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Identification of connexin43 as a functional target for Wnt signalling

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SUMMARY

Wnt mediated signal transduction is considered to regulate activity of target genes. In *Xenopus* embryos, ectopic Wnt1 and Wnt8 expression induces gap-junctional communication. During murine brain formation, Wnt1 and the gap-junctional protein connexin43 (Cx43) are co-expressed at the mid/hindbrain border, while interference with Wnt1 or Cx43 expression during embryogenesis leads to severe brain defects in the mid/hindbrain region. In PC12 cells, Wnt1 expression leads to an apparent increase in cell-cell adhesion. We investigated the effects of Wnt1 overexpression on gap-junctional communication in PC12 cells. Wnt1 expressing clones displayed an increased electrical and chemical coupling. This coincides with an increased expression of Cx43 mRNA and protein, while other connexins, Cx26, Cx32, Cx37, Cx40 and Cx45, were not up-regulated. Also, induction of Wnt1 expression in a mammary epithelial cell line leads to an increase in gap-junctional communication and Cx43 protein expression. In transient transactivation assays in P19 EC cells we found that Wnt1 and Li^+, an ion that mimics Wnt signalling, increased transcription from the rat Cx43 promoter, potentially via TCF/LEF binding elements, in a pathway separate from cAMP-induced Cx43 transactivation. The results demonstrate that Cx43 acts as a functional target of Wnt1 signalling, and Cx43 expression can be regulated by Wnt1 at the transcriptional level. Our data suggest that Wnt1-induced cell fate determination is likely to involve regulation of gap-junctional communication.

Key words: Wnt, Connexin, PC12, Transcriptional regulation

INTRODUCTION

Wnt gene products form a large family of secreted cysteine-rich glycoproteins which play important roles in directing cell fate and cell behaviour, not only during embryonic development and in adult life, but also in tumorigenesis (Nusse and Varmus, 1992). Experiments in *Drosophila*, *Xenopus*, mice and chicken indicate that the Wnt/Wg signal transduction pathway is evolutionarily conserved. Murine Wnt1 localizes at very specific sites in the embryonic nervous system and this appears essential for normal neural development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). A *Wnt1* knock-out mutation in the mouse generates severe brain defects ranging from loss of the anterior cerebellum to midbrain and posterior cerebellum (Nusse and Varmus, 1992, for review). Recent progress elucidated a signal transduction pathway downstream of Wnt1. In this pathway, Wnt1 associates to the Frizzled receptor (Yang-Snyder et al., 1996), which in turn recruits the intracellular protein dishevelled (Sokol et al., 1995; Yang-Snyder et al., 1996) leading to a downregulation of glycogen synthase kinase 3β (GSK3β) activity (Cook et al., 1996; Hedgepeth et al., 1997). GSK3β inactivation results in an increase in cytosolic β-catenin by augmenting this protein's half life (Papkoff et al., 1996; Yost et al., 1996). We and others demonstrated that members of the TCF/LEF (T cell factor/lymphocyte enhancer binding factor) family of transcription factors physically interact with β-catenin (Molenaar et al., 1996; Behrens et al., 1996; Huber et al. 1996). Nuclearly localized β-catenin/TCF complexes are supposed to regulate transcription of responsive genes by binding to the specific consensus sequence A/TA/TCAAAG, known as the TCF/LEF binding site (Van de Wetering et al., 1997; Clevers and Van de Wetering, 1997, for review). Based on genetic evidence, overexpression studies and promoter sequence analysis, several genes were found to be targets of this pathway like *Siamois*, *Ultrabithorax* and *Nodal-Related 3* (Carnac et al., 1996; Brannon et al., 1997; McKendry et al., 1997; Riese et al., 1997), while other genes are strong candidates such as the homeobox gene *Engrailed-1* and the cell adhesion molecule E-cadherin (Danielian and McMahon, 1996; Huber et al., 1996).

In a number of different cell types Wnt1 expression strengthens or induces cell-cell contacts mediated by calcium dependent adherens junctions (Bradley et al., 1993; Shackleford et al., 1993; Hinck et al., 1994). When expressed in the rat neural crest derived cell line PC12, murine Wnt1 induces a dramatic change in cell shape, from a round and

refractile appearance to a flat and closely connected, epidermoid appearance (Bradley et al., 1993; Pan et al., 1995). This is probably caused by the fact that Wnt1 induces an up regulation of the cell adhesion molecules E-cadherin, β-catenin and plakoglobin which, in addition, become localized at cell-cell junctions (Hinck et al., 1994; Papkoff et al., 1996). Furthermore, it was shown recently that Wnt1 induces a β-catenin-LEF-1 association in PC12 cells, indicating that potentially active LEF-1/β-catenin complexes are formed in PC12 cells in response to Wnt1 (Porfiri et al., 1997). Another phenomenon resulting from Wnt1 signalling, that might be linked to the enhanced cell-cell interaction, is the enhancement of intercellular communication. In *Xenopus* embryos it was found that Wnt1, and also *Xenopus* Wnt8, induced an increase in intercellular communication mediated by gap-junctions (Olson et al., 1991; Olson and Moon 1992). Gap-junctions are aggregates of intercellular channels that permit intercellular exchange of ions, small metabolites and low molecular mass signalling molecules between cells (Goodenough et al., 1996, for review). Gap-junctions obtain selective permeability by the usage of different connexin family members, all having their own more or less restrictive conductivity. Furthermore, gap-junctional permeability can be regulated by phosphorylation on tyrosine and serine/threonine residues (Goodenough et al., 1996, for review). Different connexin family members are named by their molecular mass in kDa, i.e. connexin 43 (Cx43), and have distinct expression patterns during development (Ruangvoravat and Lo, 1992).

Thus far, six connexins i.e. Cx26, Cx30, Cx32, Cx40, Cx43 and Cx45, are found to be expressed in the nervous system (listed by Goodenough et al., 1996). Interestingly, during mouse embryogenesis Cx43 is expressed as a gradient at the mid/hindbrain border which matches Wnt1 expression at this stage in the same region (Ruangvoravat and Lo, 1992). Furthermore, overexpression of Cx43 in the mid/hindbrain region prevents closing of the neural tube and leads to brain malformations (Ewart et al., 1997). Therefore the correct timing and amount of Cx43 expression at this location appears to be essential for normal neural development. Besides its neural expression, Cx43 is also expressed in several other tissues and organs, like heart, gonads, lens and skin.

