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Zebrafish *short fin* mutations in connexin43 lead to aberrant gap junctional intercellular communication

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Abstract Mutations in the zebrafish *connexin43* (*cx43*) gene cause the *short fin* phenotype, indicating that direct cell–cell communication contributes to bone length. Three independently generated *cx43* alleles exhibit short segments of variable sizes, suggesting that gap junctional intercellular communication may regulate bone growth. Dye coupling assays showed that all alleles are capable of forming gap junction channels. However, ionic coupling assays revealed allele-specific differences in coupling efficiency and gating. For instance, oocyte pairs expressing the weakest allele exhibited much higher levels of coupling than either of the strong alleles. Therefore, measurable differences in Cx43 function may be correlated with the severity of defects in bone length.
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1. Introduction

Gap junctions serve as intercellular passageways for the exchange of small molecules (⩽1200 Da in size), thereby facilitating direct communication between neighboring cells. Each gap junction channel consists of two hemichannels, or connexons. Each connexon contains six four-pass transmembrane spanning proteins called connexins. Interestingly, many connexin proteins seem to be required to fulfill the role of direct cell–cell communication. For example, there are 21 connexin genes in humans and 20 in the mouse [1]. The significance of gap junctional intercellular communication (GJIC) during development is evidenced by the large number of connexin mutations leading to human disease phenotypes [2,3]. In particular, mutations in human and mouse *CX43* cause oculodentodigital dysplasia (ODDD), a syndrome resulting in abnormalities of the craniofacial and distal limb skeleton as well as other pleiotropic phenotypes [4,5]. ODDD is transmitted as an autosomal dominant disease [4]. Mutations are typically missense mutations, which likely retain some level of function.

How might missense mutations affect the function of connexin proteins? *CX43* mutations linked to ODDD have been examined in heterologous assays [6–9]. The majority of *CX43* mutations retain the ability to assemble into gap junction plaques at the cell surface, suggesting that trafficking is not the primary cause for phenotypic variation (however, several mutant alleles result in reduced plaque formation). Indeed, defects in ionic and/or dye coupling have been revealed for all of the *CX43* mutants studied [6–9]. Therefore, the reduction in Cx43-mediated GJIC among these mutants may contribute to the development of ODDD-related phenotypes. Still, the underlying mechanism for the development of disease phenotypes remains unclear.

Mutations in zebrafish connexin genes have been identified only recently [10,11]. In particular, mutations in zebrafish *cx43* result in morphological abnormalities of the fin skeleton, suggesting that the function of *cx43* is conserved [10]. The original allele of *short fin* (*sof*^{b123}) exhibits reduced *cx43* gene expression and develops short fins due to defects in the length of bony fin ray segments. In addition to the original, non-coding sequence mutation in the *cx43* gene, three non-complementing ENU-induced mutations were identified (*sof*^{j7e1} coding for Cx43-F30V in the first transmembrane domain; *sof*^{j7e2} coding for Cx43-P191S in the second extracellular loop; *sof*^{j7e3} coding for Cx43-F209I in the fourth transmembrane domain). Previously, we found that the different missense mutations have different effects on segment length [10]. For example, homozygous Cx43-F209I alleles have only a slight effect on segment length and on fin length. In contrast, homozygous Cx43-F30V and Cx43-P191S alleles exhibit segments of similar length as the original allele (*sof*^{b123}). Since all alleles are adult viable, some level of function is predicted for each. However, as each missense mutation is found in the channel-forming region, effects on GJIC are anticipated.

The goal of this study is to determine if GJIC is affected in each mutant allele of zebrafish *cx43*, and further, if the severity of the defect is correlated with the in vivo segment length phenotype. For example, one possibility is that the efficiency of GJIC is directly related to segment length. We tested this hypothesis by monitoring the function of gap junctions using dye coupling and ionic coupling assays. Indeed, we found that the strong alleles of *cx43* (Cx43-F30V and Cx43-P191S) have more severe defects in coupling than the weakest allele (Cx43-F209I), which exhibits only moderate defects.

