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A High-throughput Gene Expression-based Screen For Factors That Modulate In-vitro Chondrogenesis Of Stem Cells Identifies Optimal Conditions And Novel Factors

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INTRODUCTION

The in vitro process of chondrogenic differentiation of mesenchymal stem cells for tissue engineering has been shown to require three-dimensional culture along with the addition of differentiating factors to the culture medium. This, however, in general leads to a phenotype lacking some of the cardinal features of native articular chondrocytes and extracellular matrix.
The factors used vary but regularly include members from the TGF beta super family and dexamethasone (DEX), sometimes in conjunction with FGF-2 and IGF-1.
The use of soluble factors to induce chondrogenesis has largely been studied on a single factor basis or with combinations of a few factors. We have combined a factorial design experiment with high-throughput digital mRNA profiling as a powerful tool to study in vitro chondrogenesis.

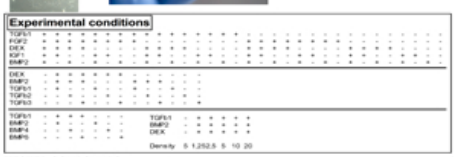
AIM OF STUDY

- to evaluate the suitability of studying in vitro chondrogenesis by factorial design and high throughput digital gene expression profiling
- to identify the optimal chondrogenic conditions
- to identify novel inhibitors of unwanted molecules in tissue engineered hyaline cartilage for further testing

METHODS

Quality control of surface markers and multipotentiality were performed on bone marrow derived MSC before embedding into alginate. A 2^5-factorial design of all combinations of TGF-b1, BMP-2, IGF-1, FGF-2 and DEX was then performed. Adapted designs for isoforms of TGF (-1,-2,-3) and BMP (-2, -4, -6) was also tested, giving a total of 48 different conditions. Furthermore, 38 different potential inhibitors of collagen type-I and -X synthesis were tested for their effect on chondrogenesis in a non-combinatorial design. Lysate or RNA was profiled using a custom probeset of 364 chondrogenesis related genes on the Nanostring nCounter platform.

Experimental setup

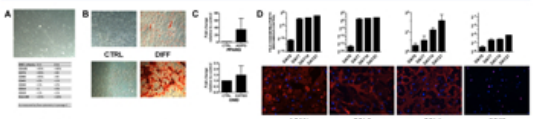


TESTED COMPOUNDS

GEFITINIB	TNFA	PD98059	MK-886	PENTIFYLLINE
IRBESARTAN	IFNG	ISOPROTERENOL	18-A-GLYCYRRHETINIC ACID	HGF
SILIBININ	AACOCF3	SAM	GLYCYRRHIZIC ACID	HALOFUGINONE
VATALANIB	DKK1	Alpha-MSH	IFNA	4-PHENYLBUTURIC ACID
PIRFENIDONE	AKTI	PGE2	IFNB	PAENOIFLORIN
NPPB	PRAVASTATIN	DLPC	SC560	PTHRP
PIOGLITAZONE	SIMVASTATIN	PENTOXIFYLLINE	GDC449	
IMIDAPRILAT	ETODOLAC	DOXYCYCLINE	T3	

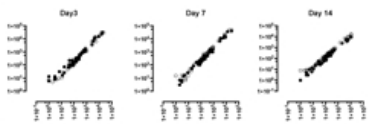
RESULTS

Quality control MSC



A. Morphology and cell surface markers of primary bone maarow derived human MSCs. **B and C.** Adipogenic (upper panel) and osteogenic (lower panel) differentiation of MSCs. **D.** Chondrogenic differentiation of MSCs

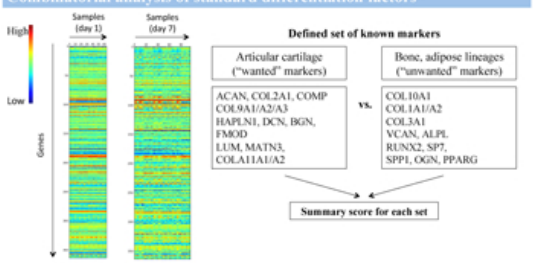
Correlation between lysates and total-RNA

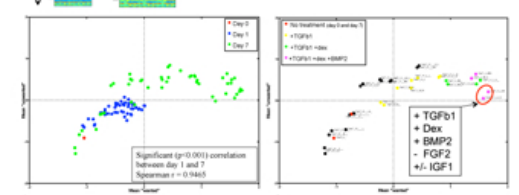


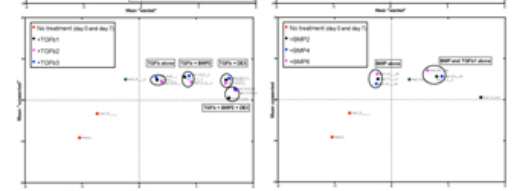
R^2	Donor 1	Donor 2
Day 3	0.9470	0.9648
Day 7	0.9586	0.9175
Day 14	0.9725	0.9559

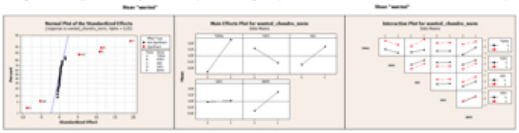
Expression profiles (Nanostring test geneset of 48 probes) were obtained from total-RNA isolated using Qiagen RNeasy and from RLT-lysates from corresponding samples in 2 donors at 3 timepoints. Correlation coefficients were deemed sufficient for subsequent experiments to be performed on lysates.

