siRNA design, knockdown efficiency and specificity

The siRNAs were designed using parameters based on papers by Reynolds *et al*. and Ui-Tei *et al*. [1, 2]. The 19 nucleotide target sequences (*fyn*-siRNA position 1096-1114, GenBank Accession U35365 and *src*-siRNA position 614-632, GenBank Accession AF157016) were synthesized into 64-65 mer oligonucleotides with BamHI/HindIII overhangs (Eurofins MWG Operon) and cloned into the expression vector pSilencer 3.0-H1 (Ambion Inc). All clones were purified using an EndoFree Plasmid Maxi Kit (QIAGEN Ltd) and sequenced (Geneservice Ltd).

Cloning of *fyn* and *src* into a C-terminal tagged GFP vector, pcDNA3.1/CT-GFP-TOPO® (Invitrogen Life Technologies), was carried out according to the manual using RT-PCR and TOPO TA cloning® technology. The cDNAs used for the RT-PCR cloning of *fyn* and *src* were obtained from rat PASMC as described previously [3] and from Geneservice Ltd (IMAGE clone 4192014), respectively. Primer pairs for cloning are as follows:

fyn sense GTTATGGGCTGTGTGCAATGTAAGGAT

fyn antisense GCAGGTTTTCACCGGGCTGATACTG

src sense GTTATGGGCAGCAACAAGAGCAAGCCCAAG

src antisense: GTAGGTTCTCCCCGGGCTGGTACTGTG

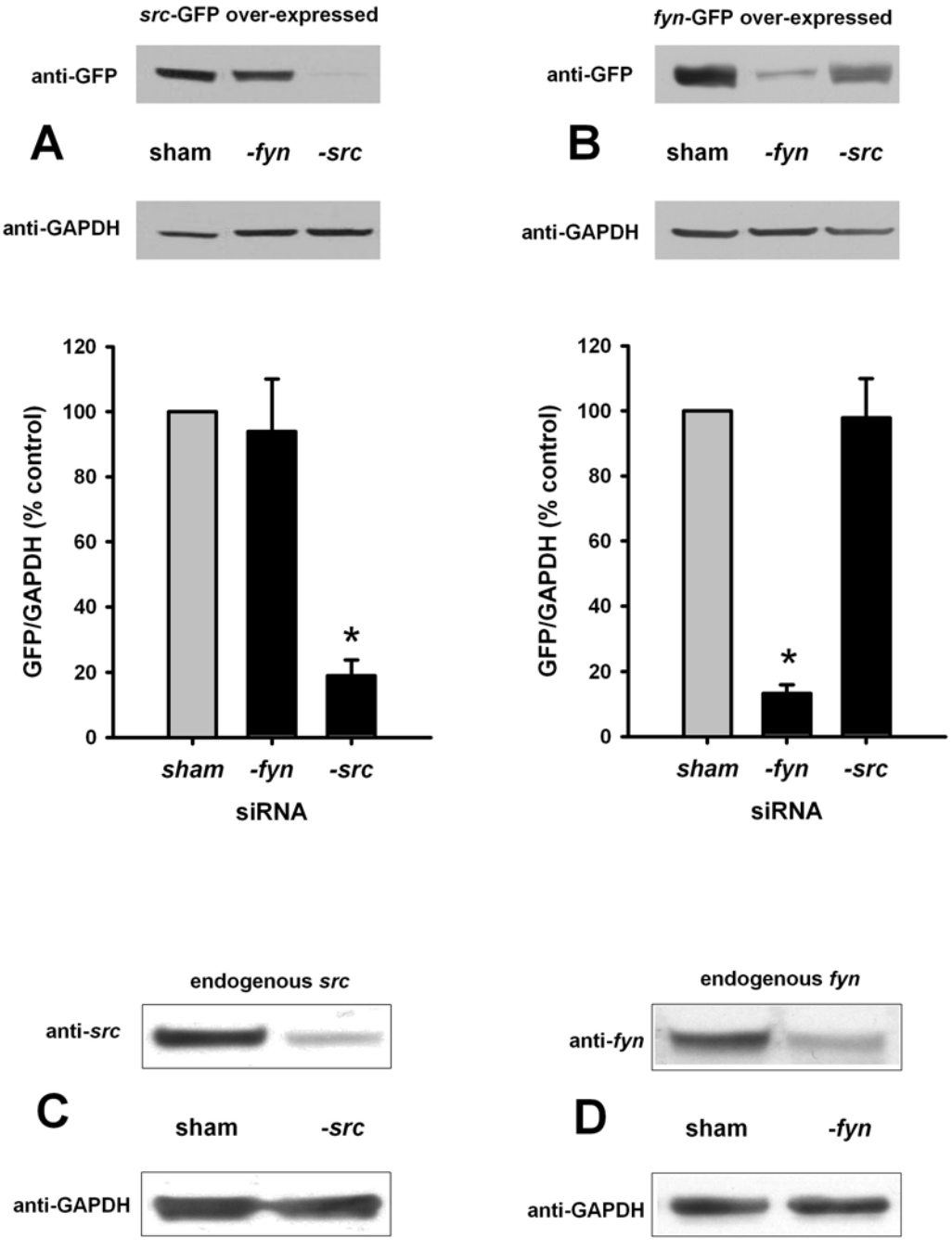
The correct clones were identified by restriction endonuclease digestion and sequencing (Geneservice Ltd). Both clones were purified using an EndoFree Plasmid Maxi Kit (QIAGEN Ltd).