The striking co-localization of Wnt1 and Cx43 messages at the mid/hindbrain border and the enhanced gap-junctional communication in Wnt1-injected *Xenopus* embryos suggests either a related induction of Wnt1 as well as Cx43 expression or an (in)direct regulation of Cx43 expression by Wnt1. Here, we describe results that identify Cx43 as a functional target for Wnt1 signalling. We show that the human, murine as well as the rat Cx43 promoter region contains several consensus TCF/LEF binding motifs, which may serve as *cis*-acting sites of Cx43 transcriptional regulation by Wnt1. By stably over-expressing *Xenopus* Wnt1 (Noordermeer et al., 1989) in PC12 cells we were able to show increased mRNA and protein levels of Cx43, leading to the formation of functional gap-junctions as measured by enhanced chemical and electrical gap-junctional communication. Also, induction of Wnt1 expression in a mammary epithelial cell line leads to an enhanced chemical coupling and increased Cx43 protein expression. Using a rat Cx43 promoter reporter construct (Yu et al., 1994) we demonstrate that in P19 embryonal carcinoma (EC) cells, transiently expressed Wnt1 induces transcription from this promoter.

MATERIALS AND METHODS

Cell culture and stable cell line generation

Stable *Xenopus* Wnt1 expressing PC12 cells were generated by co-transfection of an SV40 based expression vector containing the entire *Xenopus* Wnt1 cDNA (Noordermeer et al., 1989; Koster et al., 1996) and an expression vector containing the neomycin resistance gene. Stable clones were obtained by G418 selection (400 μg/ml) (Sigma, St Louis, MO, USA) for two to three weeks. Individual clones were isolated and cultured further in the presence of 250 μg/ml G418. Integration of the Wnt1 cDNA in the genome was checked by PCR on genomic DNA. PC12, PC12-Wnt1 and P19 EC cells were cultured in DMEM/F12 (1:1) (Gibco, Breda, The Netherlands) containing 10% FCS (Gibco). In the case of PC12-Wnt1 cells, 250 μg/ml G418 was added to the medium.

C57MG cells with a tetracyclin-repressable *Wnt1* gene expression (cell line 2-69-23) were a generous gift from K. Willert, H. Varmus and R. Nusse. 2-69-23 cells were cultured in DMEM (Gibco) containing 10% FCS (Gibco) supplemented with 250 μg/ml G418 and 50 ng/ml tetracyclin (Boehringer Mannheim). For induction studies, cells were washed with DMEM and cultivated in tetracyclin-free medium for the indicated time period.

RT-PCR

RNA from PC12 and PC12 Wnt1 cells was isolated using TriReagent (Sigma, St Louis, MO, USA). Typical yields were 200-300 μg per 5×10^6 cells, with OD 260/280 ratios between 1.75 and 1.95. cDNA was made from 1 μg of total RNA using oligo-dT and M-MLV reverse transcriptase (Gibco, Breda, The Netherlands) at 41°C for 90 minutes. Oligo sequences were 5′-GGTGCATCTTGTCTCCA-3′ and 5′-AGCAGGTGACATGACAAC-3′ for the Wnt1 sense and antisense primers, respectively. In the PCR, thirty-five cycles were run with annealing temperatures of 62°C. PCR products were analyzed on a 1.5% agarose + ethidium bromide gel run in TAE buffer.

Electrophysiology and dye injections

Cells were cultured to near confluency in 30 mm plastic Petri dishes as described above. Prior to the experiments, the culture medium was replaced by Hepes buffered culture medium. Next, cell cultures were placed on the stage of an inverted microscope (Nikon Diaphot TMF) and observed at a total magnification of ×400 using phase contrast or epifluorescence optics. Patch pipettes and microelectrodes were pulled from 1.0 mm borosilicate glass capillaries on a vertical electrode puller (Narishige PB-7) and were lowered onto selected cells in a monolayer using a hydraulic micromanipulator (Narishige M0203).

The extent of intercellular electrical communication via gap junctions was investigated qualitatively by a method described by De Roos et al. (1996) in control- and Wnt-transfected cell cultures. Briefly, the input capacitance and -resistance of semi-confluent monolayers were recorded via a single cell in semi-confluent cultures in the whole cell path clamp configuration. Patch pipettes were back-filled with a solution containing: 125 mM CsCl, 20 mM TEACl, 2 mM $MgCl_2.6H_2O$, 10 mM EGTA, 10 mM Hepes, pH 7.3, and were connected to the head stage of an EPC-7 voltage clamp amplifier (HEKA Germany). Patch pipette tip resistances, as measured in the bath, were 2-6 MΩ. After seal formation (seal resistance >5 GΩ) in the cell attached mode, the capacitance of the pipette was compensated for by transient-cancelation in the currents elicited by 2 mV voltage pulses of 5 milliseconds duration. Next, the whole cell configuration was achieved and membrane capacitance (up to100 pF) of uncoupled or very poorly coupled cells could be measured via the amplifier compensation controls using the same voltage clamp protocol as stated above. Input resistance was determined by measuring the steady state current during clamp pulses of 10-30 mV/50 ms. When the voltage clamped cells were electrically well coupled to their neighbors, total input capacitance (>>100 pF) could not be compensated and also the input resistance was greatly reduced.

To test if such electrical coupling indeed resulted from gap junctional communication, the preparation was superfused with bathing solution containing 2-4 mM of the gap junction blocker halothane.

For dye injections, microelectrodes (tip diameter <1 μm) were backfilled with either 4% Lucifer Yellow in 150 mM LiCl + 10 mM Hepes, pH 7.4, or 4% di-chlorofluorescein in 150 mM KCl + 10 mM Hepes, pH 7.4. Dye containing microelectrodes were inserted in selected cells in a monolayer. The dye was allowed to diffuse out of the pipette into the impaled cell and its adjacent cells for 2 minutes. The electrode was then retracted, the culture was viewed with epifluorescent illumination (excitation 420-490 nm, emission >520 nm) and the number of fluorescent cells including the injected one was counted.

