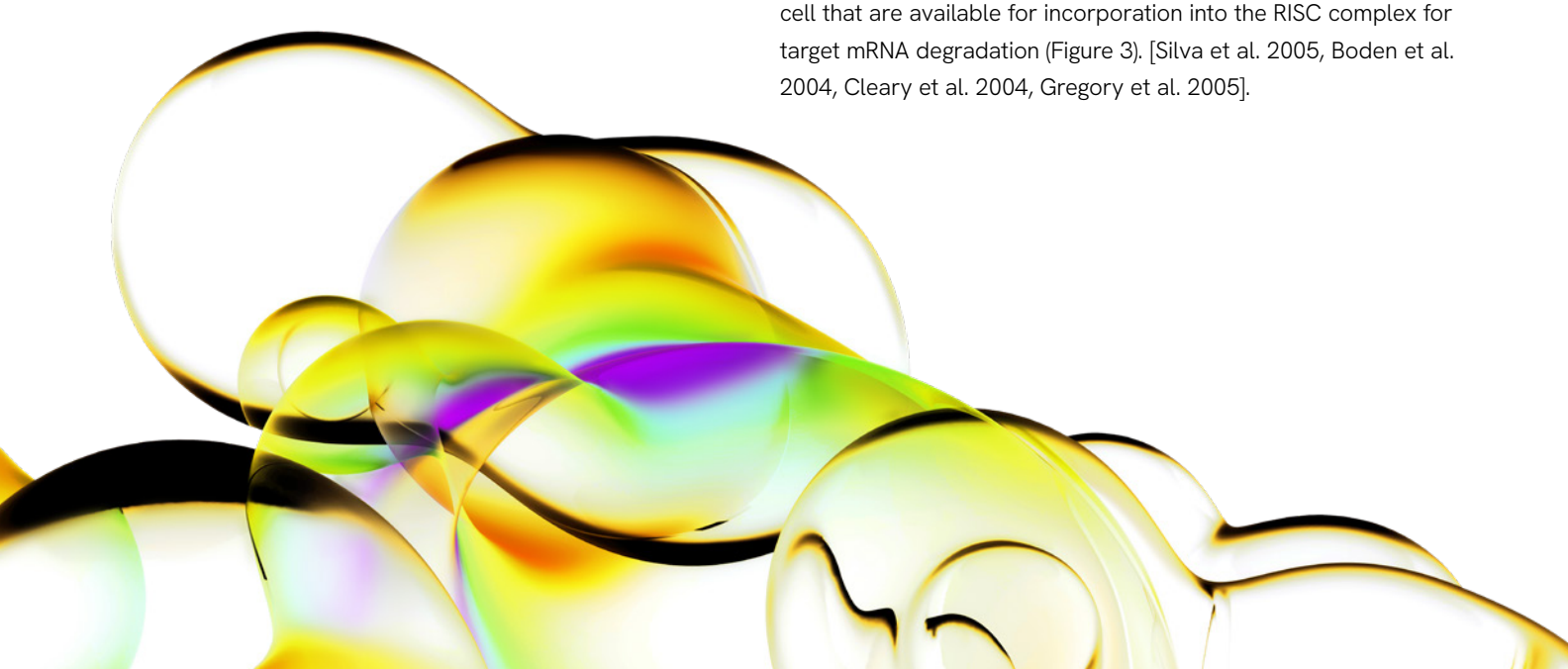
Lentiviral shRNAmir for stable and regulatable RNAi

Product description

RNA interference (RNAi) has revolutionized the study of biology and offers numerous applications in basic biology as well as in drug discovery research. Since the discovery of RNAi, several tools have been developed to enable loss-of-function studies in mammalian systems. Vector-based short hairpin RNA (shRNA) expression and synthetic short interfering RNA (siRNA) are currently the basis for all mammalian RNAi experiments (Figure 1). Each technology has specific advantages. shRNA can be used to perform many of the experiments similar to those done with siRNA. Additionally, when combined with viral delivery and integration into the genome, shRNA has the added benefit of long-term knockdown from a single treatment and the incorporation of a genetic tag (or barcode) allowing highly parallel pooled screening.

