has a respective high Quantum Yield, providing bright fluorescence and relatively low photo toxicity (Goedhart et al., 2010). The PGK promoter is known to induce constitutive gene expression in cultured cells and in transgenic mice (McBurney et al., 1994).

To allow visual single cell transcription measurements of the reporter gene in real-time, we integrated a hairpin-bearing MS2 bacteriophage sequence into the reporter gene coding DNA that upon transcriptional activation is rapidly bound with high specificity and affinity to constitutively expressed YFP-tagged MS2 bacteriophage capsid protein (Ben-Ari et al., 2010).

The reporter gene cassette contains a FRT site enabling integration of the cassette via homologous recombination with a FRT Flipase site. A series of human embryonic kidney (HEK) cells were previously created by Gierman et al, containing a FRT Flipase site and a GFP reporter gene at known genomic sites. A nested-PCR was used to identify the exact integration of the FRT Flipase containing reporter gene. The expression levels of the GFP reporter gene at these integration sites is identified in a previous study (Gierman et al., 2007)(Anink-Groenen et al. unpublished results). Our reporter gene cassette was designed such that these defined FRT Flipase containing genomic sites could be used as landing platform allowing to insert our cassette into previously examined genomic sites.

The integration of a 84x tetO tandem repeat upstream of the reporter gene enables us to modulate the epigenetic chromatin state of the reporter gene cassette via targeting of tetR binding proteins fussed to epigenetic regulatory proteins. The reporter construct, consisting of the PGK promoter, the mTurquoise reporter gene, MS2 repeats and the FRT Flipase site was synthesizes by Genescript©. The Genscript© synthesized construct was

cloned into the transfection vector pPur to add the 84x tetO tandem repeat. The final DP15 construct has a size of 11.312 kb.

