# Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition

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(Degree)
博士（医学）

(Date of Degree)
2011-03-25

(Resource Type)
doctoral thesis

(Report Number)
甲5195

(URL)
https://hdl.handle.net/20.500.14094/D1005195

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|---|---|
| 氏　　名 | 任　大勇 |
| 博士の専攻分野の名称 | 博士（医学） |
| 学 位 記 番 号 | 博い第 5195 号 |
| 学位授与の 要 件 | 学位規則第 5 条第 1 項該当 |
| 学位授与の 日 付 | 平成 23 年 3 月 25 日 |

【 学位論文題目 】

Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition(癌細胞の上皮間葉転換における Wnt5a-Ror2 シグナルの役割)

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学位論文の内容要旨

# Critical role of Wnt5a–Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition

# 癌細胞の上皮間葉転換における Wnt5a-Ror2 シグナルの役割

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## Introduction

Epithelial–mesenchymal transition (EMT) is a fundamental process during embryonic development, by which epithelial cells acquire mesenchymal, fibroblast-like phenotypes with reduced cell-cell adhesion, loss of cell polarity and increased migratory and invasive properties. Accumulating evidence demonstrate that members of the Snail-family of transcription factors, which repress transcription of *E-cadherin* gene, play critical roles in EMT during both embryonic development and tumor progression . Ectopic expression of Snail has been shown to induce EMT of epithelial cells and carcinomas . Several lines of evidence demonstrate that EMT is associated with enhanced synthesis of matrix metalloproteinases (MMPs), which degrade the extracellular matrices and thereby accelerates cell migration and invasion .

Wnt5a is a member of the Wnt-family of secreted glycoproteins that play essential roles in both developmental and physiological processes . Wnt5a primarily activates the ß-catenin-independent pathway of Wnt signaling, where Ror2, a member of the Ror-family of receptor tyrosine kinases, acts as a receptor or co-receptor for Wnt5a . Recent studies have shown that sustained or increased expression of Wnt5a and/or Ror2, which results in constitutive activation of Wnt5a–Ror2 signaling, induces expression of MMPs and thereby confers invasive properties on several types of tumor cells, including osteosarcoma, prostate carcinoma, renal cell carcinoma and melanoma cells . Furthermore, Wnt5a has been shown to induce Snail expression and EMT in melanoma cells . However, the roles of Wnt5a–Ror2 signaling in Snail-mediated EMT have not been clarified.

In this study we show that Snail induces expression of both Wnt5a and Ror2, and thereby activates Wnt5a–Ror2 signaling in A431 epidermoid carcinoma cells. Activation of Wnt5a–Ror2 signaling is dispensable for Snail-induced down- and up-regulated expression of E-cadherin and vimentin, respectively, but is essential for Snail-induced expression of MMP-2, which is critically required for highly migratory and invasive properties of Snail-expressing A431 cells. We further show that endogenous expression of Snail is required for sustained expression of *Wnt5a*, *Ror2*, and *MMP-13* in osteosarcoma SaOS-2 cells, where constitutively active Wnt5a–Ror2 signaling is responsible for *MMP-13* expression and their invasiveness . Collectively, these results suggest that Wnt5a–Ror2 signaling plays essential roles in

Snail-mediated malignant progression of tumor cells by inducing expression of MMPs in a tumor cell-type specific manner.

## Results

### Snail induces expression of Wnt5a and Ror2 in A431 cells

To investigate the role of Wnt5a and Ror2 in Snail-induced EMT, A431 epidermoid carcinoma cells were infected with retroviruses containing either empty vector (pLPCX) or mouse *Snail* cDNA in pLPCX, and puromycin-resistant transformants were pooled to eliminate clonal variations. Ectopic expression of Snail resulted in disappearance and appearance of *E-cadherin* and *vimentin*, respectively, and in morphological alterations from an epithelial/cuboidal to a mesenchymal/fibroblastic shape. We examined the expression levels of *Wnt5a* and *Ror2* as well as their respective relatives, *Wnt5b* and *Ror1*, in these transformants. Marked increase in mRNA levels of *Wnt5a* and *Ror2* were detected in Snail-expressing A431 cells (Snail/A431), compared to control cells (pLPCX/A431). In agreement with their mRNA levels, protein levels of Wnt5a and Ror2 in Snail/A431 cells were apparently higher than those in pLPCX/A431 cells as detected by immunoblotting. Anti-Ror2 immunostaining revealed increased expression of Ror2 in both the cytoplasm and cell membrane of Snail/A431 cells .