We have further refined this technology with the creation of genome-scale microRNA-adapted shRNA libraries. shRNAmir are expressed as primary-miRNA (pri-miRNA) transcripts. These constructs were created by redesigning the well-studied human miRNA, miR-30, to express an artificial siRNA/miRNA. The stem of the primary miR-30 transcript was replaced with gene-specific duplexes for different target genes (Figure 2). This design does not affect normal miRNA maturation and allows endogenous miRNA processing to produce mature siRNAs. The shRNAmir design harnesses endogenous enzymatic processing by the RNase III Drosha, which increases subsequent Dicer recognition and specificity. shRNAmir triggers enter the RNAi pathway ahead of either shRNA or siRNA, and are processed by both Drosha and Dicer, leading to more siRNAs produced in the cell that are available for incorporation into the RISC complex for target mRNA degradation (Figure 3). [Silva et al. 2005, Boden et al. 2004, Cleary et al. 2004, Gregory et al. 2005].



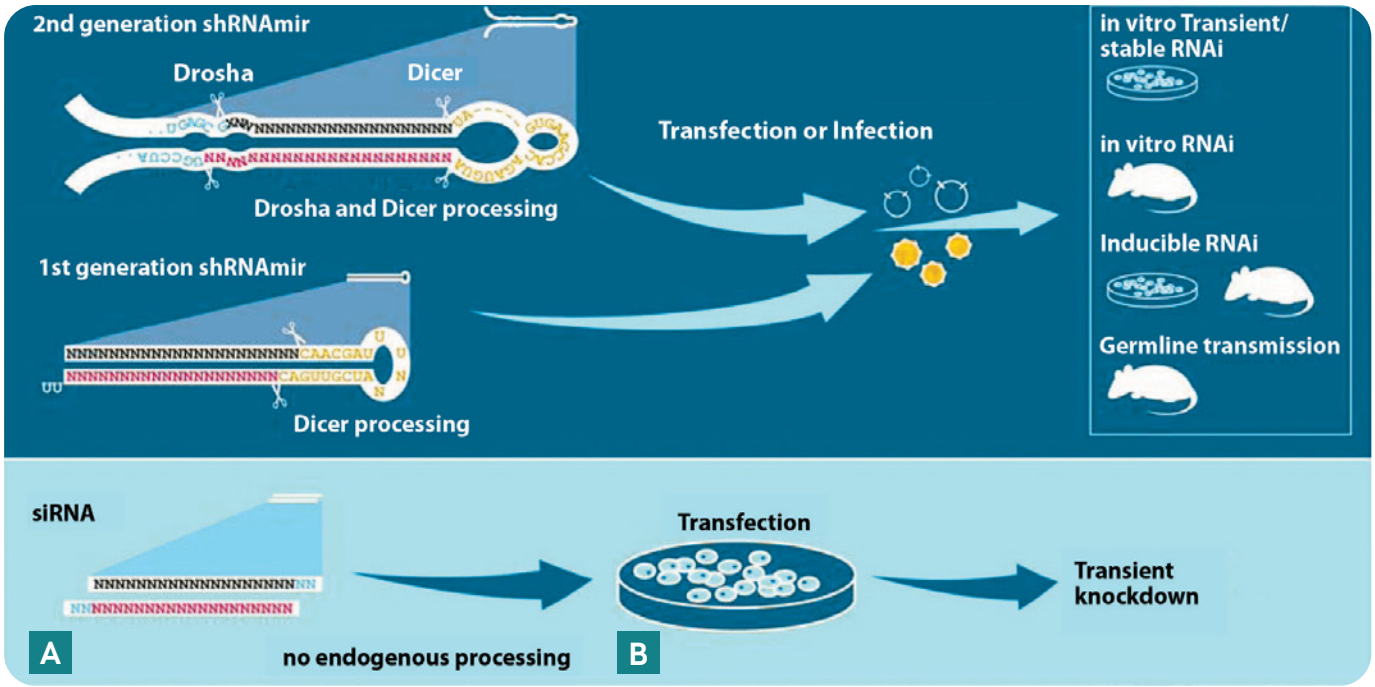


Figure 1: Evolution of RNAi triggers broadens RNAi applications.

