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## Wnt signalling in development and disease

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# Wnt4 : A strong modulator of myogenesis.

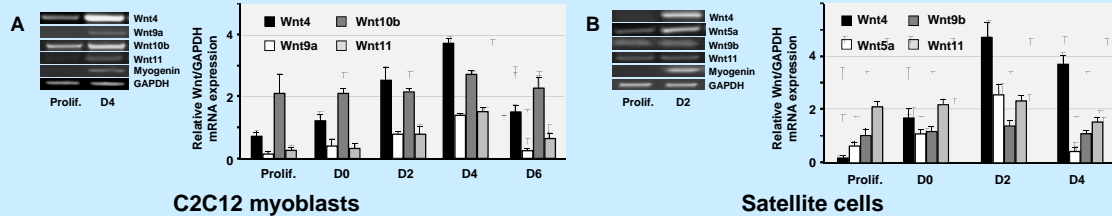
## Its interaction with myostatine expression and pathway

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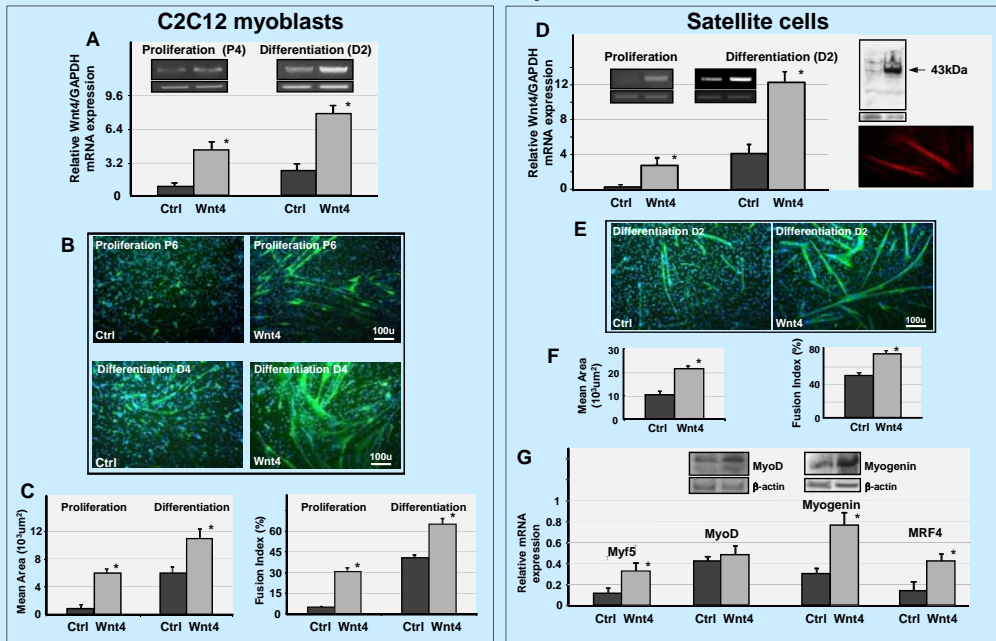
**INTRODUCTION:** While many data are accumulating concerning the functions of Wnt proteins during embryonic muscle development, the knowledge of the implications of Wnt signaling in adult muscle homeostasis and more specifically in the control of proliferation to differentiation is much more speculative. We thus focused on the roles of Wnt during C2C12 myoblasts and satellite cells differentiation. Wnts expression profiling indicates clearly that Wnt4 is strongly induced during differentiation of the both cellular types. We examined the myogenic effects of Wnt4 by modulating its expression levels during C2C12 myoblasts and satellite cells differentiation. Moreover, we investigated the regulation of the myogenic inhibitor myostatin by Wnt4.