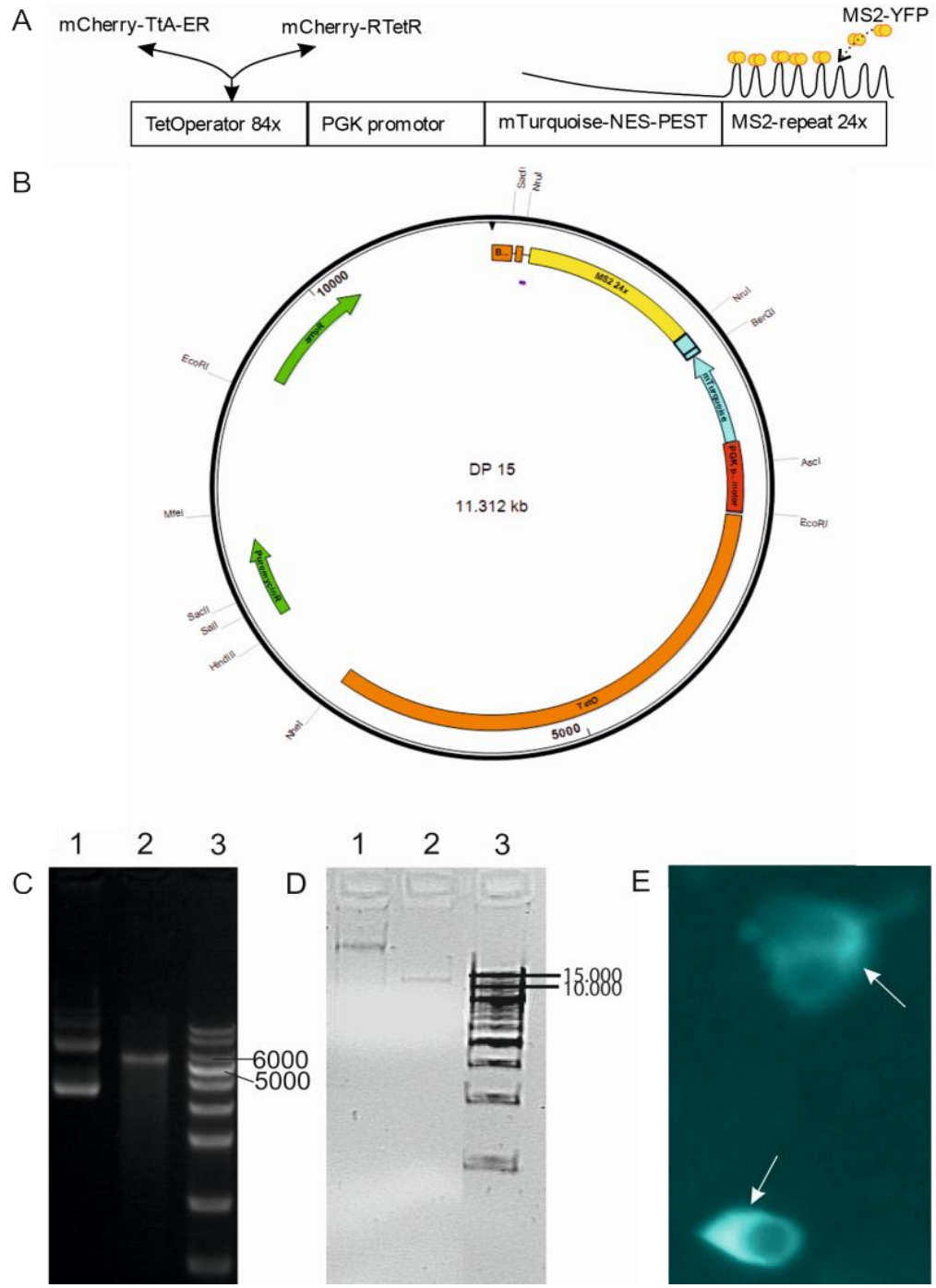

Figure 1: Reporter gene cassette

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1A) The reporter gene cassette containing a 84x tandem tetO repeat sequence allowing targeting of the tetR and rtetR constructs (i.e. Cherry tagged TtA containing an ER binding domain and Cherry tagged rtetR). The mTurquoise reporter gene containing a nuclear export signal (NES) and a PEST degradation signal as well as a 24x MS2 repeat is driven by a PGK promoter. The MS2 hairpin repeat allows microscopic detection of the MS2-tagged transcripts at the reporter gene cassette via the binding of YFP tagged MS2 binding protein.

1B) The engineered transcription unit and the tetO 84x tandem repeat cloned in a pPur plasmid. The entire DP15 construct has a size of 11.312 kb. The MS2 24x repeat sequence (yellow), the mTurquoise-NES-PEST gene (blue), the 84x tetO tandem repeat (orange) and the ampicillin and puromycin resistance genes (green) are indicated. The unique restriction sites of DP15 are shown.

1C) Restriction digestion analysis of the Genescript© synthesized construct consisting of the PGK promoter, mTurquoise reporter gene, MS2 repeats and FRT Flipase site. An agarose gel analysis of the HindIII restriction digested Genescript© synthesized construct is shown. The construct is cloned into the Puc57 vector exhibiting a combined size of 5622 bp (insert: 2912 bp and puc57: 2710 bp). Lane 1 shows the undigested vector, lane 2 the construct digested by HindIII. Lane 3 shows a restriction digestion DNA 'KB' ladder provided by Genescript.

1D) Agarose gel analysis of the NheI restriction digested DP15 construct. Lane 1 shows the entire non-digested DP15 construct. In lane 2 the DP15 construct is digested with NheI. NheI cuts DP15 immediately adjacent to the tetO 84x tandem repeat. The single band shows that the DP15 construct is intact and approximately 12 kb. Lane 3 shows the restriction digestion DNA 'large' ladder provided by MRC Holland.

1E) Widefield image of Hek H100 cells transfected with the DP15 construct. The mTurquoise-NES-PEST reporter gene is located in the cytoplasm of the cells. Arrows depict three cells expressing the DP15 construct in the cytoplasm one day after transfection.

