IDENTIFICATION AND TISSUE-SPECIFIC CHARACTERIZATION OF NOVEL SHOX-REGULATED GENES IN ZEBRAFISH HIGHLIGHTS SOX FAMILY MEMBERS AMONG OTHER GENES

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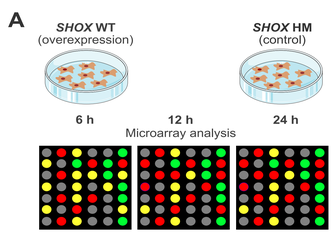
Introduction

SHOX deficiency causes a spectrum of clinical phenotypes related to skeletal dysplasia and short stature, including Léri-Weill dyschondrosteosis, Langer mesomelic dysplasia, Turner syndrome, and idiopathic short stature. SHOX controls chondrocyte proliferation and differentiation, bone maturation, cellular growth arrest and apoptosis via transcriptional regulation of its direct target genes *NPPB*, *FGFR3* and *CTGF*[1]. However, our understanding of SHOX-related pathways is still incomplete. To elucidate the underlying molecular mechanisms and to better understand the broad phenotypic spectrum of SHOX deficiency, we aimed to identify novel SHOX targets.

Methods and Results

We analyzed differentially expressed genes in *SHOX-overexpressing* human fibroblasts (NHDF), and confirmed the known SHOX target genes *NPPB* and *FGFR3* among the most strongly regulated genes, together with 143 novel candidates. Altogether, 23 genes were selected for further validation, first by whole-body characterization in developing *shox-deficient* zebrafish embryos, followed by tissue-specific expression analysis in three *shox-expressing* zebrafish tissues: head (including brain, pharyngeal arches, eye, and olfactory epithelium), heart, and pectoral fins. Most genes were physiologically relevant in the pectoral fins, while only few genes were also significantly regulated in head and heart tissue. Interestingly, multiple *sox* family members (*sox5*, *sox6*, *sox8*, and *sox18*) were significantly dysregulated in *shox-deficient* pectoral fins together with other genes (*nppa*, *nppc*, *cdkn1a*, *cdkn1ca*, *cyp26b1*, and *cy26c1*), highlighting an important role for these genes in shox-related growth disorders. Network-based analysis integrating data from the Ingenuity pathways revealed that most of these genes act in a common network.



B

6h		12h		24h	
SOX8	6.4	*HES5*	20.2	*SOX8*	44.6
HES5	2.5	*SOX8*	16.2	*HES5*	36.1
NUAK2	2.3	*RRAD*	11.5	*VGF*	22.0
RGS16	2.2	*ARC*	11.3	*ARC*	19.8
BCOR	2.1	*CYP26B1*	8.3	*RRAD*	17.7
SNF1LK	2.1	*VGF*	7.4	*CYP26B1*	17.3
HSPA6	2.1	*FGFR3*	7.1	*RASD1*	15.2
LOH3CR2A	1.9	*SOX18*	6.4	*NPPB*	12.2
ARC	1.9	*KRT17*	5.1	*FGFR3*	10.5
TRIB1	1.9	*RASD1*	4.4	*KRT17*	9.9

Figure. 1. Microarray analysis of *SHOX* overexpressing wild type (WT) and mutant (HM) normal human dermal fibroblast (NHDF) cells. (A) Overview of experimental design. **(B)** Top 10 regulated genes in *SHOX* overexpressing NHDF cells after 6, 12, and 24 h. Identical colors indicate the same genes at different time points. Relative expression ratios are indicated next to the gene's name.

References

[1]Marchini A, Ogata T, Rappold GA. A Track Record on SHOX: From Basic Research to Complex Models and Therapy. Endocr Rev. 2016

Gene	6h	12h	24h
ARC	1.9	11.3	19.8
BMP4	1	1	1
CDKN1A	1.2	2.9	5.4
CDKN1B	1.4	0.7	1
CDKN1C	1.1	1.8	5.1
CYP26A1	1.1	1.1	1
CYP26B1	1	8.3	17.3
CYP26C1	1	1.1	1
FGFR3	1.7	7.1	10.5
HES5	2.5	20.2	36.1
KRT17	1.4	5.1	9.9
NPPA	1	1.1	1.4
NPPB	1	1.7	12.2
NPPC	1	0.9	1
RASD1	1.5	4.4	15.2
RRAD	1	11.5	17.7
SHOX2	1	1	1
SOX5	1	1	1
SOX6	1.2	1	1
SOX8	6.4	16.2	44.6
SOX9	1	0.8	0.8
SOX18	1.1	6.4	9.0
VGF	1.1	7.4	22.0