### Wnt Expression Profile



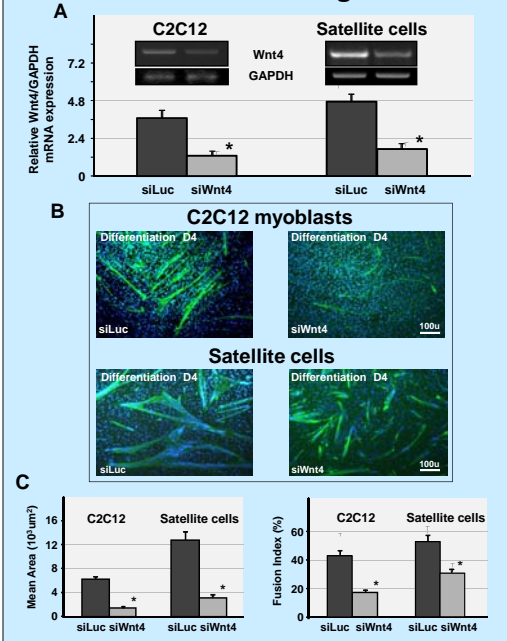
Wnt4 expression is induced during C2C12 and satellite cells differentiation. (A) RT-PCR was performed on total RNA extracted from C2C12 myoblasts at proliferative state (Prolif.) and 4 days (D4) after switch to differentiation culture medium (DM). Quantization of expression of activated Wnt genes was made by sqRT-PCR at proliferative state (Prolif.) and at day0 (D0), day2 (D2), day4 (D4) and day6 (D6) after switch to DM. (B) RT-PCR was performed on total RNA extracted from satellite cells at proliferative state (Prolif.) and 2 days (D2) after switch to DM. Quantization of expression of activated Wnt genes was made by sqRT-PCR at proliferative state (Prolif.) and at day0 (D0), day2 (D2) and day4 (D4) after switch to DM. Expression levels were normalized to the level of GAPDH expression. Histograms are presented as the mean  $\pm$  SEM for 5 independent experiments.

### Wnt4 overexpression



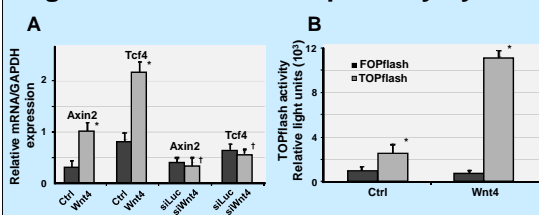
Wnt4 exhibits an acute myogenic activity on C2C12 and satellite cells. (A, D) Ectopic expression of Wnt4 in stably transfected C2C12 cells and in transiently transfected satellite cells. Expression of mouse Wnt4 was measured by sqRT-PCR on proliferative or differentiated C2C12 myoblasts (A) and satellite cells (D). In satellite cells, Wnt4 was detected by Western blot and immunofluorescence using an anti-Wnt4 antibody (D). Histograms are presented as the mean  $\pm$  SEM for 5 independent experiments. (B, C, E, F, G) Effects of Wnt4 overexpression on C2C12 myoblasts and satellite cells differentiation. Fluorescent images obtained with an anti-troponin T antibody on stably transfected C2C12 myoblasts (B) and transiently transfected satellite cells (E) with empty (Ctrl) or Wnt4-containing (Wnt4) pcDNA3 expression vector in proliferation or differentiation medium. Nuclei were labeled by DAPI. Mean area of myotubes and fusion index of Ctrl or Wnt4 C2C12 myoblasts (C) and satellite cells (F). The bar charts show the mean  $\pm$  SEM from 6 independent experiments. \* $P < 0.01$ . sqRT-PCR and Western blot analysis of Myf5, MyoD, myogenin and MRF4 expression in satellite cells transfected with Ctrl or Wnt4 pcDNA3 expression vector 2 days after switch to DM (G). Histograms are presented as the mean  $\pm$  SEM for 6 independent experiments. \* $P < 0.05$ . GAPDH primers were used as control for PCR quantization and  $\beta$ -actin as a loading control for Western blot.