The 84x tetO tandem repeats are known to exhibit genomic instability via recombination events (Al-Allaf et al., 2012). The tetO sequence and eventually the created DP15 construct are therefore cultured in specialized Stbl2^{TM} competent *E.Coli* cells. The Stbl2^{TM} cells are highly efficient chemically competent bacterial cells that contain a unique set of markers to allow stable cloning of direct repeats and retroviral sequences and tandem array genes. We noticed that even in these Stbl2^{TM} cells the tetO repeat is unstable. Figure 1C and D illustrate the agarose gel testing of respectively the synthesized construct and the full DP15 construct. Figure 1C shows the cassette integrated in a Puc57 vector resulting in a band of 5622 bp after restriction digestion with HindIII. Figure 1D shows

that the NheI restriction digested construct results in a band of 11.312 kb, illustrating that the DP15 construct remained intact after multiplying the construct in Stbl2^{TM} cells.

We tested the transient expression of the DP15 construct in Hek H100 cells and visualized the expression of the mTurquoise reporter gene in living cells using a wide field microscope equipped with CCD camera. Figure 1E shows 2 typical representations of cells transfected with the DP15 construct. Since the mTurquoise reporter gene is tagged with a NES, the fluorescent protein is observed in the cytoplasm of the transfected cells. The next step is to integrate the DP15 construct into the FRT Flipase site of the Hek H100 cells and perform selection and correct single integration of clones with the inserted construct. Due to time constraints we did not manage to create clones with the singly integrated DP15 construct.

Targeting epigenetic regulatory proteins

To modulate the transcriptional activity of the reporter gene, we created fusion proteins consisting of tetR fluorescently tagged regulatory proteins. The tetR-tetO binding can be reversed at minute time-scale upon treating the cells with tetracyclin or doxycyclin (Gossen and Bujard, 1992) allowing to determine the effects of the targeted regulatory proteins upon their binding or release from the tetO binding sequences (tet-on or tet-off induction). The tandem tetO repeat sequences can also be used to visualize the compaction of the chromatin structure upon targeting tetR tagged proteins (Brink et al., 2013; Tumbar et al., 1999; Verschure et al., 2005). We used mCherry-tTA-ER (a gift from SM Janicki (Rafalska-Metcalf et al., 2010) consisting of the mCherry tagged transactivator tTA and the ligand binding domain of the estrogen receptor (ER) to modulate the nuclear versus cytoplasmic localization of the targeting construct upon addition of Tamoxifen.

The commercially available TtA and reverse TtA (rTtA) are fusions of the tetR and rtetR, respectively with the acidic activation domain (AAD) of VP16. We annotated the tetR binding protein and the VP16 AAD (Figure 2A) in the sequence of rTtA and we designed a PCR to obtain only the rtetR domain from this construct. We fused the rtetR to mCherry in a low expression vector entitled p3'SS, containing a polyomaF9 promoter. We used this

vector to create a fusion construct consisting of rtetR tagged mCherry fused to either Heterochromatin 1β (HP1β) protein or methyl-CpG binding protein 2 (MeCP2) (Figure 2B). Both HP1 and MeCP2 are proteins are known to be particularly enriched at pericentromeric chromatin. HP1 is a chromatin-binding protein that bridges via its chromo- and chromoshadow domain H3 lysine 9 tri-methylated histones with other chromatin associated proteins thereby advancing the 'spreading' of a transcriptionally silent heterochromatin structure (Verschure et al., 2005). MeCP2 was originally found to bind methylated DNA and to act as a transcription repressor (Nan et al., 1997). The regulatory protein targeting constructs were designed to modulate the transcriptional activity of the reporter gene cassette. Figure 2C shows an agarose gel with the mCherry-lacR vector that was used to replace lacR with rtetR. The newly cloned mCherry-rtetR, mCherry-rtetR-MeCP2 and mCherry-rtetR-HP1 were digested at a unique site in the rtetR construct to discriminate the presence of rtetR instead of lacR, shown in Figure 2C, lane 1. To verify the presence of HP1β (700bp) and MeCP2 (1400bp) all constructs were digested with Asc1, as shown in Figure 2C, lane 3 upon performing a single digestion in the backbone of the vector.

To test the created targeting proteins we used U20S 2-3-6 cells containing a reporter gene cassette consisting of tetO tandem repeats, as well as an adjacently placed lacO tandem repeat (Rafalska-Metcalf et al., 2010). We transiently co-transfected the rtetR constructs together with a lacR-EGFP construct (Brink et al., 2013; Verschure et al., 2005), to verify the binding properties of the rtetR constructs.