Northern blotting and hybridization

Total RNA was isolated as described above. 25 μg of total RNA was denaturated at 65°C in running buffer (20 mM MOPS, 1 mM EDTA and 5 mM sodium acetate) containing 50% (w/v) formamide and 6% (w/v) formaldehyde. Subsequently RNA was loaded on a 1% agarose slab gel containing 6% formaldehyde and transfered to Qiabrane membrane (Qiagen, Westburg, Leusden, The Netherlands). Hybridization with 100 ng probe was performed in 50% (w/v) formamide, 5× SSC, 0.1% (w/v) SDS, 2× Denhardt's solution, 0.1 mg/ml salmon sperm DNA, 10 mM EDTA and 20 mM sodium-phosphate, pH 6.5, at 42°C overnight. Probes were labelled using a multiprime labelings set (Rediprime, Amersham) and 1[α-^{32}P]dCTP (Amersham) resulting in specific activities around 4×10^8 dpm/μg DNA. After hybridization blots were washed twice in 2× SSC, 0.1% (w/v) SDS, once in 1× SSC, 0.1% (w/v) SDS and once in 0.5× SSC, 0.1% (w/v) SDS at room temperature. Signal was visualized and quantified using a PhosphoImager (Molecular Dynamics).

Immunofluorescence

Cells were grown on coverslips for two days till semi-confluence. Cells were washed twice with phosphate buffered saline (PBS) and fixed in 3.5% formaldehyde in PBS for 30 minutes, subsequently permeabilized with 0.3% Triton X-100 in PBS for 5 minutes and quenched with 50 mM glycine in PBS for two times 10 minutes. Non-specific binding was blocked by incubation in NET-gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.05% NP40, 0.25% gelatin, 0.02% NaN_3) for two times 10 minutes. Cx43 antibody (Transduction labs, Lexington, KY, USA) was diluted in NET-gel and coverslips were incubated for one hour in this dilution. Primary antibody was detected with CY3-conjugated sheep anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA) for one hour. Non-bound antibodies were removed by 6 washes with NET-gel and cells were embedded in Mowiol. Cells were viewed on a Zeiss Axiovert 135 M microscope and photographs were taken.

Immunoprecipitation and western blot detection

Cells, cultured to near confluency in 60 mm dishes, were washed twice with PBS at 4°C and lysed in 1 ml cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin). Lysates were incubated on ice for at least 5 minutes at 4°C and clarified by centrifugation at 14,000 ***g*** for 10 minutes at the same temperature. 1 μg Cx43 antibody (Transduction labs, Lexington, KY) was added to the lysate containing 1 mg protein, and mixed for 4 hours at 4°C. Protein G-agarose was added for 16 hours to precipitate the immune complexes. The precipitate was washed four times with RIPA and then the proteins were dissolved in Laemmli sample buffer and resolved by 12.5% SDS-PAGE and subsequently electroblotted on Immobilon-P membrane (Amersham). Blots were blocked with 2% milk powder in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20) for 1 hour, incubated with Cx43 antibody diluted in TBST for 1 hour, and peroxidase-conjugated secondary antibody for 1 hour. After antibody incubations the blots were washed 6 times 5 minutes in TBST. Secondary antibody was detected using enhanced chemiluminescence (Amersham).

Transient transfections and luciferase assay

For transient-transfections assays, P19 EC cells were seeded in 35 mm dishes and transfected by the calcium-phosphate method. Cells were transfected with a total of 10 μg DNA, consisting of a mixture of 2 μg of luciferase reporter plasmid, 1 μg of pSG5LacZ and the indicated expression constructs. pSG5 was added to obtain a final amount of 10 μg plasmid. 16 hours post-transfection, DNA containing medium was removed, and replaced by normal culture medium. Cells were stimulated as indicated in the legend of the figures. 40 hours post-transfection, cells were harvested and assayed for luciferase activity using the Luclite luciferase gene reporter assay kit (Packard, CT), according to the manufacturer's protocol. Luciferase values were obtained in a Topcount liquid scintillation counter (Packard, CT). β-Galactosidase activity was measured by incubating cell lysate with ONPG (*o*-nitrophenyl-β-D-galactopyranoside; Sigma) in sodium phosphate buffer (Pfahl et al., 1990). Measurements were performed in the linear range of the enzymatic reaction (between OD 0.1 and 1.0) at 420 nm. Luciferase values were corrected for transfection efficiency by deviding them by the β-galactosidase values.

RESULTS

Wnt1 expression enhances gap-junctional communication and stimulates Cx43 expression

Previous studies demonstrated that expression of mouse Wnt1 in the rat PC12 cells leads to increased levels of β-catenin, plakoglobin and E-cadherin resulting in an enhanced cell-cell adhesion (Shackleford et al., 1993; Bradley et al., 1993). To test if the increased physical association would be accompanied by a functional electrical and chemical coupling between the adhering cells, we transfected PC12 cells with *Xenopus Wnt1* (Noordermeer et al., 1989) and a neomycin resistance gene and selected for stable transfectants. To check for Wnt1 expression we performed RT-PCR on total RNA extracts of wild-type and Wnt1 transfected clones. Oligonucleotides were designed to cover almost the entire coding region of the Wnt1 cDNA. Using these oligonucleotides, a single band of the expected size (1,100 bp) was observed in three independent Wnt1 expressing clones designated as Wnt1-2, Wnt1-6 and Wnt1-8, respectively (Fig. 1). No signal was observed using cDNA from untransfected

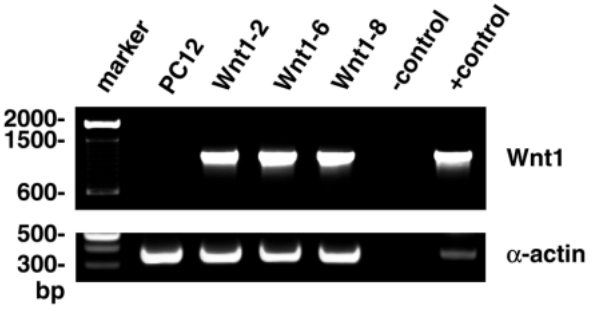

Fig. 1. Characterization of *Xenopus* Wnt1 expression in wild-type and stably transfected PC12 cells lines. PCR using primers against *Xenopus* Wnt1 and rat α-actin was performed on cDNA made of total RNA from wild-type PC12 cells (PC12) or Wnt1 transfected clones (Wnt1-2, Wnt1-6, Wnt1-8). As a negative control no cDNA was added, as positive control 1 pg of *Xenopus* cDNA was used.