Table. 1. Genes selected for validation. Blue: novel targets; green: known SHOX-associated genes; grey: additional members of gene families

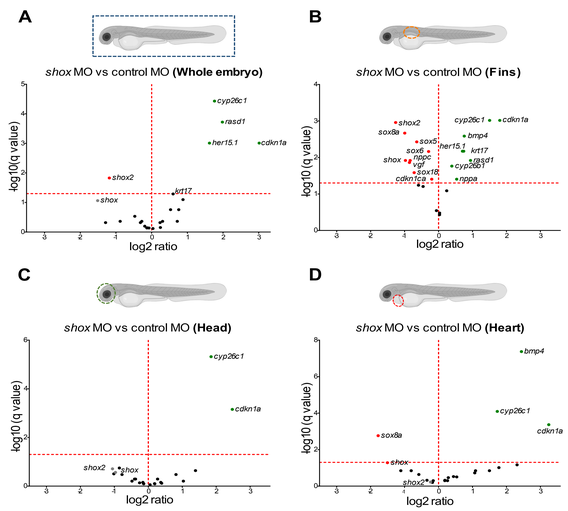


Figure 2. Tissue-specific validation of putative Shox target genes in zebrafish embryos after shox knockdown. Volcano plot presentations of expression ratio of selected shox target genes in **A)** whole zebrafish tissue (n=6 shox MO, n=6 control MO), **B)** fins (n=3 shox MO, n=3 control MO), **C)** head (n=4 shox MO, n=4 control MO), and **D)** heart (n=4 *shox* MO, n=5 control MO) at 55 hpf after morpholino-mediated knockdown of *shox* compared to controls. Genes above the red horizontal dashed line are significantly regulated. Genes shown to the left of the dashed vertical line are down-regulated, those on the right are up-regulated. MO, morpholino.

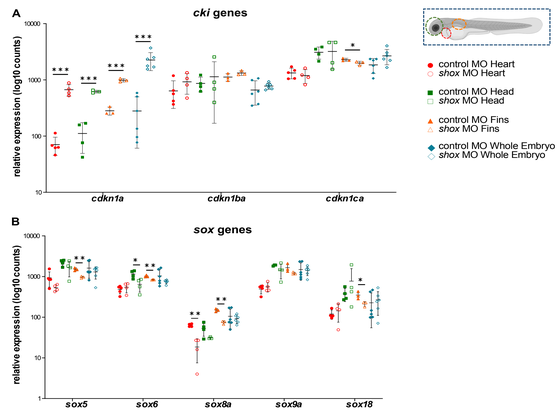


Figure 3. Tissue-specific analysis of gene families upon morpholino-mediated *shox* knockdown in zebrafish embryos 55 hpf compared to controls. Relative expression levels for **A)** the cyclin-dependent kinase inhibitor (cki) family members *cdkn1a*, *cdkn1ba*, *cdkn1ca*; **B)** members of the SRY-related HMG-box (sox) genes *sox5, sox6, sox8a, sox9a* and *sox18* in heart (red; n = 4-5), head (green; n = 4), fin (orange; n = 3) or whole embryo (blue; n = 6).

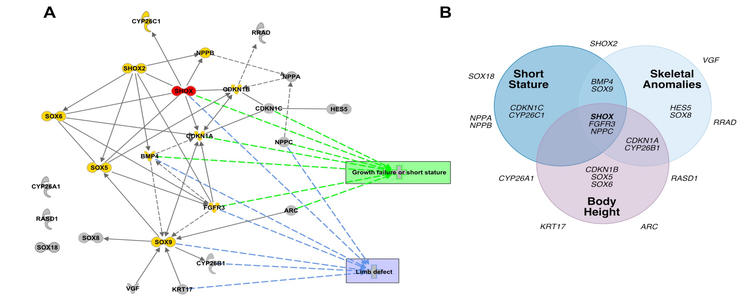


Figure 4. Network-based pathway analysis of SHOX-regulated genes using the Ingenuity pathway analysis software revealed that most of the SHOX-regulated genes act in a common network **(A)**. Gene-disease association based on the DisGeNET database (www.disgenet.org/home/) revealed associations between SHOX and 23 SHOX-regulated genes with respect to skeletal phenotypes **(B)**.

Conclusion

Our results provide novel insights into the genetic pathways and molecular events leading to the clinical manifestation of *SHOX* deficiency.

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