Ror2 and Dvl proteins (Dvl2 and Dvl3) in Snail/A431 cells were found to exhibit slower electrophoretic mobilities relative to those in pLPCX/A431 cells , and these mobility shifts were sensitive to calf intestinal alkaline phosphatase (CIP) treatment , indicating that Ror2 and Dvl proteins were highly phosphorylated in Snail/A431 cells relative to those in pLPCX/A431 cells. These results suggest that Snail-induced expression of Wnt5a and Ror2 leads to the activation of Wnt5a–Ror2 signaling, thereby inducing phosphorylation of Ror2 and Dvl proteins.

We tested the possibility that cell shape changes during Snail-mediated EMT by itself might regulate expression of Wnt5a and Ror2. To this end, pLPCX/A431 cells were treated with anti-E-cadherin blocking antibody (SHE78-7), which induces cell shape changes by inhibiting cell-cell adhesion , a phenomenon similar to that observed during EMT. However, this treatment for up to 2 days failed to induce expression of Wnt5a, Ror2, and Snail , indicating that loss of E-cadherin-dependent cell-cell adhesion by itself is not sufficient to induce expression of Wnt5a and Ror2



in Snail/A431 cells.

### Wnt5a–Ror2 signaling is involved in Snail-induced expression of MMP-2

We found a drastic increase in expression of *MMP-2* and its gelatinolytic activity in Snail/A431, but not pLPCX/A431 cells , while both expression and gelatinolytic activity of MMP-9 were marginally detected in both cells. The finding that *MMP-2*, but not *MMP-1*, *-9*, and *-13*, can be induced by Snail in A431 cells suggests an important role of MMP-2 in invasive phenotypes of Snail/A431 cell.

We knocked down *Snail* expression in Snail/A431 cells. *Wnt5a* expression was suppressed only weakly, expression of *Ror2* and *MMP-2* was drastically inhibited by *Snail* knockdown. These findings suggest that expression levels of Ror2 and MMP-2 are directly correlated with those of Snail irrespective of its effects on inducing EMT, while expression levels of Wnt5a are more associated with phenotypes of EMT regulated by Snail, such as expression of *vimentin*.

We next examined whether Wnt5a and Ror2, induced by Snail, are involved in the induction of MMP-2 expression in Snail/A431 cells. To this end, we knocked down the expression of *Wnt5a* and *Ror2* by using two siRNAs with different sequences for the respective target genes. Interestingly, immunoblotting analysis showed that *Ror2* siRNAs suppressed expression of not only Ror2, but also Wnt5a drastically, while *Wnt5a* siRNAs failed to affect Ror2 expression in Snail/A431 cells. These findings suggest that Ror2 might positively regulate Wnt5a expression. Since we could not detect significant effect of *Ror2* siRNAs on *Wnt5a* mRNA levels in Snail/A431 cells , Ror2 might regulate Wnt5a expression at least partly at the translational and/or post-translational levels. Immunoblotting analysis also revealed that the delayed electrophoretic mobilities of Dvl proteins were inhibited by treatment with either *Wnt5a* or *Ror2* siRNA . In addition, *Wnt5a* siRNAs inhibited the delayed electrophoretic mobility of Ror2 . These findings indicate that Wnt5a–Ror2 signaling, activated by Snail-induced expression of Wnt5a and Ror2 in a cell-autonomous manner, can be abrogated by treatment with either *Ror2* or *Wnt5a* siRNA. Semi-quantitative and quantitative RT-PCR analyses demonstrated down-regulation of *MMP-2* mRNA levels in Snail/A431 cells following treatment with either *Ror2* or *Wnt5a* siRNA. Furthermore, gelatin zymography showed substantial reduction of MMP-2 protein levels in the conditioned media from the siRNA-treated Snail/A431 cells. In contrast, induced expression of *vimentin*,

suppressed expression of *E-cadherin* , and the mesenchymal morphology of the cells , induced by Snail were all unaffected by treatment with these siRNAs. These results suggest that Wnt5a–Ror2 signaling, activated by Snail-induced expression of Wnt5a and Ror2, up-regulates MMP-2 expression, without affecting the typical characteristics of EMT. Ectopic expression of both Wnt5a and Ror2 had marginal effect on either *Snail*, *E-cadherin* or *vimentin* mRNA levels in A431 cells , indicating that expression of Wnt5a and Ror2 is not sufficient to induce EMT of A431 cell. It can be assumed that this discrepancy may be due to differences in cell types used.

