HA-Neogenin cDNA subcloning

Full length mouse neogenin cDNA in pYX-Asc was purchased from Open Biosystems, and was removed from the vector with *NotI/SalI*, and then ligated into *NotI/EcoRI* sites of pcDMA3. To generate HA tagged neogenin (HA-Neogenin), a fragment containing Ig k-chain leader sequence and HA epitope was applified from pDisplay vector (Invitrogen) by PCR using AccuPrime *Pfx* SuperMix (Invitrogen) with primers 5′-GTAGGCGTGTACGGTGGGAG-3′ (primer 1) and 5′-GAGCCGCTCTTCGTGGCCGCGGCGCCGGACGCAGCATAATCTGGAACATCATATG GATAGTCACC-3′ (primer 2). An upstream fragment corresponding to the N-terminus of mature neogenin protein was amplified by from the pcDNA3 plasmid containing neogenin using primers 5′-CGGCCACGAAGAGCGGCTC-3′ (primer 3) and 5′-TGGAGTTGGCTTCCCAGT GACTTCAC-3′ (primer 4). The two fragments were fused together using primers 1 and 4. The amplicon was digested with *KpnI/NheI* and ligated into *KpnI/NheI* sites of the pcDNA3 plasmid containing neogenin.

Immunoprecipitation

HEK293 cells were transfected with HA-Neogenin, Flag-HJV or both using Effectene. 48 hrs later, cell were lysed with 10 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton-100, and protease inhibitors (Roche). The lysates were incubated overnight at 4°C with ANTI-FLAG M2 Affinity Gel (Sigma, Saint Louis, Missouri) which had been blocked 4 h with 1% BSA in Tris buffer. M2 beads were washed with the Tris-HCl buffer four times. The beads were mixed with reducing Laemmli sample buffer and subject to immunoblotting with HA antibody.

Western blotting

Hep3B cells or HEK293 cells transfected with BMPRII-GFP, ActRIIA-Myc, ActRIIB-CFP, HA-ALK2, ALK3, HA-ALK6 or HA-Neogenin plasmid in combination with their siRNA were lysed with 10 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton-100, and protease inhibitors (Roche). Cell lysates were separated by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-GFP (Invitrogen), anti-Myc, anti-HA (Santa Cruz Biotechnology, Inc.), anti-ALK3 (Santa Cruz Biotechnology, Inc.), anti-neogenin (Santa Cruz Biotechnology, Inc.), or anti-Flag M5 antibody (Sigma), followed by the horseradish peroxidase-conjugated secondary antibodies. Antibody binding was detected with chemiluminescence reagent (NEN Life Sciences Products, Boston, MA). Membranes were stripped in 0.2 M glycine, pH 2.5, 0.5% Tween 20 for 20 mins, and re-probed with monoclonal anti-β actin antibody Chemicon).