

## The mechanism behind decorin's effect on myoblast differentiation: interaction among decorin, myogenic genes, myostatin, follistatin, and P21

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**Introduction:** Previously, we have demonstrated that decorin can improve injured skeletal muscle healing by both blocking the fibrotic effect of transforming growth factor beta (TGF-beta) and enhancing muscle regeneration (1,2). We now are working to identify the underlying mechanisms by which decorin enhances muscle regeneration. We have determined that decorin increases muscle cell fusion and up-regulates myogenic gene expression during myoblast differentiation. However, the mechanism by which decorin stimulates the signaling pathway responsible for this up-regulated differentiation remains unknown. In the study reported here, we genetically modified myoblasts to express the decorin gene to investigate the mechanisms behind decorin's effect on muscle regeneration.

**Materials and methods:** Cell culture: C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 10% horse serum (HS). To promote differentiation, the cells then were cultured for up to 6 days in DMEM supplemented with 2% HS, at which point the myotubes were counted. All cells were grown at 37°C in 5% CO<sub>2</sub>.

Gene transfer: Lipofectamine was used to transfect C2C12 cell with an AAV-mDecorin plasmid containing a mouse decorin sequence and a CMV promoter. The transfected C2C12 cells, *CD cells*, were selected by treatment with G418 (500 mg/mL) for 2 weeks. The expression of decorin by the CD cells was verified by western blot analysis.

Western blot analysis: C2C12 and CD cells were lysed when the cultures reached 70% confluence. Protein samples were separated on a 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel, transferred to nitrocellulose membranes, and analyzed by immunostaining. The primary antibodies used for this process were anti-decorin (gift from Dr. Fisher, NIH) at 1:1000; anti-myf5, anti-myf6, anti-myoD, anti-myogenin, and anti-myostatin at 1:1000; and anti-myosin, anti-desmin, and anti-P21 at 1:2000 for 1 hour at room temperature. The secondary antibody, anti-rabbit-HRP (1:5000), was applied for 1 hour. Peroxidase activity was determined by enhanced chemiluminescence (ECL), and positive bands were detected on X-ray film.

**Results:** 1. Decorin-transfected C2C12 cells expressed high levels of decorin. After selection with G418, CD cells expressed high levels of decorin protein, as demonstrated by western blot analysis.

2. Decorin increased myoblast differentiation by increasing the expression of myogenic proteins in vitro. In comparison with nontreated C2C12 cells, CD cells exhibited greatly enhanced differentiation and fusion.

Western blot analysis revealed increased expression of the myogenic proteins myf5, myf6, myoD, and myogenin in the CD cell population. However, desmin expression by the 2 cell populations remained similar.

3. Decorin up-regulated P21 and follistatin but down-regulated myostatin expression in myoblasts. We detected a low level of P21 and follistatin expression in the C2C12 cells; however, we observed high expression of both P21 and follistatin in the CD cells. In addition, C2C12 cells expressed a detectable level of myostatin whereas CD cells did not.

**Discussion:** The results reported here demonstrate that decorin up-regulates P21, an important cyclin-dependent kinase (cdk) inhibitor, but down-regulates myostatin, a negative regulator of skeletal muscle mass, in myoblasts (3,4). The up-regulation both of P21 and of other myogenic genes, including MyoD, in decorin-expressing muscle cells is a potential mechanism by which decorin promotes muscle cell differentiation and muscle regeneration; indeed, research has shown that myoD plays a central role in the differentiation of muscle cells (5). Moreover, decorin's up-regulation of follistatin, an antagonist of myostatin that down-regulates myostatin, might help to accelerate skeletal muscle healing after injury (6). In sum, our results indicate that decorin influences muscle healing by up-regulating the expression of both P21 and myogenic proteins, including MyoD, while down-regulating the expression of TGF-beta1 and myostatin.

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