We visualized the tetO array of the mCherry-rtetR transfected U2OS 2-6-3 cells with the confocal microscope (Figure 2D). Upon addition of doxycyclin the rtetR construct is released from the tetO repeat, whereas eGFP-lacR binding at the lacO array is maintained since lacR-lacO binding is unaffected by the presence of doxycyclin. We show that mCherry-rtetR binds to the tetO array in presence of doxycylin and that mCherry-rtetR is released from the tetO array after removing doxycyclin from the medium. We show the presence of eGFP-lacR at the lacO array both in the presence and absence of doxycyclin. Figure 2C and D show the presence of the mCherry-rtetR construct fused to MeCP2 or HP1 at the reporter gene cassette in the nucleus before addition of doxycyclin.

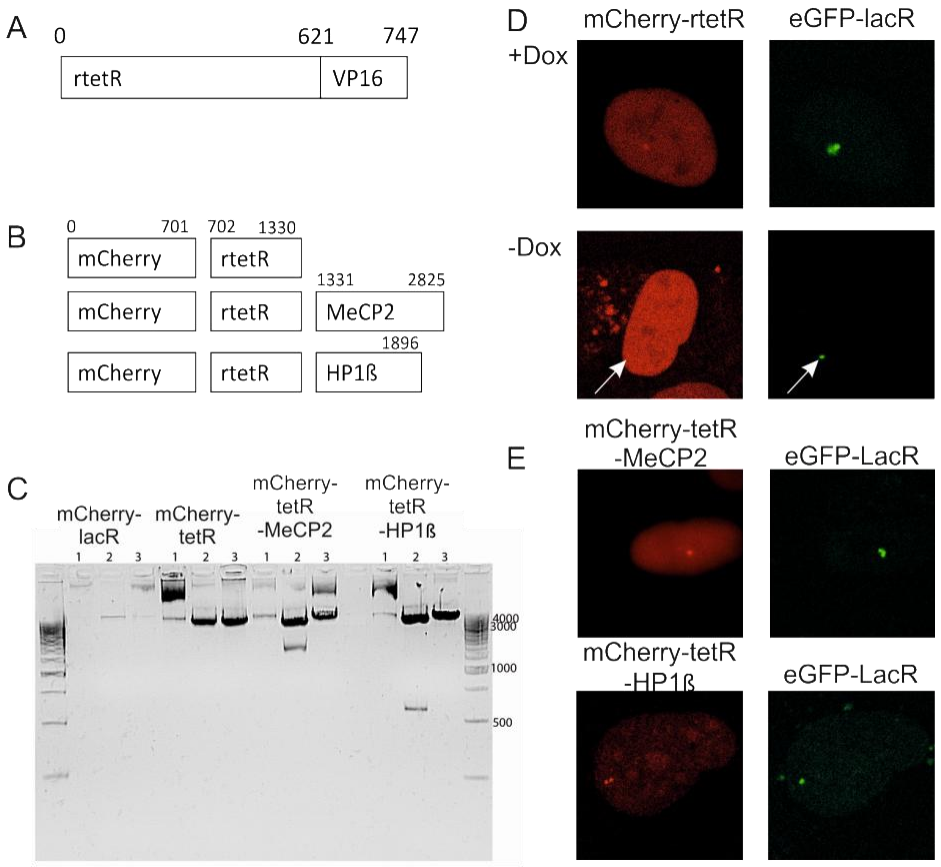

Figure 2: Targeting constructs

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2A) Representation of the rTtA construct. The rTtA construct consists of the rtetR and 3x the VP16 AAD domain exhibiting sizes of 621 and 126, respectively. Primers were designed to PCR only the rtetR domain, this part of the construct is used for the creation of the rtetR constructs represented in Figure 2B.

2B) A representation of the mCherry-rtetR tagged constructs. The targeting constructs were created to modulate the epigenetic chromatin structure of the DP15 reporter gene cassette and hence to measure the effect on the transcriptional activity of the mTurquoise reporter gene.