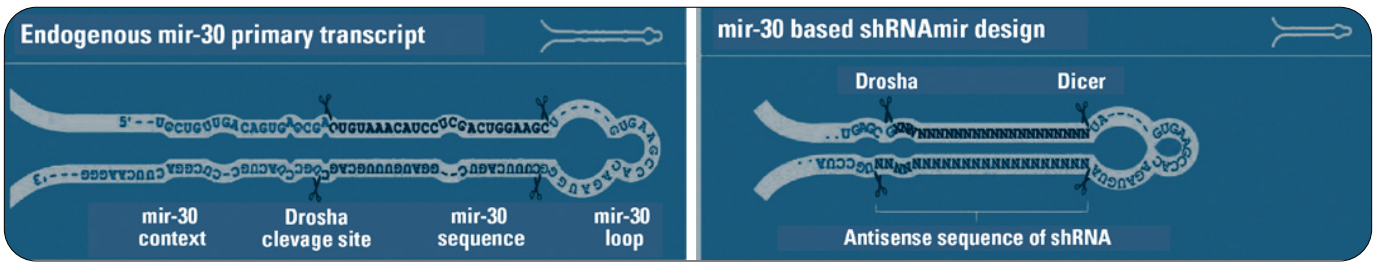


Figure 2: shRNAmir design is based on the primary microRNA-30 (mir-30) transcript. **A**. Endogenous mir-30 primary transcript. **B**. shRNAmir expressed from a miR-30 context. The mature mir-30 sequence has been replaced with a gene specific duplex. This design allows specific processing by Drosha and Dicer and active loading into the RISC complex.

Additionally, the lentiviral vector format can transduce hard-to-transfect cell lines like primary and non-dividing. These lentiviral vectors in combination with shRNAmir design offer a powerful tool for RNAi studies.

Whole genome shRNAmir lentiviral libraries have been created using both the GIPZ™ lentiviral vector (Figure 4) that incorporates green fluorescent protein (GFP) to track shRNAmir expression, and the TRIPZ™ lentiviral vector (Figure 5), which shares many of the benefits of GIPZ and includes a tetracycline (doxycycline) inducible promoter that produces tightly regulatable RNAi.

This tech note summarizes data provided by Rigel Therapeutics from their pilot experiments conducted to evaluate the GIPZ and TRIPZ Lentiviral shRNA Genome Scale Libraries. Seventy-eight GIPZ shRNAmir constructs designed against 19 target genes were transduced into multiple cell lines at low multiplicity of infection (MOI). The GIPZ shRNAmir exerted knockdown effects at the both RNA and protein level. Knockdown of the targeted genes with GIPZ shRNAmir mimicked known cellular phenotypes induced by small molecule inhibitors. The GIPZ shRNAmir were also employed to dissect the components in a signaling pathway using a SMAD reporter system. Their findings support previous data showing that the shRNAmir constructs produced effective knockdown at low copy.

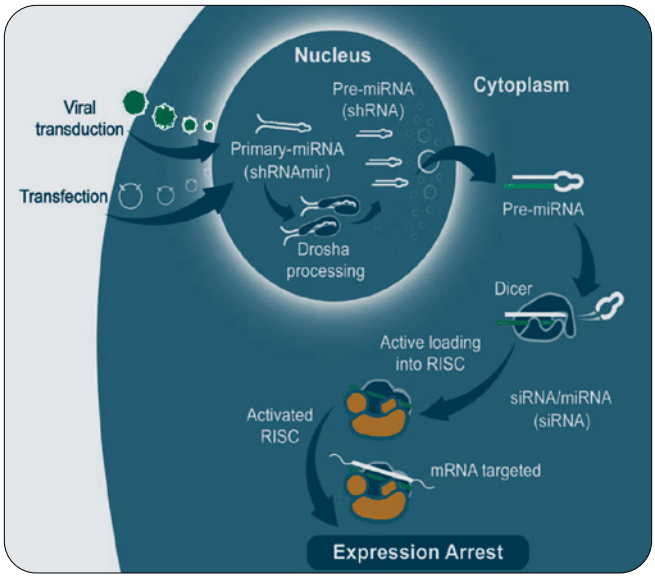


Figure 3: shRNAmir is processed via the endogenous microRNA pathway. 1) shRNAmir triggers modeled on primary miRNA are processed by Drosha and Dicer to produce mature siRNA targeting a complementary mRNA. 2) shRNA triggers are approximately modeled on precursor miRNA and are processed by Dicer to produce mature siRNA. 3) Chemically synthesized siRNA enter the RNAi pathway post-Dicer cleavage and incorporate into RISC to target complementary mRNA. All three RNAi triggers use the endogenous RNAi pathway but have distinct entry points.

