# Development and automation of a fibrotic phenotypic screening using a High Content Screening approach

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## 1-INTRODUCTION

A common feature of chronic kidney disease is the fibrotic status which progressively settles in over years. To address this issue, a phenotypic screening was developed to identify molecules able to specifically block the fibrotic activity of TGFβ1 and acting downstream its receptors.

## 2-VALIDATION OF THE RAT KIDNEY FIBROBLAST MODEL: NRK-49F

Micro-array studies showed that upon stimulation by TGFβ1, NRK-49F cells developed a fibrotic response as exemplified by the increase of extracellular matrix gene expression such as collagens and fibronectin.

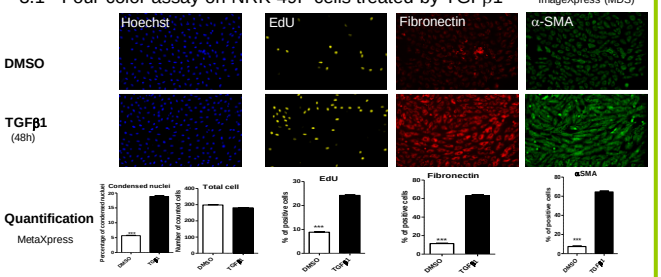
| Time | Number of genes differentially expressed P <0.01 | Number of genes differentially expressed P <0.01 + ILog2(ratio)I>0.6 |
|---|---|---|
| 6 h | 8,800 | 1,066 |
| 24 h | 12,763 | 2,176 |
| 48 h | 13,948 | 2,797 |

| Gene Name | Sequence Description | 6 h | | 24 h | | 48 h | |
|---|---|---|---|---|---|---|---|
| | | Fold Change | P*-value | Fold Change | P*-value | Fold Change | P*-value |
| Tgfb1 | Transforming growth factor, beta 1 | 1.6 | 1.5E-03 | 2.2 | 2.1E-06 | 2.5 | 3.9E-07 |
| Tgfb2 | Transforming growth factor, beta 2 | 1.0 | 6.5E-01 | 1.8 | 7.3E-07 | 1.4 | 2.0E-04 |
| Tgfb3 | Transforming growth factor, beta 3 | 2.1 | 1.4E-07 | 2.1 | 5.2E-08 | 1.5 | 5.2E-05 |
| Col1α2 | Collagen, type I, alpha 2 | 0.9 | 4.4E-01 | 1.8 | 5.8E-04 | 1.9 | 1.6E-05 |
| Col3α1 | Collagen, type III, alpha 1 | 1.2 | 4.8E-01 | 2.9 | 1.0E-06 | 3.6 | 2.4E-05 |
| Col4α1 | Collagen, type IV, alpha 1 | 1.4 | 2.8E-03 | 2.3 | 7.3E-08 | 2.9 | 2.5E-09 |
| Fn1 | Fibronectin 1 | 1.4 | 5.0E-02 | 2.1 | 1.0E-04 | 1.7 | 6.3E-04 |
| Tnc | Tenascin C | 1.6 | 2.1E-03 | 4.5 | 5.8E-10 | 4.0 | 9.7E-10 |
| Has2 | Hyaluronan synthase 2 | 2.5 | 4.2E-08 | 4.8 | 1.0E-11 | 7.4 | 3.5E-13 |
| Ctgf | Connective tissue growth factor | 3.2 | 1.2E-08 | 2.5 | 2.6E-07 | 1.3 | 2.4E-02 |
| Vegfa | Vascular endothelial growth factor A | 1.4 | 4.2E-03 | 2.4 | 1.4E-07 | 2.3 | 2.2E-07 |
| Fgf2 | fibroblast growth factor 2 (basic) | 2.3 | 2.5E-04 | 3.1 | 3.2E-06 | 4.1 | 9.3E-07 |
| Serpine1 | Serpin peptidase inhibitor (plasminogen activator inhibitor type 1) | 11.3 | 2.7E-10 | 14.0 | 5.1E-11 | 4.9 | 1.8E-08 |

## 3- HIGH CONTENT SCREENING ASSAY

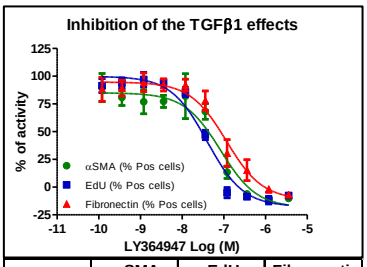
### 3.1 - Four color assay on NRK-49F cells treated by TGFβ1

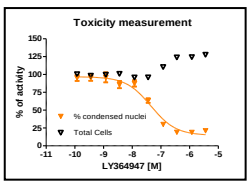
Pictures: ImageXpress (MDS)