2C) Agarose gel of the restriction enzyme digestions of mCherry-lacR, mCherry-rtetR, mCherry-rtetR-MeCP2 and mCherry-rtetR-HP1β to check the correct cloning of the fusion proteins. The following restriction digestions are shown: 1) NruI digestion makes a single cut in the rtetR protein, 2) AscI digestion isolates the insertion of HP1β (~700bp) or MeCP2 (~1400bp) and 3) SacII makes a single cut in the plasmid backbone.

2D) Confocal images of U2OS-2-6-3 cells containing a 84x tandem tetO repeat transfected with mCherry-rtetR (red) and eGFP-lacR (green). The cells treated with doxycycline (+dox) for 24 hours and the cells treated with doxycyclin for 24 hours are subsequently cultured in dox depleted for 1 hour (-dox). +Dox treated cells show that the rtetR is bound to the tetO-array. -Dox treated cells show that rtetR is lost from the tetO array. EGFP-lacR binding at the lacO array is shown in both +dox and -dox treated conditions.

2E) Confocal images of U2OS 2-6-3 cells transfected with either mCherry-rtetR-MeCP2 or mCherry-rtetR-HP1β and eGFP-lacR. The reporter gene cassette is visualized by the binding of eGFP-lacR to the lacO array and the tetO array is visualized by the binding of mCherry-rtetR-MeCP2 or mCherry-rtetR-HP1β.

Discussion

In the present study we created an engineered reporter gene cassette that should enable the measurement of transcription dynamics in a single gene in single cells, using real-time microscopy imaging. To visualize synthesized transcripts a MS2 repeat was placed immediately adjacent to a mTurquoise-NES-PEST reporter gene. To measure the effect of modulating the chromatin composition on transcriptional dynamics in single cells, we created a set of tetO targeting proteins fusing rtetR to mCherry or to the regulatory proteins MeCP2 and HP1β. In addition, we designed the reporter gene cassette such that it can be integrated in a single genomic location using FRT Flipase integration. We show the molecular design of the DP15 cassette and its cytoplasmic localization in Hek H100 cells. Moreover, we show the molecular design of mCherry rtetR tagged MeCP2 and HP1 epigenetic regulatory proteins and their nuclear localization in U2OS 2-6-3 cells. We

discuss the design of our reporter gene cassette containing cell line compared to the current state-of-the-art to measure mRNA with high temporal and spatial resolution.

Transcription cell population measurements

Biochemical transcription cell population measurements (e.g. time-resolved quantitative RT-PCR, chromatin immunoprecipitation, ChIP) have generated multiple models of transcription regulation that are often interpreted in terms of sequential molecular events (Coulon et al., 2013). The general view is that recruitment of different molecular partners progressively stabilizes the eventually formed protein complex and facilitates recruitment of other factors in a static and well-ordered manner. The complication of such population snapshot measurements is that transient events and events occurring only in a subset of cells are not identified. Typically live-cell experiments provide kinetic aspects of transcription regulation over a broad range of time scales that are much shorter than most gene-induction population studies.

An indirect approach to uncover temporal gene expression variability among genes is to analyse the steady-state distribution of fluorescent reporter protein levels in sub cell populations. For example first performing flow cytometry cell population selection and subsequently count the distribution of protein levels (Hoppe et al., 2014; Viñuelas et al., 2013). In addition, nowadays single cell biochemical high-throughput analysis technologies such as single cell RNA-seq, are becoming more common and reliable (Stegle et al., 2015). A second indirect approach to determine transcriptional dynamics regards measuring the fluorescent protein levels in single cells using time-lapse microscopy and reconstruct the RNA time-traces (Harper et al., 2011; Suter et al., 2011). These studies revealed that a significant amount of cell-cell variability in gene expression exists. Such noise in gene expression is shown to correlate with gene functioning, promoter features and genomic positioning of a gene. Information about cell-cell transcription variability and hence cell population heterogeneity, not only impacts fundamental research but is also important for medical applications (Navin et al., 2011). Cellular heterogeneity in gene

activity is an important factor to infer tumor cell behavior, specially with respect to the upcoming field of personalized medicine (Murtaza et al., 2013).