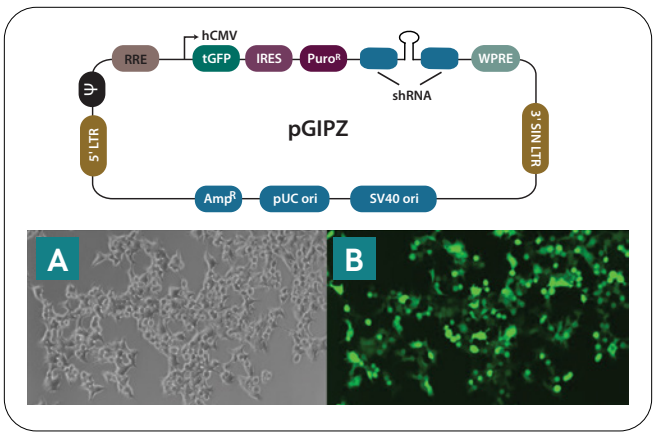


Figure 4: The pGIPZ vector expresses turboGFP (tGFP) as a marker of shRNAmir expression. **A**. Vector map highlighting the components of pGIPZ. shRNAmir is constitutively expressed as a polycistronic vector expressing tGFP, puromycin resistance marker (Puror) and the shRNAmir. **B**. HEK293T cells transduced by GIPZ at a low MOI. Green fluorescence is an indicator that the shRNAmir-containing transcript is being expressed.

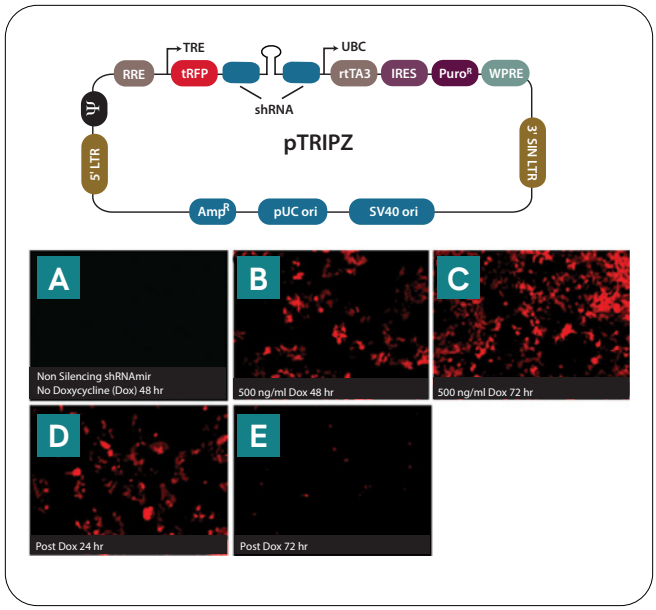


Figure 5: **A.** The TRIPZ vector incorporates many of the same elements of the GIPZ lentiviral backbone including a fluorescent marker (tRFP) of shRNAmir expression. Additionally, it contains all of the components necessary for inducible expression of the shRNAmir. **B-D**. tRFP expression is shown as a marker of controlled, inducible expression from this vector. Transduced cells were fed media containing 500 ng/mL doxycycline (Dox) and maximal induction was seen within 72 hours. **E.** Subsequently, The cells were washed and fed medium without doxycycline. Seventy-two hours later tRFP reflected a minimal level of expression.

Gene expression knockdown

Seventy-eight shRNAmir in either the GIPZ or the TRIPZ lentiviral vectors were evaluated and percent remaining mRNA expression was measured by qPCR. H1299 human lung carcinoma cells were transduced at an MOI of 1-2, selected with puromycin for 48 hours and gene expression was measured by qPCR. Three independent experiments were performed for each shRNAmir. Previous work done in concert with the National Cancer Institute showed that the GIPZ library was capable of achieving ≥ 70% knockdown in approximately 2 of 3 hairpins. The findings here were similar. In H1299, 72% of the shRNA were shown to knockdown gene expression by ≥ 70% (Figure 6). Overall this validated the previous expectations of the library for consistency and level of knockdown.