wild-type PC12 cells. Without a reverse transcriptase reaction no PCR product was found (data not shown). As a positive control for the PCR reaction, *Xenopus* cDNA was used yielding a product of the same length as the cDNA from the Wnt1 clones. When performing RT-PCR using primers directed against the ubiquitous expressed α-actin gene, a band of the expected size was found in equivalent amounts in both Wnt1 and untransfected PC12 cell lines. These results clearly indicate that, at least at the mRNA level, Wnt1 is ectopically expressed in the PC12-Wnt1 cell lines. Morphologically, the *Xenopus* Wnt1 expressing cells resemble PC12 cells expressing mouse Wnt1 (Bradley et al., 1993; Zheng et al., 1996), i.e. flattened and organized in clusters. Furthermore, our Wnt1 overpressing cells do not differentiate upon NGF treatment which coincides with a decrease in the duration of NGF-induced MAP-kinase activation (data not shown), as shown already by others (Shackleford et al., 1993; Pan et al., 1995).

To see if the apparent increase in cell-cell adhesion due to ectopic Wnt1 expression would also lead to an enhanced intercellular communication via gap-junctions, we analyzed electrical and chemical coupling in the different cell lines. The extent of electrical gap-junctional communication was analyzed by measuring electrical coupling of wild-type PC12 and Wnt1-6 cells. Cells were considered as uncoupled when the input resistance (R_{in}) was ≥0.5 GΩ and membrane capacity was ≤50 pF and when no changes in these values were observed upon treatment with the anaesthetic halothane, a commonly used drug known to close gap-junctions, thereby forming a good means to identify electrical coupling. As an example for these experiments, Fig. 2A shows the electrical recording of coupled Wnt1-6 cells, which uncouple following halothane treatment (Fig. 2B). Typically, for gap-junctional communication, this effect can be reversed by removal of the drug (Fig. 2C). Non or poorly coupled cells display an average R_{in} of 1.1 and 3.6 GΩ for PC12 and Wnt1-6 cells, while R_{in} for coupled cells was 0.1 GΩ (Table 1). Furthermore, the incidence of electrical coupling was increased in Wnt1-6 cells when compared to wild-type PC12 cells (Table 1).

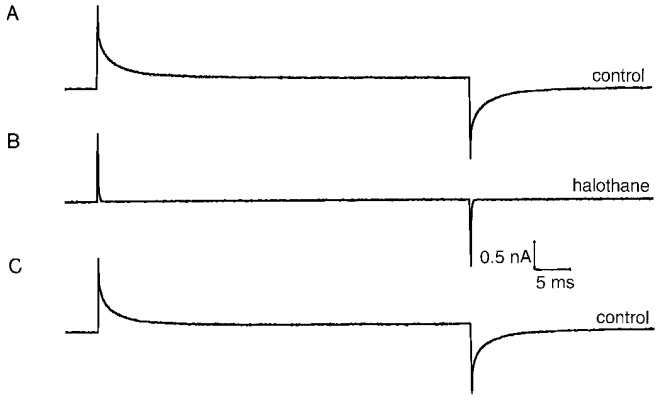

Fig. 2. Example of electrical coupling in a monolayer of PC12-Wnt1-6 cells. Current response measured in one patch clamped cell in this monolayer to 10 mV/50ms voltage clamp pulses. (A) In control bathing solution current showed slow capacitive transient (τ=17 ms) and reached a steady state level of 145 pA, corresponding to an input resistance (R_{in}) of 0.07 GΩ. These figures indicate that the patch clamped cell was electrically well coupled to its neighbours. (B) Application of halothane to bathing solution resulted in drastic decrease of capacitive time constant (<0.1 ms) and steady state current (4 pA→R_{in} = 2.5 GΩ), indicating total uncoupling of the patch clamped cell. (C) Uncoupling effect of halothane is reversible upon reperfusion with control solution.

Table 1. Electrical coupling in wild-type and Wnt1 expressing PC12 cells

	Rin (GΩ) poorly/uncoupled cell clusters	Rin (GΩ) well coupled cell clusters)	N_{total}	$N_{coupled}$	% Coupled
PC12	1.1±9.3	0.1	7	1	14
Wnt1-6	3.6±1.4	0.1±0.2	11	7	64

Extent of electrical coupling was measured in wild-type and Wnt1-6 PC12 cells. Cells were determined as uncoupled when R_{in} ≥0.5 GΩ and Cm ≤50 pF and when no change in R_{in} and Cm was observed following halothane treatment. Only R_{in} values are presented. N_{total} states the number of cells measured. $N_{coupled}$ is the number of cells determined as coupled by the above mentioned criteria. Data represent mean ± s.d.

We further measured metabolic coupling by injecting Lucifer Yellow or diChloroFluorescein into one cell. Only cells that were positioned within a large cluster of at least fifty visibly linked cells were injected. Two minutes post-injection, spreading of the dye into other cells was scored under UV illumination. Individual scores were subsequently pooled into 5 different so-called coupling groups: 0-2 coupled cells were scored as having minimal coupling, 3-5 as low coupling, 6-8 as moderate coupling, 9-11 as good coupling or 12 cells and more as extensive coupling. In wild-type PC12 cells, coupling was only observed to maximally 2 cells (Table 2). In contrast, Wnt1-2, Wnt1-6 and Wnt1-8 cells displayed an increase in the number of coupled cells with coupling of up to 15 cells (Table 2). The two different dyes yielded similar results. To exclude the possibility that gap-junctional communication is upregulated due to clonal selection rather than resulting from Wnt1 expression in the PC12 cell lines, we used the C57MG

Table 2. Dye coupling in wild-type and Wnt1 expressing PC12 cells

	#Coupled cells					
	0-2	3-5	6-8	9-11	≥12	N
Lucifer Yellow						
PC12	100	0	0	0	0	17
Wnt1-2	87	7	3	3	0	31
Wnt1-6	56	25	6	13	0	16
Wnt1-8	86	0	14	0	0	21
DiChloroFluorescine						
PC12	100	0	0	0	0	33
Wnt1-2	91	7	0	0	2	46
Wnt1-6	80	9	9	0	3	35
Wnt1-8	81	13	3	0	3	32

Dye coupling of wild-type PC12 and Wnt1 expressing PC12 cells. Individual scores were pooled into 5 different coupling groups: 0-2 coupled cells as minimal coupling, 3-5 as low coupling, 6-8 as moderate coupling, 9-11 as good coupling or 12 cells and more as extensive coupling. Numbers in coupling groups are percentages of injected cells belonging to the specific coupling group. N determines the total number of dye injections for each dye and clone.