TGFβ1 induces phenotypic changes in NRK-49F quantifiable by imaging

### 3.2 - Validation of the assay with a TGFβ receptor (ALK5) inhibitor: LY364947

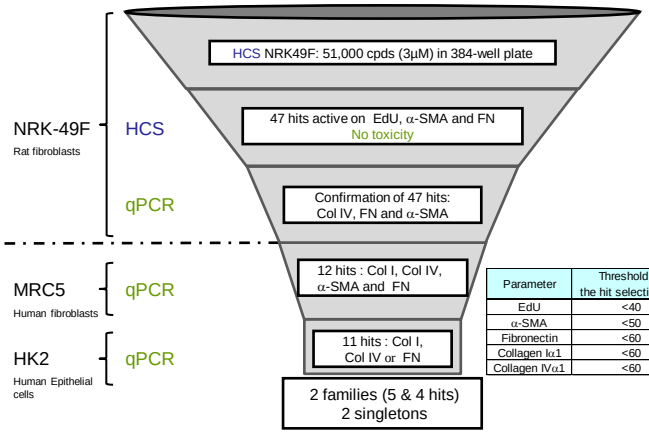



| | αSMA | EdU | Fibronectin |
|---|---|---|---|
| IC50 (M) | 8.32E-08 | 3.98E-08 | 1.14E-07 |

ALK5 inhibitor blocks the fibrogenic effects of TGFβ1, in dose dependent manner, without toxicity.

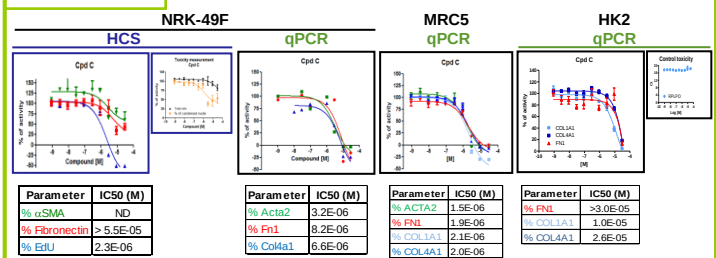
## 4- SCREENING AND HIT SORTING




| Parameter | Threshold for the hit selection (%) |
|---|---|
| EdU | <40 |
| α-SMA | <50 |
| Fibronectin | <60 |
| Collagen Iα1 | <60 |
| Collagen IVα1 | <60 |

- Out of the 47 hits selected from HCS, all were confirmed by qPCR in NRK-49F cells, suggesting that our compounds did not regulate fibronectin and αSMA at the post-translational level.
- mRNA induction of collagen IVα1 by TGFβ1 was also blocked by the 47 hits in NRK-49F cells.
- 12 hits prevented the "fibrotic" induction by TGFβ1 in human lung fibroblasts cell line, MRC5.
  - These compounds were also active on collagen Iα1 induced by TGFβ1.
- 11 hits were also active on human kidney epithelial cells (HK2) suggesting that these compounds target conserved fibrotic effectors.

### Example of hit




| Parameter | IC50 (M) |
|---|---|
| % αSMA | ND |
| % Fibronectin | > 5.5E-05 |
| % EdU | 2.3E-06 |

| Parameter | IC50 (M) |
|---|---|
| % Acta2 | 3.2E-06 |
| % Fn1 | 8.2E-06 |
| % Col4a1 | 6.6E-06 |

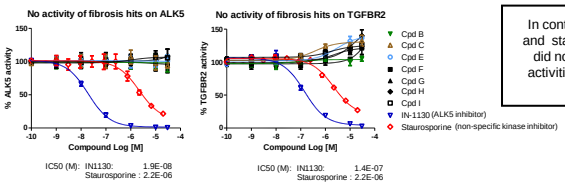
| Parameter | IC50 (M) |
|---|---|
| % ACTA2 | 1.5E-06 |
| % FN1 | 1.9E-06 |
| % COL1A1 | 2.1E-06 |
| % COL4A1 | 2.0E-06 |

| Parameter | IC50 (M) |
|---|---|
| % FN1 | >3.0E-05 |
| % COL1A1 | 1.0E-05 |
| % COL4A1 | 2.6E-05 |

This hit was active on the three cell types without showing cell toxicity

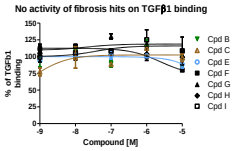
## 5- HITS DID NOT ACT AT THE TGFβ1 RECEPTOR LEVEL

### 5.1 - Biochemical assay of TGFβ receptor Kinase inhibition using $^{33}$P ATP




In contrast to IN-1130 and staurosporine, hits did not block kinase activities of ALK5 and TGFβR2

### 5.2 - $^{125}$I-TGFβ1 binding competition assay on 3T3 cells




As aimed, our hits were not able to block kinase activities of ALK5 and TGFβR2, nor the TGFβ1 binding to its receptors.

## 6 - CONCLUSIONS

- We successfully developed and performed a fully automated fibrotic HCS screening on 51k compounds with 4 readouts
- 47 hits were selected for their capacity to block the TGFβ1 effects on proliferation, differentiation and matrix production.
- In human fibroblasts and epithelial cells, 11 hits retained full antifibrotic activity, suggesting that these molecules are acting on important and conserved fibrotic pathways activated by TGFβ1.
- None of the hits were acting at the TGFβ receptor level, suggesting innovative targets or mechanisms of action.

Our HCS strategy has allowed us to identify innovative hits with anti fibrotic activity *in vitro*. These hits can also be used as tools to identify therapeutic targets involved in fibrotic process.