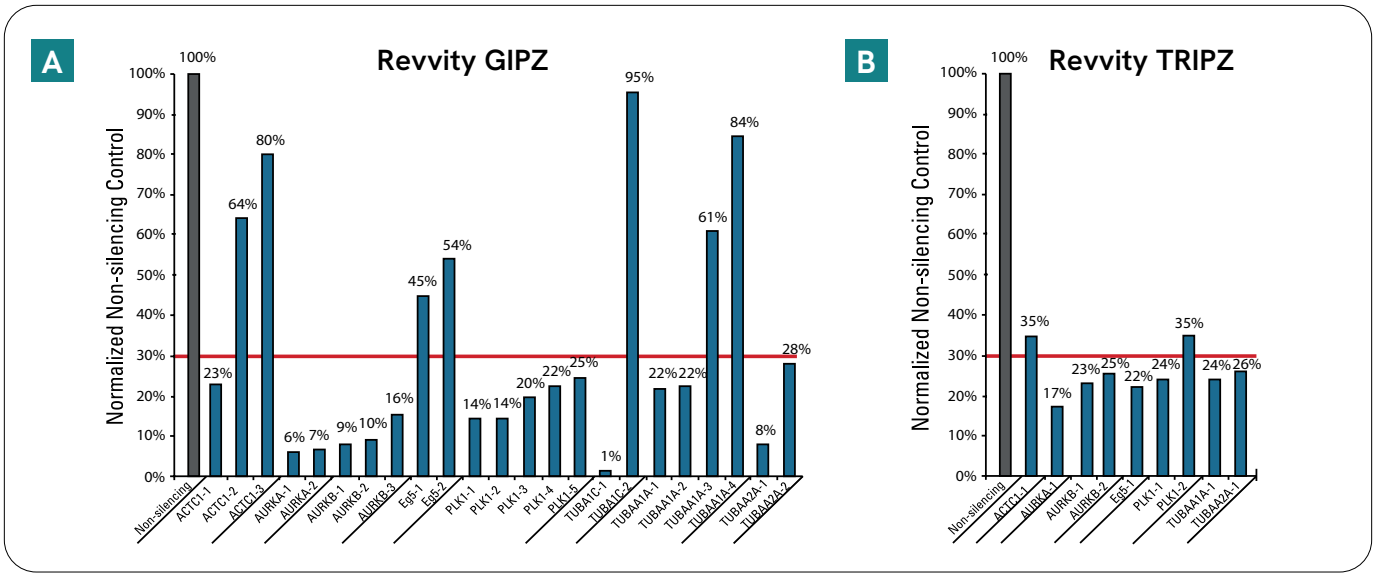


Figure 6: Demonstration of knockdown efficiency. In H1299, a human lung carcinoma cell line, 72% of the shRNA were shown to knock down gene expression by ≥ 70%. The red line denotes this level of knockdown. Multiple hairpins target each gene either in **A**. GIPZ or **B**. TRIPZ. All samples were measured using qPCR. The value shown is an average of three independent experiments.

Constitutive and inducible knockdown at the protein level

mRNA knockdown is a quick and useful validation of shRNAmir function. However, the resulting decrease in protein is usually what is thought to alter phenotypes. The cell surface marker AXL was targeted in HeLa cells and changes in protein level were measured by a fluorescent immunocytochemistry assay using FACS analysis to determine the ability of GIPZ and TRIPZ to knockdown protein levels. Every shRNAmir in both vectors provided > 70% knockdown of the RNA in HeLa cells (data not shown). The option of inducible shRNAmir expression provides experimental flexibility, and the uninduced state is an ideal control for many experiments. However, one concern about inducible expression systems is the possibility of a basal expression level in the absence of the inducing agent (tetracycline in this case) which is commonly referred to as 'leaky' expression.

There are two main components on the pTRIPZ vector enabling induction: the tetracycline response element (TRE) and the transactivator. The transactivator expressed from pTRIPZ is rtTA3, a modified form optimized for increased response to tetracycline without increasing background. TRIPZ demonstrated tightly controlled expression. The level of protein expression is compared between untransduced cells and cells that are transduced but no tetracycline ('OFF') has been added to induce expression of the shRNAmir. These protein levels should be the same if there is no unwanted expression of the shRNAmir, and this is what was found by using a FACS assay and measuring AXL (Figure 7).