Transfection efficiency as well as efficiency and specificity of knockdown were first confirmed in HEK-293 cells, cultured in DMEM media containing 10% FCS. Cells were co-transfected with 2µg of either GFP-tagged *fyn* or GFP-tagged *src* (to over-express the respective target proteins) and 0.5µg of *fyn*-siRNA, *src*-siRNA (to knockdown the respective target proteins) or sham (scrambled) siRNA, using Calcium Phosphate co-precipitation. Cells were harvested at 48 hours post-transfection. A control transfection using pmaxGFP showed >90% transfection efficiency, determined by fluorescence microscopy. Protein extracts were probed with 1:2000 mouse monoclonal anti-GFP (Roche Diagnostics Ltd) and 1:5000 mouse monoclonal anti-GAPDH (Abcam plc) and HRP-conjugated anti-mouse secondary (Sigma). As shown in Figure-I, over-expressed *src*-GFP protein was only knocked down by *src*-siRNA (panel **A,** *P<0.05 vs. sham transfected, n=3) and over-expressed *fyn*-GFP protein was only knocked down by *fyn*-siRNA (panel **B**, *P<0.05 vs. sham transfected, n=3). Sham transfection was without effect.

Pulmonary artery smooth muscle cells (PASMC) were transfected using the Basic Nucleofector® Kit for Primary Smooth Muscle Cells and the nucleofector device (Amaxa Biosystems). After 72 hours in culture the transfection efficiency was >90%, determined using pmaxGFP provided in the kit and confirmed by fluorescence microscopy. Knockdown of endogenous *src* and *fyn* in PASMC was similar to that in HEK-293 cells

and confirmed by western blot with anti-*src* (Cell Signalling) and anti-*fyn* (Santa Cruz) antibodies (Figure-I, panels **C** & **D**, respectively, representative of two determinations).

Figure-I



References

(**1**) Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. *Nat Biotechnol* 2004 March;22(3):326-30.

(**2**) Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res* 2004;32(3):936-48.

(**3**) Knock GA, Shaifta Y, Snetkov VA, Vowles B, Drndarski S, Ward JP, Aaronson PI. Interaction between src family kinases and rho-kinase in agonist-induced Ca^{2+}-sensitization of rat pulmonary artery. *Cardiovasc Res* 2008 February 1;77(3):570-9.