Table 3. Dye coupling in 2-69-23 cells

Induction	Coupled cells	N
+tet	2.2±0.5	19
−tet 24 hours	4.3±1.0*	18
−tet 48 hours	7.9±1.2**	19

Chemical coupling in 2-69-23 cells in which Wnt1 is repressed (+ tet) or induced for either 24 hours (−tet 24 hours) or 48 hours (−tet 48 hours). Junctional transfer was indexed by the number of cells to which the fluorescent dye Lucifer Yellow was transferred upon injection of a test cell (mean ± s.e.). N determines the number of dye injections for each condition.
*P<0.1 +tet versus −tet 24 hours.
**P<0.01 +tet versus −tet 48 hours.

mammary tumor derived cell line 2-69-23 which can be induced to overexpress murine Wnt1 (Korinek et al., 1998). Transient expression of Wnt1 in C57MG cells leads to an increased stability of β-catenin (Papkoff et al., 1996). In 2-69-23 cells, ommision of tetracyclin results in the expression of Wnt1. Under this condition we find, as expected, translocation of β-catenin from the plasma membrane to the cytoplasm and nucleus (data not shown). Induction of Wnt1 in this cell line leads to an increase in gap-junctional communication after 24 hours and becomes even further enhanced after 48 hours as determined by spreading of the dye Lucifer Yellow (Table 3). Our results show that, at least in PC12 and C57MG cells, Wnt1 expression is accompanied by an enhanced gap-junctional communication.

The observed increased gap-junctional communication could be caused by regulation mechanisms at several levels. We first analyzed if the expression of gap-junctional genes was altered in the Wnt1 expressing cells. Therefore, we analyzed the Wnt1 cell lines for the expression of different connexins using RT-PCR. We used primers designed for Cx26, Cx32, Cx37, Cx40, Cx43 and Cx45 to screen for expression of these connexins. Except for the Cx43 primers, we could not detect any product of the correct length for the other connexins (data not shown). Interestingly, the amount of Cx43 PCR product yielded from Wnt1 cells derived RNA preparations, appeared to be increased compared to wild-type PC12 cells (data not shown). To quantitate differences in Cx43 message, we performed northern blotting. As illustrated in Fig. 3, Cx43 mRNA can be detected in total RNA preparations from wild-type PC12 cells. The amounts of Cx43 mRNA are increased in all three Wnt1 clones. Corrected for GAPDH levels, the induction values are 3.7-, 6.6- and 6.0-fold for Wnt1-2, Wnt1-6 and Wnt1-8 cell-lines, respectively. As a positive control P19EC cells were used which have been shown to express Cx43 (Belliveau et al., 1997).

To compare Cx43 protein levels, cell lysates were prepared from PC12 and Wnt1 cell-lines. Subsequently, Cx43 was immunoprecipitated from 1 mg of total protein for each lysate, and immunoprecipitates were analyzed for Cx43 levels by western blot. As can be seen in Fig. 4A, Cx43 protein is expressed in non-transfected PC12 cells (lane 1). Cx43 protein levels are clearly increased in Wnt1 expressing cell-lines (lane

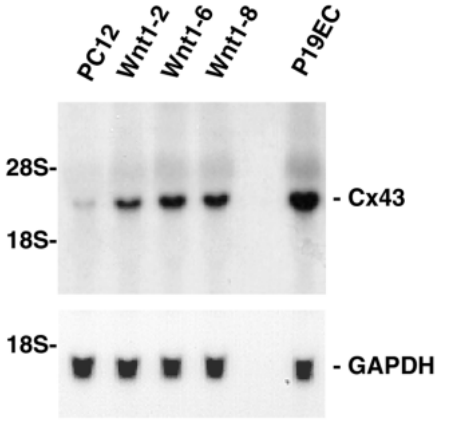

Fig. 3. Cx43 mRNA levels are increased in Wnt1 expressing PC12 cells. Total RNA was isolated from PC12, Wnt1-2, Wnt1-6, Wnt1-8 and P19 EC cells. 25 μg samples were run on a 1% agarose/6% formaldehyde gel and transferred to Qiabrane filter. Hybridization was performed with a rat Cx43 probe, obtained by random priming of a 1.0 kb fragment of rat Cx43 cDNA. To correct for differences in RNA loading, the same blot was stripped and rehybridized with a mouse GAPDH probe. Positions of 28S and 18S ribosomal RNA and Cx43 and GAPDH messages are indicated.