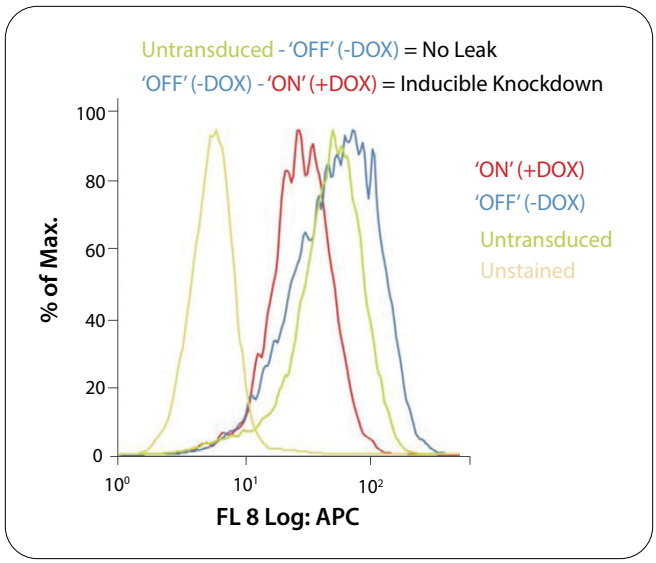


Figure 7: Decrease in protein level without 'leaky' expression shown by FACS analysis. Immunocytochemistry was used to detect the AXL protein by FACS. The leftshift of the peaks denotes a decrease in the AXL protein level after knockdown with TRIPZ shRNA targeting AXL. The untransduced and 'OFF' sample primarily overlap suggesting very little uninduced or leaky expression. The left-shifted red peak compared to the blue shows the knockdown.

Phenotypic validation

A high content screen ultimately relies on phenotypic changes. In this example, H1299 cells were transduced with constructs from the GIPZ library targeting genes associated with cell cycle progression and cancer. Many of the phenotypes presented as polyploidy including endoreduplication, a cellular state in which the genome of the cell is duplicated but it does not under go mitosis. The polyploidy phenotype can present as one very large nucleus or multinucleated phenotypes and matched very well with previous data using siRNA or small molecule described in the literature (Figure 8, see page 4).

The library was able to mimic known cellular phenotypes induced by small molecule inhibitors for every target gene chosen, thereby validating its use as a screening tool with a phenotypic assay. Any of these results could be incorporated as a positive control into a high content screen for additional novel genes inducing the phenotype.

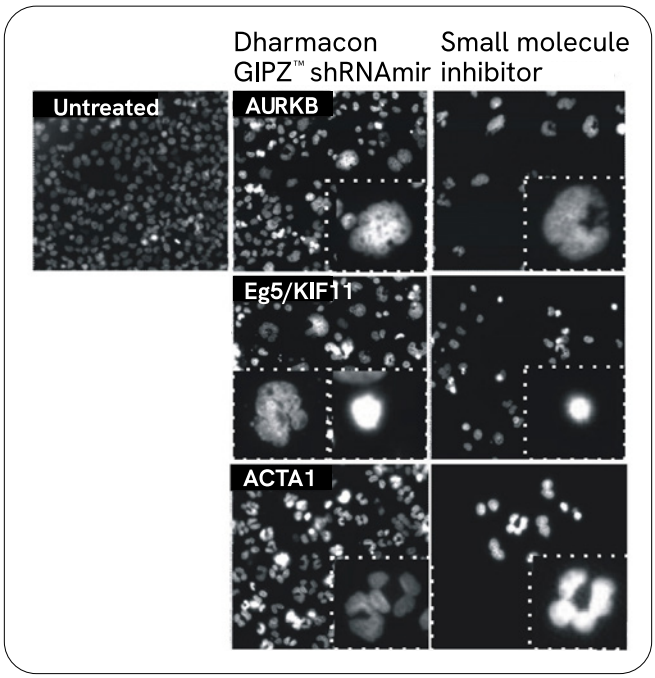


Figure 8: Knockdown of the targeted genes produces phenotypes known to result from disrupting the cell cycle. AURKB, and PLK1 knockdown produced an endoreduplication phenotype. EG5 knockdown produced the expected preprometaphase 'stellate' phenotype and endoreduplication. Knockdown of ACTA1 produced a multinucleated phenotype.