### Wnt4 silencing



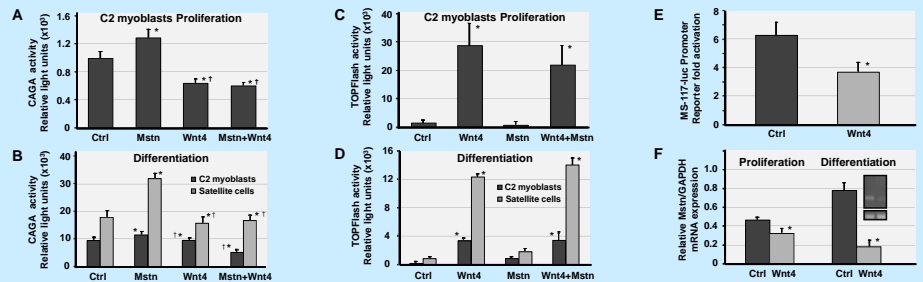
Wnt4 silencing inhibits myogenic activity on C2C12 and satellite cells. (A) Effect of siRNA transfection on Wnt4 gene expression during C2C12 and satellite cells differentiation. Wnt4 expression level was normalized with GAPDH expression. Cells transfected with siRNA specific to Luciferase were used as a control (siLuc). Histograms of each experiment are presented as the mean  $\pm$  SEM for 5 independent experiments. \* $P < 0.005$ . (B) Differentiation of C2C12 and satellite cells transfected with siWnt4 and siLuc. Fluorescent images were obtained by an immunostaining with monoclonal anti-troponin T antibody. Nuclei were stained by DAPI. (C) Mean area of myotubes and fusion index of C2C12 and satellite cells in DM. Histograms of each experiment are presented as the mean  $\pm$  SEM for 5 independent experiments. \* $P < 0.01$ .

### Regulation of canonical pathway by Wnt4



Wnt4-mediated activation of the Wnt canonical pathway in satellite cells. (A) Transcriptional expression of Axin2 and Tcf4 in satellite cells 2 days after switch to DM in cells transfected with empty (Ctrl) or Wnt4-containing expression vector (Wnt4) or siRNA specific to Luciferase (siLuc) or Wnt4 (siWnt4). Expression was measured by sqRT-PCR. GAPDH primers were used as control. Results of each experiment are presented as the mean  $\pm$  SEM for 4 independent experiments. \* Significantly different from the control ( $P < 0.05$ ); † significantly different from Wnt4 transfected cells ( $P < 0.05$ ). (B) Activity of the reporter gene TOPflash and negative control FOPflash in differentiated satellite cells (D2) transfected with empty (Ctrl) or Wnt4-containing expression vector (Wnt4). Histograms are presented as the mean  $\pm$  SEM for 4 independent experiments. \* $P < 0.01$ .

### Regulation of Myostatin promoter activity and expression by Wnt4



Inhibition of Mstn-mediated CAGA reporter activity and Mstn expression by Wnt4. (A, B, C, D) C2 and satellite cells were transiently transfected with the CAGA and TK-Renilla reporters (A, B) and TOPflash and TK-Renilla reporters (C, D). Cells were co-transfected with either empty (Ctrl) or Wnt4-containing (Wnt4) expression vector or treated with recombinant Mstn (Mstn). Luciferase activity was measured and normalized to the TK-Renilla. C2 cells were analyzed in proliferation medium (A, C) or 2 days after switch to DM (B, D). Experiments were performed in triplicate and error bars represent SEM. \* Significantly different from the untreated control ( $P < 0.05$ ); † significantly different from treated with Mstn alone ( $P < 0.05$ ). (E) C2 myoblasts were transiently transfected with the MS1177 myostatin promoter construct and either empty (Ctrl) or Wnt4-containing (Wnt4) expression vector. Fold induction of MS1177-luc promoter reporter was calculated by normalizing firefly luciferase activity to Renilla luciferase 3 days after switch to DM. The average of three experiments is shown, and error bars represent SEM \* $P < 0.05$ . (F) Transcriptional expression of Mstn in proliferative and differentiated C2 myoblasts transfected with Control or Wnt4-containing vector. \* $P < 0.05$ .

**Conclusions:** Our results, based on Wnts expression profiling, Wnt4 overexpression and Wnt4 siRNA-mediated inhibition experiments showed that Wnt4 has a strong myogenic activity. In addition, we showed (i) that Wnt4 inhibits the signaling pathway induced by the myogenic inhibitor myostatin and (ii) that Wnt4 negatively regulates myostatin expression. Taken together, our results indicate that Wnt4 is a strong modulator of myogenesis and this effect could be associated with an inhibition of myostatin activity.