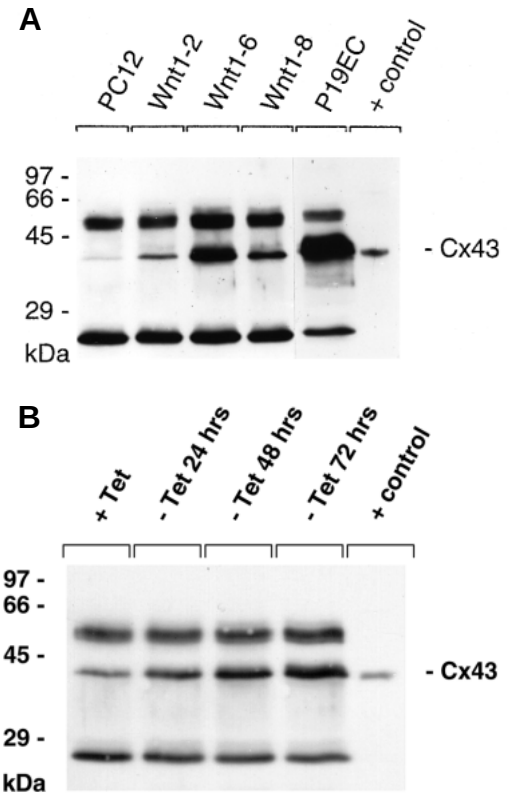

Fig. 4. Cx43 protein levels are increased in Wnt1 expressing PC12 and 2-69-23 cells. (A) Cx43 was immunoprecipitated from 1 mg of total cell lysate from PC12, Wnt1-2, Wnt1-6, Wnt1-8 and P19 EC cells. Precipitated Cx43 was subsequently detected by western blotting and Cx43 antibody incubation. As a positive control total rat brain lysate (Transduction labs) was used. (B) 2-69-23 cells were either left on 50 ng/ml tetracyclin (+tet), or Wnt1 was induced by ommision of tetracylin for 24, 48 and 72 hours respectively. Subsequently, immunoprecipitations were performed as in A. Position of Cx43 is indicated on the right, molecular mass markers are indicated on the left. Bands at ~50 and ~20 kDa result from the immunoprecipitating antibody.

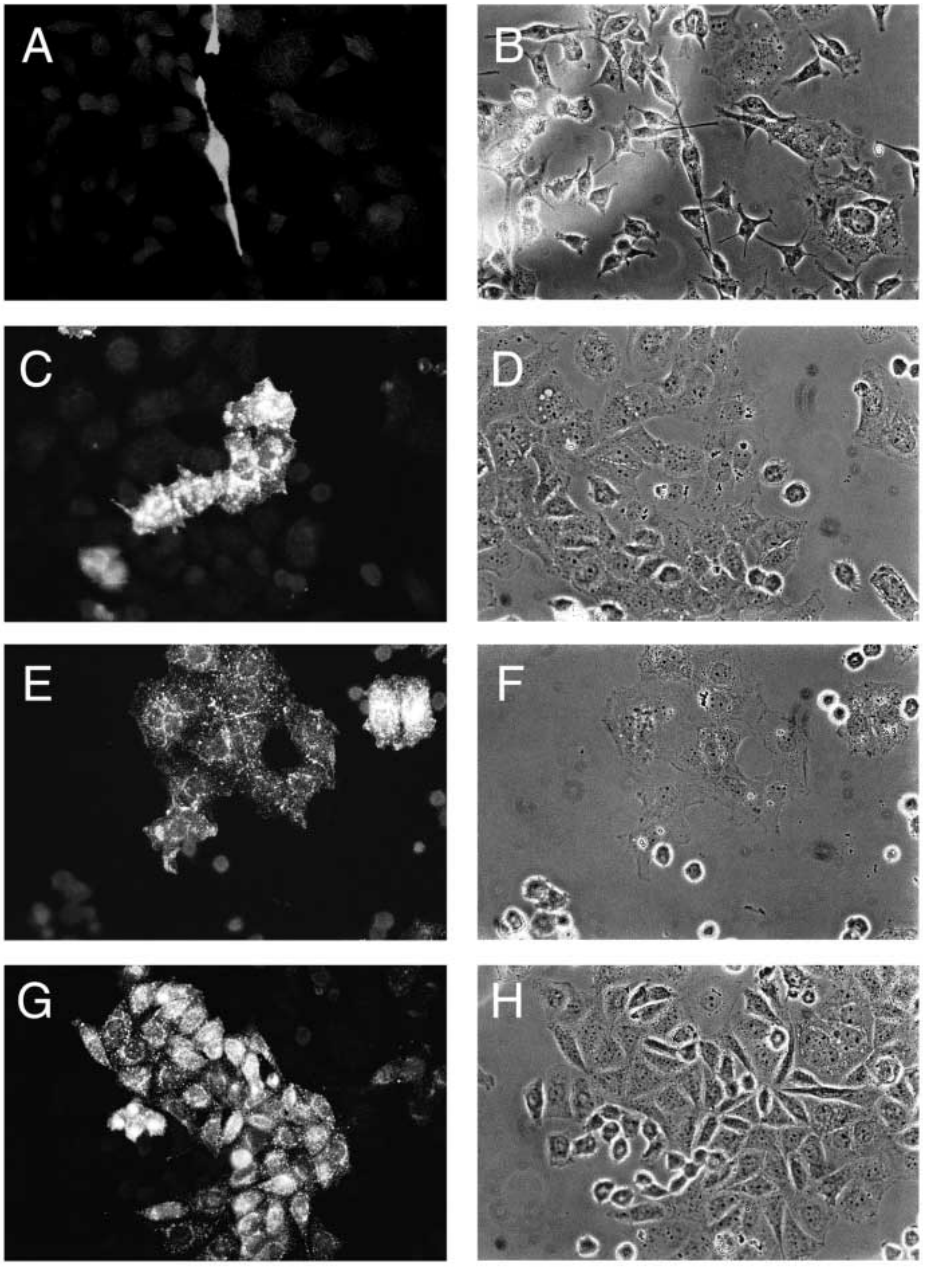

Fig. 5. Cx43 expressing PC12-Wnt1 cells are clustered. Cx43 was detected in situ by indirect-immunofluorescence microscopy on PC12 cells (A), Wnt1-2 (C), Wnt1-6 (E) and Wnt1-8 (G). Corresponding phase contrast images are given in B, D, F and H, respectively.

2-4), with clone Wnt1-6 displaying the highest level of Cx43 expression. As shown in Fig. 4B, induction of Wnt1 in 2-69-23 cells also leads to an increase in Cx43 protein levels already after 24 hours. At later time points the levels of Cx43 protein are even further increased. This increase in Cx43 expression in this cell line correlates with an increased gap-junctional communication (Table 3).