**Wnt5a–Ror2–MMP-2 pathway is crucial for invasive properties of Snail/A431 cells**

Snail/A431 cells exhibited significantly higher migratory and invasive abilities than pLPCX/A431 cells . To examine the role of MMP-2 in Snail-induced cell migration and invasion, we treated Snail/A431 cells with the MMP-2 inhibitor I (OA-Hy), a selective inhibitor of MMP-2, or *MMP-2* siRNA . Migration and invasion of Snail/A431 cells were abrogated by inhibiting MMP-2 activity or *MMP-2* expression , showing that MMP-2 is responsible for drastically enhanced migratory and invasive properties of Snail/A431 cells. Similarly, migration and invasion of Snail/A431 cells were significantly reduced by siRNA-mediated suppressed expression of either *Ror2* or *Wnt5a* . Taken together, these results suggest that Wnt5a–Ror2 signaling mediates Snail-induced cell migration and invasion by up-regulating MMP-2 expression.

**Endogenous Snail is required for sustained expression of Wnt5a and Ror2, leading to induction of MMP-13 expression in SaOS-2 cells**

SaOS-2 cells are of mesenchymal origin and express Snail endogenously, raising a possibility that endogenously expressed Snail may mediate constitutive expression of Wnt5a and Ror2 in SaOS-2 cells. To test this possibility, we knocked down expression of endogenous *Snail* in SaOS-2 cells. We found that suppressed expression of *Snail* substantially inhibits expression of both *Wnt5a* and *Ror2* . Furthermore, *Snail* knockdown also reduced expression of *MMP-13*, but not *MMP-2* expression .These results indicate that endogenous expression of Snail is responsible for sustained expression of *Wnt5a*, *Ror2*, and *MMP-13*, but not *MMP-2*, in SaOS-2



cells. In agreement with these results, knockdown of *Ror2* in SaOS-2 cells failed to affect expression of *MMP-2*, but inhibited expression of *MMP-13* . In addition, expression of endogenous *Snail* and *vimentin* was unaltered by *Ror2* knockdown , suggesting that Snail-induced expression of Wnt5a and Ror2, which leads to activation of Wnt5a–Ror2 signaling, is critical for up-regulated expression of *MMP-13* in SaOS-2 cells.

In summary, we have demonstrated here that ectopic expression of Snail induces expression of both Wnt5a and Ror2, thereby leading to activation of Wnt5a–Ror2 signaling in A431 cells. Activation of Wnt5a–Ror2 signaling is essential for Snail-induced expression of MMP-2, which is critically required for highly migratory and invasive properties of Snail/A431 cells. We have further shown that endogenous expression of Snail is required for sustained expression of *Wnt5a*, *Ror2*, and *MMP-13* in osteosarcoma SaOS-2 cells. These results suggest that Wnt5a–Ror2 signaling plays essential roles in Snail-mediated malignant progression of tumor cells by inducing expression of MMPs in a tumor cell-type specific manner. These present findings shed light on our understanding of the role of Wnt5a–Ror2 signaling in Snail-mediated malignant progression of tumors. Further study will be required to clarify the regulatory mechanism(s) underlying Snail-induced expression of Wnt5a and Ror2 and its role in tumor invasion/metastasis *in vivo*.