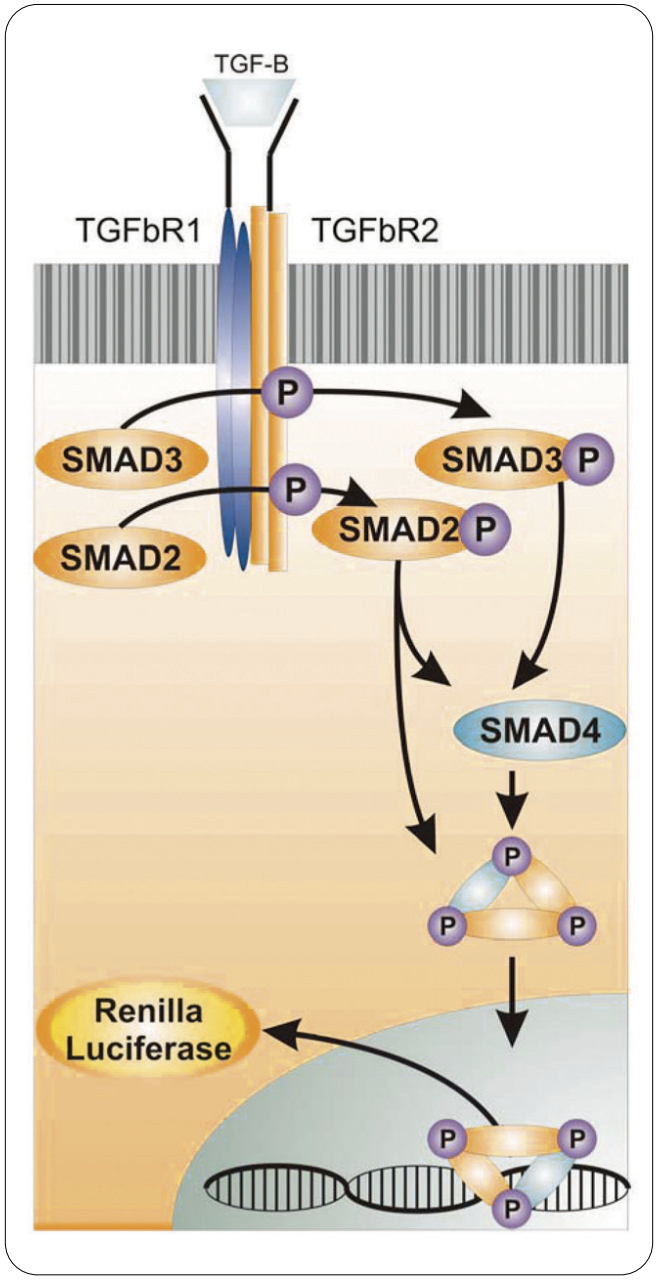


Figure 9: The cell line HeLa/ SMAD-gRL was engineered to express luciferase when stimulated with TGF-β. The cell line allowed luciferase to be used as a readout for signaling through this pathway. Unknown genes' contribution to this pathway can now be correlated to luciferase activity. Genes without redundancy in the pathway will result in a stronger disruption in luciferase activity when knocked down. The converse is also true. This is reflected in the luciferase readout of TGFbR1 which is required for signaling and the SMADS that have redundant partners with alternate downstream signaling. (Adapted from Rigel's data presentation.)

Pathway analysis

One of the more sophisticated experiments that can be done with RNAi is to dissect the components in a signaling pathway. Changes in many genes' expression can be difficult to detect directly. Using a reporter system can alleviate this by linking expression of a reporter protein (for example, luciferase) to a promoter that is known to be activated when the pathway of interest is involved. The well characterized TGF-β pathway was used as a model. TGF-β, a cell signaling molecule involved in proliferation and differentiation in many cell types regulates gene expression through SMAD pathway. Signaling through this pathway initiates with the TGF-β-receptor binding TGF-β and involves many elements of the SMAD signaling pathway. SMADs represent a broad family of genes that can overlap in their contribution to signaling [reviewed in Derynk, R. et al. 2003]. A cell line (HeLa/SMAD-gRL) was designed to expresses luciferase from a SMAD response element-based promoter (Figure 9) coupling TGF-β signaling activity to luciferase activity, a functional readout more amenable to a high throughput assay. Dose response curve was first generated by stimulation of this pathway without disrupting gene expression (Figure 10a). After each gene suspected to be in the pathway was knocked down the resulting dose response curve was compared back to the control (Figures 10 b-d). Changes in luciferase activity were used to determine the relative contribution of many genes in the TGF-β/SMAD signaling pathway. Knockdown of the TGF-β receptor, TGFbR1, significantly disrupted luciferase expression (Figure 10b). Knockdown of SMAD2 and SMAD4 (Figures 10c and 10d respectively) resulted in a significant but incomplete disruption of the reporter. This reflects the redundant nature of multiple SMADs with overlapping signaling roles.