Finally, we compared the expression of Cx43 protein in wild-type and Wnt1 expressing PC12 cell populations by immunofluorescence microscopy. While in wild-type PC12 cells Cx43 is occasionally expressed in some cells; in most cases these cells are surrounded by Cx43 negative cells (Fig. 5A). In contrast, PC12 cells expressing Wnt1 more often show Cx43 expression while positive cells are usually located in clusters of up to 15 cells (Fig. 5C,E,G). Expression levels of Cx43 per cell are very similar for each cell in a cluster. Remarkably, the borders between expressing and non-expressing cells are sharp with respect to Cx43 expression, while morphologically no differences between these cells can be observed. In Wnt1 cells Cx43 localizes as dots at cell-cell borders, a conformation typical for integration in gap-junctions. Furthermore, Cx43 was found intracellularly as dots around the nucleus, presumably in the ER and Golgi. Also, the intensity of immunostaining of Cx43 is generally lower and more smooth in appearance in wild-type cells in comparison with Wnt1 expressing cells. The percentage of Cx43 positive cells in the different cell populations was also determined. It was found that 2.6±1.7% (mean ± s.d.) of the PC12 cells express Cx43, while 10.2±7.9%, 19.7±5.7% and 12.4 ±4.4% of the Wnt1-2, Wnt1-6 and Wnt1-8 express Cx43, respectively. These data suggest that Wnt1 expression leads to an increase in Cx43 expression in clusters of cells.

Induction of Wnt1 signalling pathway leads to increased Cx43 promoter activity in P19 cells

Given the effect of Wnt1 expression in PC12 on Cx43 mRNA levels, Wnt1 signalling probably activates Cx43 expression at the transcriptional level. Since Wnt1 exerts some of its downstream effects via β-catenin/TCF and β-catenin/LEF dependent regulation of transcription, we performed a computer search for TCF/LEF regulatory elements in the rat Cx43 promoter (Yu et al., 1994). As depicted in Fig. 6, this promoter contains two TCF/LEF binding consensus sequences in opposite orientation. TCF/LEF site 1 is located at −1,394 bp while site 2 is located at −714 bp with respect to the transcription start site. A third consensus sequence is found in the first intron following a non-translated short exon. Two similar TCF/LEF motifs are also found in the human and mouse Cx43 promoter partly at similar positions, and a third one is located in the first intron (Geimonen et al., 1996; Chen et al., 1995). In addition, Cx43 promoters contain many other *cis*-acting elements regulating transcription, like AP1, AP2, CRE and ERE elements (Yu et al., 1994; Chen et al., 1995; Geimonen et al., 1996). To determine if transcriptional activation of the rat Cx43 promoter is directly regulated by Wnt1 signalling, we assayed the activity of the promoter in a luciferase-reporter construct (Yu et al., 1994). To perform the transactivation assays, we chose P19 EC rather than rat PC12 cells since transfection efficiencies are generally very low in PC12, not allowing quantification of the results. Furthermore, P19 EC cells already display a considerable amount of Cx43 expression (see Figs 3 and 4) and thus activation of the Cx43 reporter construct will occur in a physiologically relevant setting. Indeed, when expressed in non-treated P19 EC cells, the reporter displays some basal activity (Fig. 7). Co-transfection of Wnt1 expression vector and Cx43-luc leads to a low but reproducible increase of luciferase activity. Recently, it has been described that Wnt1 signalling can be mimicked by Li^+ treatment. The action of Li^+, like Wnt1, leads to an inhibition of GSK3β (Stambolic et al., 1996; Hedgepeth et al., 1997).

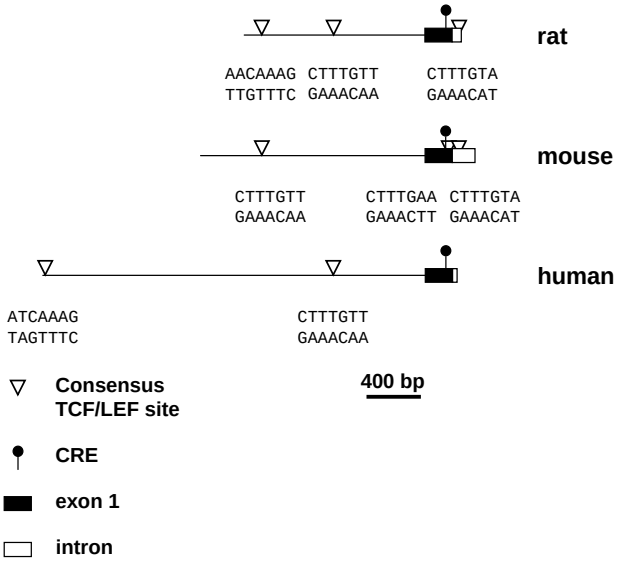

Fig. 6. Schematic representation of rat, mouse and human Cx43 promoters, first exons and parts of the first intron region. Consensus TCF/LEF binding sites (sequences) and cyclic AMP responsive elements are indicated.

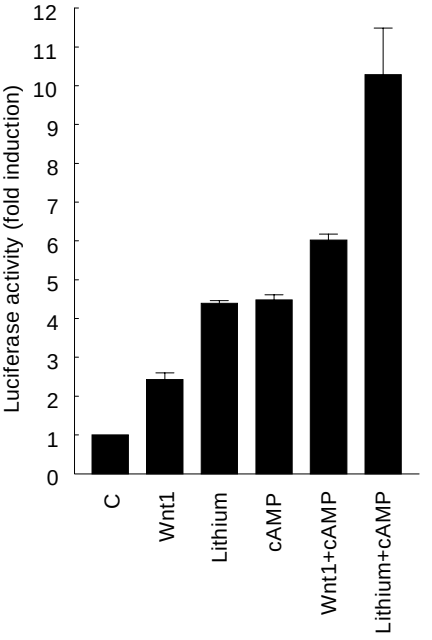

Fig. 7. Wnt1 and lithium enhance Cx43 promoter activity in P19 EC cells. P19 EC cells were transfected with the rat Cx43-promoter-luciferase and pSG5LacZ alone (C) or co-transfected with Wnt1 expression construct. Where indicated stimulations with 10 mM lithium or 1 mM dbcAMP were performed for 16 hours. Cells were lysed and luciferase and galactosidase activities were determined. See Materials and Methods for further details.