**Experimental procedures**

**Plasmids, antibodies, and reagents**

The mouse *Snail* cDNAs obtained from RIKEN FANTOM clones (Yokohama, Japan) was cloned into pCMV-Tag4 vector (Stratagene, La Jolla, CA) to generate cDNA encoding flag-tagged Snail (Snail-flag). The cDNA for Snail-flag was subcloned into retroviral vector *pLPCX* (Clontech, Mountain View, CA) to generate *pLPCX-Snail*. An anti-Ror2 antibody was prepared as described (Kani *et al.* 2004). The following antibodies were purchased commercially: mouse monoclonal antibodies against E-cadherin (67A4; Santa Cruz Biotechnology, Santa Cruz, CA and SHE78-7; Takara, Otsu, Japan), Snail (L70G2, Cell Signaling Technology, Danvers, MA), Dvl3 (sc-8027; Santa Cruz Biotechnology), α-tubulin (CP06; Calbiochem, La Jolla, CA); rabbit polyclonal antibodies against Dvl2 (3216; Cell

Signaling Technology); goat polyclonal antibodies against Wnt5a (AF645, R&D Systems, Minneapolis, MN). MMP-2 inhibitor I (OA-Hy) was obtained from Calbiochem.

**Small interfering RNA (siRNA)**

The following target sequences were used for the respective siRNAs: *Wnt5a* #1 (5'-TAACCCTGTTCAGATGTCA-3');*MMP-2*(5'-GGAGAGCTGCAACCTGTTT-3');*Snail*#1(5'-GCCTGGGTGCCCTCAAGAT-3');*Snail*#2(5'-AGGACTCTAATC CAGAGTT-3');mouse-*Snail*(5'-GATGCACATCCGAAGCCAC-3');*Dvl2*(5'-CAT GGAGAAGTACAACTTC-3');*Dvl3*(5'-GTTCTTCTTCAAGTCTATG-3').Sequenc es of *control* siRNA #1 and siRNAs for *Ror2* #1, *Ror2* #2, and *Wnt5a* #2 were previously described (Enomoto *et al.* 2009). The *control* siRNA #2 (Medium GC Duplex #2) and *Ror2* siRNA #2 were 25-bp Stealth™ siRNAs synthesized by Invitrogen (Carlsbad, CA). All other 21-bp siRNAs were synthesized by Sigma (St Louis, MO).

**Cell culture, retroviral infection, and transfection**

A431 and SaOS-2 cells were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS). Packaging cell line Plat-A, provided by T. Kitamura, was maintained in DMEM containing 10% FBS, 1 μg/ml puromycin (Sigma), and 10 μg/ml blasticidin (Sigma). Plat-A cells were transfected with *pLPCX* or *pLPCX-Snail* using Lipofectamine 2000 (Invitrogen). After 48 hr, viral supernatant was collected and filtered through 0.45-μm size pore. A431 cells were infected with the viral supernatant in the presence of 4 μg/ml polybrene (Sigma) for 24 hr. After infection, cells were selected with 1 μg/ml puromycin for 2 weeks before analysis. For siRNA transfection, we used Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

**Immunoblotting, phosphatase treatment, and immunofluorescence microscopy**

Whole cell lysates were prepared and subjected to immunoblot analysis as described (Nishita *et al.* 2006). For dephosphorylation of Dvl2, cells were lysed in dephosphorylation buffer [50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl flouride, 10 μg/ml leupeptin]. Lysates were treated with 20 units of calf intestinal alkaline phosphatase (CIP) at 37°C for 60 min. For



dephosphorylation of Ror2, whole cell lysates were prepared and subjected to immunoprecipitation with anti-Ror2 antibody as described (Nishita *et al.* 2006). Immunoprecipitates were washed with dephosphorylation buffer and treated with 20 units of CIP at 37°C for 60 min. CIP-treated samples were subjected to immunoblot analysis with the respective antibodies. For immunofluorescence analysis, cells were fixed and stained with the respective antibodies or Alexa Fluor 647-phalloidin (Invitrogen) as previously described (Nishita *et al.* 2006). Fluorescent images were obtained using a laser scanning confocal imaging system (LSM510; Carl Zeiss MicroImaging, Inc., Thornwood, NY) and processed using Photoshop CS (Adobe San Jose, CA).

**Transwell migration and invasion assays**

Cells ($2 \times 10^4$ cells) treated or untreated with the respective siRNAs for 72 hr were suspended in 100 μl DMEM. For cell migration assay, cells were loaded onto the upper well of the Transwell chamber (8-μm pore size; Corning, Corning, NY) that was precoated on both sides with 0.1% fibronectin (Sigma). For invasion assay, cells were loaded onto the upper well of the Transwell chamber that was precoated with Matrigel (BD Biosciences, San Jose, CA) on an upper side of the chamber. The lower wells were filled with 600 μl of conditioned medium collected from confluent monolayers of L cells that had been cultured in DMEM containing 5% FBS. After incubation for 5 hr for migration assay or 22 hr for invasion assay, migrating cells on the lower face of the membrane were stained and counted under a microscope Biozero BZ-8000 (KEYENCE, Osaka, Japan) as previously described (Nishita *et al.* 2006).