Spatial localization of transcripts

RNA detection using in situ hybridization provides a method to quantify RNA levels in fixed cells, allowing single cell and also high-resolution detection when coupled with ultrathin sectioning and electron microscopy (Weil et al., 2010). With single molecule RNA FISH (smRNA FISH) multiple fluorescent probes are hybridized to a single mRNA enabling single molecule transcript detection levels (Raj et al., 2008). smRNA FISH revealed that a large cell-to-cell heterogeneity exists in transcript levels. Based on these observations it suggested that genes exhibit pulsitile behavior fluctuating between 'on' and 'off' states, i.e. transcriptional bursting (Rybakova et al., 2015; Suter et al., 2011). Currently, smRNA FISH is optimized such that it allows fast, multi-plexed automated and also high throughput transcript analysis (Battich et al., 2013). FISH sequencing of RNA (FISSEQ) or multiplexed error-robust FISH (MERFISH) are new technologies for visualizing RNA at subcellular single molecule level providing information on the localization of thousands of RNA species, including splice variants and single nucleotide polymorphisms (Chen et al., 2015; Lee et al., 2014).

Transcription dynamics

Transcription dynamics can be measured in cells expressing a reporter gene cassette such as designed in the present study (Bertrand et al., 1998; Larson et al., 2009; Rafalska-Metcalf and Janicki, 2007). Transcript tagging is enabled via genomic integration of a reporter gene encoding the RNA of interest with stem bacteriophage stem loop sequences such as MS2, PP7 or λ-phage N-protein boxB that bind with high affinity to their fluorescently tagged coat protein (Bertrand et al., 1998). Combining two transcript tagging systems allows live-cell imaging of two mRNA species simultaneously (Hocine et al., 2013; Lange et al., 2008). The initial mammalian cell lines expressing MS2 tagged transcripts exhibited multiple copies of a reporter gene cassette (Rafalska-Metcalf et al.,

2010). Such a multicopy reporter gene cassette integration improves detection of MS2 fluorescently tagged transcripts but exhibits the drawback that transcriptional measurements represent averaging of the transcript levels of the multicopied reporter genes (Rafalska-Metcalf and Janicki, 2007). Synchronized expression of the reporter genes within a multicopy reporter gene cassette enables to approximate the transcription dynamics of single genes. Previous studies showed that targeting of the acidic amino acid domain (AAD) of a transcriptional activator (i.e. VP16) to a multicopy reporter gene cassette boosts synchronous transcriptional activation of multicopy reporter genes (Rafalska-Metcalf et al., 2010). The MS2 transcript tagging has nowadays also been extended to a single gene copy integration for instance using the Flp-In system (Yunger et al., 2010) and even as an endogenous knock-in mouse harboring one tagged allele of the β-actin gene (Lionnet et al., 2011).

A major drawback of microscopic single cell and especially of real-time transcription measurements is that they are time-consuming and technically demanding. In general, microsopic detection methods have constraints regarding the production of large data sets. Moreover, both biological and technical variability between the cells further complicates single cell microscopical data interpretation. Moreover, experimental cell modulation tools, can induce large cell-to-cell biological variability in the response time of cells. Also experimental technical factors such as variations in culture conditions, the exact temperature of the set-up and timing in sample preparations can induce experimental variability. The reproducibility of the set-up of single cell microscopic measurements is essential such that multiple experiments can be compared. Akthar et al. (2013) developed a multiplexing approach to enable parallel monitoring of transcriptional activity of thousands of randomly integrated reporter genes. This multiplexing enables massive upscaling of reporter gene transcriptional screening at various genomic sites. In addition, the approach enables the screening of different kind of constructs for instance constructs with different promoters and other sequential differences.