Treatment of the Cx43-luc transfected cells with 10 mM Li^+ for 16 hours leads to an increase in luciferase activity of 4 times over control levels. The stronger induction of Cx43 reporter by Li^+ as compared to Wnt1 might be explained by the fact that Li^+ acts further downstream in the signalling cascade. Although Wnt1 and lithium both induce transcriptional activation of the Cx43 promoter, we cannot exclude the possibility that *cis*-acting elements other than the potential TCF/LEF binding sites are involved. Previous studies have demonstrated that Cx43 transcription is induced by cAMP and by cAMP inducing agents probably via a cAMP responsive element (CRE) (Mehta et al., 1992; Schiller et al., 1992). When Cx43-luc transfected cells are treated with 1 mM dibutyryl-cAMP for 16 hours, the amount of luciferase activity increases to 4 times the basal activity, indicating that cAMP induces Cx43 transcription also in P19 EC cells.

To test if Wnt1 and cAMP signalling act in a synergistic or additive fashion, we stimulated Cx43-luc expressing cells with Li^+ and cAMP together, or co-expressed Cx43-luc and Wnt1 followed by cAMP treatment and compared luciferase activities with those of cells treated with only one stimulus. As can be seen from Fig. 7, the cAMP response is additional to Wnt1 and Li^+ responses. This indicates that in P19 EC cells, Wnt1 and cAMP signalling pathways act in parallel on the Cx43 promoter.

DISCUSSION

We used the neural crest derived cell line PC12 to test the effects of Wnt1 expression on intercellular communication.

The overexpression of *Xenopus* Wnt1 in this cell line leads to similar changes in cell morphology as seen for murine Wnt1. One of the most profound changes of Wnt1 expressing PC12 cells in contrast to wild-type PC12 is the tendency to grow in closely connected groups. Here we show that the connected clusters of cells are functionally coupled via gap-junctions, giving the cells the potential to coordinate their behaviour by a free exchange of ions, for example calcium, and small second messengers, such as cAMP or cGMP. Earlier studies describing PC12 cells overexpressing murine Wnt1 indicated that these PC12 cells displayed an increased cellular adhesion probably mediated by E-cadherin (Bradley et al., 1995). Although the PC12 XWnt1 cells resemble PC12 cells overexpressing murine Wnt1 by morphological and biochemical criteria, we did not observe an increase in adhesion complexes formed by β-catenin/E-cadherin complexes (data not shown). This indicates that the increased gap-junctional communication and Cx43 expression is not a secondary result from increased cell-cell interaction mediated by the β-catenin/E-cadherin complexes.

In parallel with the increased coupling by gap-junctions we find that the gap-junctional protein Cx43 shows elevated expression levels in Wnt1 expressing PC12 clones and 2-69-23 cells. Interestingly, an increase in Cx43 expression has also been observed by the overexpression of quail Wnt11 in a mesodermal cell line (Eisenberg et al., 1997). This indicates that the increase in Cx43 expression observed by us might reflect a more basal function among the different Wnt family members. Thus far no expression of Cx26, Cx32 and Cx43 was observed in PC12 cells (Eghbali et al., 1991). Of the tested connexins, we could only find a signal by RT-PCR using Cx43 primers, thereby confirming and expanding prior observations that PC12 does not express Cx26, Cx32, Cx37, Cx40 and Cx45. Yet, at the protein level only very low amounts of Cx43 could be detected in PC12 cells when compared to, for instance, P19 EC cells. This low level of expression could be easily missed by sub-optimal detection techniques. Surprisingly, upon immunofluorescence localization of Cx43 in Wnt1 expressing cells we observed that only a subpopulation of the cells expressed the protein, while others were negative. As a result, sharp bounderies in cell clusters are formed with respect to Cx43 expression and likely in gap-junctional communication, in otherwise morphological indistinguisable cell populations. Interestingly, this type of expression pattern is similar to that found in PC12 cells expressing the carboxy-terminal region of amyloid precursor protein (Nagy et al., 1996). The mechanism for this patched distribution of expression in the population remains unknown but it may be regulated by positive or negative feedback loops initiated by Cx43 protein itself or by gap-junctional communication within these clusters of connected cells. The clusters of Cx43 expressing cells, which are likely also functionally coupled, might act synchronously in developmental processes such as cell differentiation and migration, thereby forming developmental units of cells.

From the comparison of Cx43 expression as measured at the RNA and protein levels (Figs 3, 4A) it appears that the increase in Cx43 protein can not be entirely caused by an increase in transcriptional activity from the Cx43 promoter. This suggests that Wnt1 signalling may also regulate the Cx43 protein level at post-transcriptional levels. Injection of Wnt1 or Wnt8 mRNA in a fertilized *Xenopus* egg before the first cleavage results in an increased gap-junctional communication in the ventral part of the 32-cell stage embryo (Olson et al., 1991; Olson and Moon 1992). Since transcription only starts at the mid blastula transition the induction of gap-junctional communication by both Wnts must be regulated at post-transcriptional levels. This could be directly through maternal connexin mRNA stabilization, regulated translation or connexin protein stabilization, or more indirectly by affecting cell adhesion processes.

The relation between Wnt-induced Cx43 expression and enhanced gap-junctional communication in embryonic development is still unclear. Interestingly, Cx43 is expressed in discrete regions of the mouse embryo known to undergo extensive induction (Ruangvoravat and Lo, 1992). In the 10.5-day mouse embryo Cx43 mRNA is distributed as a gradient in regions spanning the mid/hindbrain border in a pattern which is very similar to that seen for Wnt1 mRNA (Wilkinson et al., 1987). Wnt1 knock-out mice lack the cerebellum and varying parts of the midbrain (Thomas and Capecchi, 1990; McMahon and Bradley, 1990), although no defects in this region have been reported for the Cx43 knock-out mouse (Reaume et al., 1995). However, mice over-expressing Cx43 exhibit cranial neural tube defects, probably by a perturbation or delay in neural tube closure (Ewart et al., 1997). Importantly, embryos with a mild neural tube defect exhibited their defect at the mid/hindbrain border, which might indicate that this region is most vulnerable for misregulation of Cx43 expression. Our results suggest that Wnt mediated cell fate determination in the mid/hindbrain border region, as well as in other developmental systems invoking Wnt signalling, may be mediated by regulation of gap-junctional communication.

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