**Gelatin zymography**

Cells treated or untreated with the respective siRNAs for 72 hr were incubated with DMEM without FBS for 24 hr. Conditioned media, normalized by cellular protein contents, were concentrated by ultrafiltration using Microcon YM-10 (Millipore, Bedford, MA). The concentrated conditioned media and cell lysates were subjected to gelatin zymography using gelatin-zymography kit (Primary Cell Co., Sapporo, Japan) according to the manufacturer's instructions.

**RT-PCR and quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) and treated with RNase-free DNase I (Invitrogen). Total RNA (0.5 μg) was subjected to RT-PCR using SuperScript One-Step RT-PCR with Platinum Taq system (Invitrogen). *18S rRNA* was used as an internal control. For qRT-PCR, total RNA (1 μg) was reverse transcribed using Prime Script 1st strand cDNA synthesis kit (Takara), and real-time PCR was performed on the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) using LightCycler 480 SYBR Green I Master mix (Roche Diagnostics). Relative mRNA levels were determined after normalization by *GAPDH* mRNA levels.

神戸大学大学院医学系研究科（博士課程）

| 論文審査の結果の要旨 | | | |
|---|---|---|---|
| 受付番号 | 甲 第2162号 | 氏　名 | 任 大勇 |
| 論文題目 Title of Dissertation | Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition<br><br>癌細胞の上皮間葉転換における Wnt5a-Ror2 シグナルの役割 | | |
| 審査委員 Examiner | 主　査 Chief Examiner 古瀬幹夫<br>副　査 Vice-examiner 片岡徹<br>副　査 Vice-examiner 松尾雅文 | | |

（要旨は１，０００字～２，０００字程度）

Epithelial–mesenchymal transition (EMT) is a fundamental process during embryonic development, by which epithelial cells acquire mesenchymal, fibroblast-like phenotypes with reduced cell-cell adhesion, loss of cell polarity and increased migratory and invasive properties. Accumulating evidence demonstrate that members of the Snail-family of transcription factors play critical roles in EMT during both embryonic development and tumor progression. It has been shown that ectopic expression of Snail can induce EMT of epithelial cells and carcinomas, and that EMT is associated with enhanced synthesis of matrix metalloproteinases (MMPs), that accelerate cell migration and invasion.

Wnt5a is a member of the Wnt-family of secreted glycoproteins that play essential roles in developmental and physiological processes. Wnt5a primarily activates non-canonical Wnt signaling, where Ror2 receptor tyrosine kinases acts as a receptor for Wnt5a. It have been shown that sustained or increased expression of Wnt5a and Ror2, resulting in constitutive activation of Wnt5a–Ror2 signaling, can induce expression of MMPs and confer invasive properties on several tumor cells. Furthermore, Wnt5a has been shown to induce Snail expression and EMT in melanomas. However, the roles of Wnt5a–Ror2 signaling in Snail-mediated EMT remain unclear.

In this study it was found that ectopic expression of Snail can induce expression of both Wnt5a and Ror2, thereby activating Wnt5a–Ror2 signaling in A431 epidermoid carcinoma cells. Activation of Wnt5a–Ror2 signaling is dispensable for Snail-induced down- and up-regulated expression of E-cadherin and vimentin, respectively, but is indispensable for Snail-induced expression of MMP-2, which is required for highly migratory and invasive properties of the cells. I also found that endogenous expression of Snail is required for sustained expression of Wnt5a, Ror2, and MMP-13 in osteosarcoma SaOS-2 cells, where constitutively active Wnt5a–Ror2 signaling is critical for MMP-13 expression and invasiveness. The results suggest that Wnt5a–Ror2 signaling plays essential roles in Snail-mediated progression of tumor cells by inducing MMP expression in a tumor cell-type specific manner.

The candidate, having completed studies on the roles of Wnt5a-Ror2 signaling in tumor progression following EMT, and having advanced the knowledge in the area of non-canonical Wnt signaling, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).