Genomic integration of reporter gene cassette

Our reporter gene cassette is designed to be singly integrated in any mammalian cell line or transgenic mouse making use of the FRT Flipase site via homologous recombination. The main reason to use the FRT-Flipase recombinant cloning method for integration of our engineered reporter gene cassette was the availability of a large set of Hek H100 clonal cell lines containing an FRT site integrated in pre-determined genomic sites with known transcriptional activity (Gierman et al., 2007). Nowadays the CRISPR/Cas9 system provides a very efficient way to induce homologous recombination (Boettcher and McManus, 2015; Hsu et al., 2014; Liu et al., 2015). A major advantage of the CRISPR/Cas9 method could be to fuse a MS2 repeat directly to an endogenous gene instead of integrating a large reporter gene cassette. A possible challenge of the CRISPR/Cas9 method for transcription dynamics measurements might be interference of the guide RNA with MS2 hairpin loops. Another drawback of using the CRISPR/Cas9 for genomic integration is their off target integration.

Conclusion

A substantial component of transcriptional variability has been linked to molecular dynamics occurring at the local gene level involving the epigenetic chromatin state. To study the contribution of epigenetic regulation on transcription dynamics at single gene level it is inevitable to perform dedicated real-time microscopy imaging but also to create testable quantitative and predictive models. To minimize technical fluctuations affecting cell-cell variability measurements it is important to use a well defined experimental platform and to perform multiple measurements within a relatively short time frame. We show the design, engineering and transient expression of our constructed reporter gene cassette in living Hek H100 cells. As a next step we plan to integrate the reporter gene cassette via homologous integration in the human genome of Hek H100 cells. We created epigenetic regulatory targeting constructs and show their ability to bind and release from the tetO array by doxycyclin treatment.

Materials & Methods

ISOLATION OF RTETR

The TetOn advanced construct was annotated using DNAstar. PCR was performed with following primers: forward primer: tgtacagcAtgtctagactggacaagagc, Reverse primer: ggcgcgccccgccgctttcgcactttgc @62°C. Taq polymerase from MRC Holland was used in an applied biosystem gene amp PCR system 2700.

CONSTRUCTION OF DP15

The reporter gene cassette was created in two stages. The promotor, gene and signal sequences together with the MS2 repeat and the FRT site were synthesized by Genescript© as one construct. The tetO repeat was cloned into the pPur transfection vector. Subsequently, the Genescript© synthesized construct was cloned into pPurTetO1.84.

RESTRICTION DIGESTION AND GEL ANALYSIS

Restriction digestion was performed according to the conditions provided by Fermentas. Fermentas enzymes and buffers were used for exactly one hour at 37°C. 1% TBE or TAE agarose (Sigma) gels were run at 90V until DNA was properly separated (Biorad electrophoresis) (60-90 min). Images of the gels were made using a Genius imager. DNA was cut from the gel and isolated using Fermentas purification kit and ligated 16-20 hours at 16°C with Roche T4 ligase.

TRANSFECTION AND CELL CULTURE

DNA was extracted using Fermentas miniprep kit, and transfected with Invitrogen Lipofectamin 2000 using the Invitrogen provided conditions. U2OS 2-6-3 cells were cultured in Glutamax DMEM, supplemented with 10% Tet-approved FCS serum (Clontech), 1% PS (Gibco). Hek H100 cells were cultured in Glutamax DMEM supplemented with 10% FCS serum and 1% PS (Gibco). For imaging the cells were grown in Mattek dishes or Alcian Blue coated coverslips. Coverslips are fixed in 4% formaldehyde diluted in PBS and embedded in Vectashield. Mattek dishes were washed and covered in Microscopy Medium and imaged in real-time.

MICROSCOPY

Fixed cells were imaged using a Zeiss LSM510 (Zeiss, Germany), equipped with a Zeiss plan neofluar 636/1.25 NA oil objective. We used multitrack scanning, employing an UV laser (364 nm), an argon laser (488 nm) and a helium-neon laser (543 nm) to excite DAPI staining and green and red fluorochromes. Emitted fluorescence was detected with BP 385-470, BP 505–550 and 560 LP filters. Three-dimensional (3D) images were scanned at 512 by 512 pixels averaging 4 times using a voxel size of 200 nm axial and 60 nm lateral.

Zeiss S100 axiovert was used for wide-field imaging of the reporter protein, using a 40 x 1.3 n/a oil objective. mTurquoise was excited with a HBO 50 HG mercury lamp ~430 nm. CCD camera Images were taken using and an axiocam 1CM (Zeiss, Germany).