Combinatorial analysis of standard differentiation factors







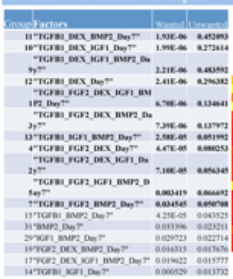


Analysis using a wanted and unwanted geneset identified larger separative differences after longer exposure though with a highly significant correlation between day 1 and day 7. The condition of TGFb1, DEX and BMP2 +/- IGF1 had the most optimal ratio between wanted and unwanted genes. Only minor effects of isoforms of TGF or BMP could be identified.

Factorial analysis of main effects and interactions analysed on day 7 found significant positive main effects for TGFb1, BMP2 and DEX and a negative effect of FGF2 on both wanted and unwanted gene sets. Significant interactions were found for combinations of TGFb1 and BMP2 showing non-additive, non-synergistic effect on both gene sets, and for TGFb1 and DEX showing a synergistic effect on wanted genes. The combination of FGF2 with DEX or FGF with DEX and TGFb1 both had synergistic effects on the expression of unwanted genes.

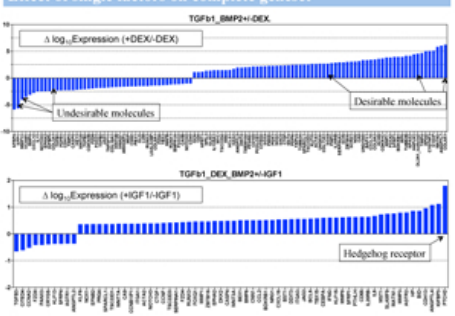
Geneset enrichment analysis

Group	Factors	Wanted	Unwanted
	11^TGFB1_DEX_BMP2_Day7^	1.93E-06	0.452093
	10^TGFB1_DEX_IGF1_Day7^	1.99E-06	0.272614
	^TGFB1_DEX_IGF1_BMP2_Da		
	9y7^	2.21E-06	0.483592
	12^TGFB1_DEX_Day7^	2.41E-06	0.296382
	^TGFB1_FGF2_DEX_IGF1_BM		
	1P2_Day7^	6.70E-06	0.134641
	^TGFB1_FGF2_DEX_BMP2_Da		
	3y7^	7.39E-06	0.137972
	13^TGFB1_IGF1_BMP2_Day7^	2.58E-05	0.051992
	4^TGFB1_FGF2_DEX_Day7^	4.47E-05	0.080253
	^TGFB1_FGF2_DEX_IGF1_Da		
	2y7^	7.10E-05	0.056345
	^TGFB1_FGF2_IGF1_BMP2_D		
	5ay7^	0.003419	0.066692
	7^TGFB1_FGF2_BMP2_Day7^	0.034545	0.050708
	15^TGFB1_BMP2_Day7^	4.25E-05	0.043523
	31^BMP2_Day7^	0.033396	0.023231
	29^IGF1_BMP2_Day7^	0.029723	0.022714
	19^FGF2_DEX_BMP2_Day7^	0.016515	0.017676
	17^FGF2_DEX_IGF1_BMP2_Day7^	0.019622	0.015777
	14^TGFB1_IGF1_Day7^	0.000529	0.013732



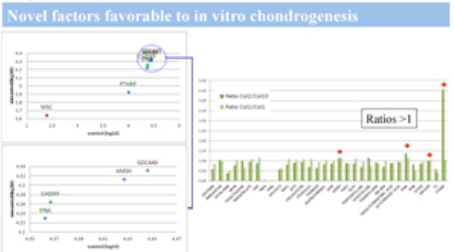
Conditions in bold are enriched for wanted genes and **not** enriched for unwanted genes. Heatmap of the top 10 conditions visually confirms ranking.

Effect of single factors on complete geneset



Analysis of differentially expressed genes between conditions of interest allows for biological interpretation.

Novel factors favorable to in vitro chondrogenesis



Based on favorable ratios of collagen expression and wanted/unwanted genes compared to MSCs undergoing differentiation in the standard condition (TGFb1, BMP2 and DEX) four factors have been chosen for further investigation.

CONCLUSION

Factorial design with high throughput gene expresssion profiling can be used to investigate differentiation cocktails directly on lysates of cells in alginate.

A cocktail of TGFb1, BMP2 and DEX is the most efficient from a gene expression perspective, but all factors beneficial to wanted genes do also increase unwanted genes. Eliminating one of these factors severely decreases expression of wanted genes, indicating that perfect differentiation to articular cartilage may not be achievable with the currently used factors.

Four factors that may improve in vitro chondrogenesis were selected from the screen and are being investigated further.