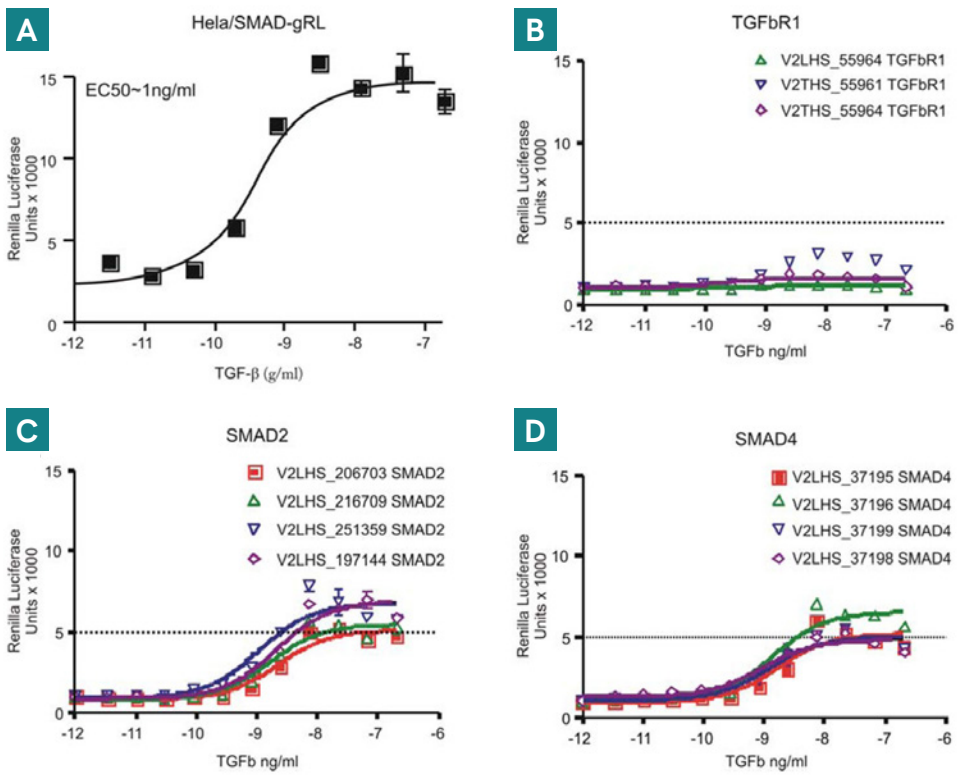


Figure 10: Luciferase as a read out for SMAD signaling using the cell line HeLa/SMAD-gRL. **A**. Control dose response curve was generated from the HeLa/SMADgRL cell line without disrupting the pathway. SMAD genes known to participate in this signaling pathway were knocked down using multiple hairpins to each gene (their clone IDs are shown in the legend). A decrease in the luciferase output suggests a disruption in the SMAD signaling pathway. The response curves were compared to the control. Multiple hairpins to three of the target genes are shown here **B**. TGFbR1, the TGF-β receptor, **C**. SMAD2 and **D**. SMAD4.

This data provides benchmarks for luciferase activity while disrupting genes known to be involved in the pathway. Going forward, using the current data to gauge participation in TGF-β signaling pathway, this assay is amenable to a high throughput screen of the entire genome. Knockdown of the TGF-β receptor, TGFbR1, significantly disrupted luciferase expression (Figure 10b). Knockdown of SMAD2 and SMAD4 (Figures 10c and 10d respectively) resulted in a significant but incomplete disruption of the reporter. This reflects the redundant nature of multiple SMADs with overlapping signaling roles. This data provides benchmarks for luciferase activity while disrupting genes known to be involved in the pathway. Going forward, using the current data to gauge participation in TGF-β signaling pathway, this assay is amenable to a high throughput screen of the entire genome.

Conclusion

The ability of the GIPZ and TRIPZ lentiviral shRNAmir constructs to produce effective knockdown has been evaluated at the mRNA and protein level as well as in functional analysis. qPCR results confirmed not only that a majority of the constructs in this library are capable of inducing sufficient knockdown (~ 2 out of 3), but that this can be achieved at low MOIs in most cases. This has significant advantages for the customer who is interested in knockdown of a single gene or the customer who is interested in a larger scale screening tool:

1. One of the main concerns of an RNAi experiment is the possibility for off-target effects. The ability to achieve significant knockdown at low MOIs will reduce the possibility of concentration dependent off-target effects.
2. Fewer resources can be used to perform more experiments.
3. Any downstream event dependant on knockdown will be more robust. For example, this was shown for the TGF-β pathway (Figures 9 and 10). Known phenotypes caused by small molecule inhibitors were mimicked and the essential components of the pathway were able to be distinguished from the redundant components.

References

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For more information:

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