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2. Materials and methods

2.1. Generation of constructs for GJIC assays

The coding sequence from each allele (wild-type, *sof*^{tz1} coding for Cx43-F30V, *sof*^{tz2} coding for Cx43-P191S, and *sof*^{tz3} coding for Cx43-F209I) was amplified using oligonucleotides with EcoRI sites engineered at the 5′ ends (cx43-E2F TCGCAGAATTCGATGGGT-GACTGGAGTGCG); (cx43-E2R-CGACAGAATTCGGACGTC-CAGGTCATCAGG). Amplified products were subcloned into the EcoRI site of pEGFP-N1 (Clontech) and sequenced to ensure that additional errors were not introduced during amplification. The coding sequences were also subcloned into pCS2+. Alleles expressed in pEGFP-N1 were used for dye coupling assays in HeLa cells. Sense RNA transcribed from the pCS2+ vector was injected into *Xenopus* oocytes for ionic coupling assays.

2.2. Cell culture and transfection of HeLa cells

HeLa cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, penicillin and streptomycin (Gibco-BRL). The cells were plated to 50% confluency in 35 mm dishes and transfected with 0.5 μg of plasmid DNA using the Effectene reagent (Sigma). Transfection efficiency was determined 24 h later by visualizing live cells for Cx43-EGFP expression with a fluorescence microscope.

2.3. Microinjection of HeLa cells using propidium iodide

Pairs of HeLa cells that had been transfected with each of the *cx43* alleles subcloned in the pEGFP-N1 vector were microinjected using the Eppendorf FemtoJet microinjector and Eppendorf InjectMan® NI2 micromanipulator. One cell from each pair was injected with propidium iodide (PI; 1 mg/mL, MW = 668.4) and a total of 50 cell pairs were injected. After transfer had proceeded for 15 min, the cells were viewed for fluorescence using a Nikon Eclipse TE2000-E microscope fitted with the Endow GFPBP (Zeiss) filter for the visualization of EGFP. The number of injected cells able to transfer dye was determined as a percentage in each of three experiments. The cells were determined to have successfully transferred dye if the dye was detectable in the non-injected cell of a pair. Three independent experiments were carried out, the percentage of cells that shared dye was determined per experiment, and the standard deviation was calculated. Student's *t*-tests were completed to determine if differences in dye coupling (between each mutant allele and wild-type Cx43) were significant.

2.4. In vitro transcription, oocyte microinjection and pairing

Wild-type and mutant *cx43* constructs were linearized using the NotI restriction site of pCS2+, and transcribed using the SP6 mMessage mMachine RNA protocol (Ambion, Austin, TX). Adult *Xenopus* females were anesthetized with ethyl 3-aminobenzoate methanesulfonate and ovarian lobes were surgically removed and digested for 2 h in a solution containing 50 mg/ml collagenase B, and 50 mg/ml hyaluronidase in modified Barth's medium (MB) without Ca^{2+}. Stage V–VI oocytes were collected and injected first with 10 ng of antisense *Xenopus* Cx38 oligonucleotide to eliminate endogenous connexins [12,13]. Antisense oligonucleotide treated oocytes were then injected with wild-type Cx43, Cx43-F30V, Cx43-P191S, or Cx43-F209I cRNA transcripts (5 ng/cell), or H_2O as a negative control. The vitelline envelopes were removed in a hypertonic solution (200 mM aspartic acid, 10 mM HEPES, 1 mM $MgCl_2$, 10 mM EGTA, and 20 mM KCl at pH 7.4), and the oocytes were manually paired with the vegetal poles apposed in MB with Ca^{2+}.

2.5. Dual-cell whole-cell voltage clamp

Gap junctional coupling between oocyte pairs was measured using the dual whole-cell voltage clamp technique [14]. Current and voltage electrodes (1.2 mm diameter, omega dot; Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with solution containing 3 M KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4. Voltage clamp experiments were performed using two GeneClamp 500 amplifiers controlled by a PC-compatible computer through a Digidata 1320A interface (Axon Instruments, Foster City, CA).

For measurements of junctional conductance, both cells in a pair were initially clamped at −40 mV to eliminate any transjunctional potential. One cell was then subjected to alternating pulses of ±20 mV, while the current produced by the change in voltage was recorded in the second cell. The current delivered to the second cell was equal in magnitude to the junctional current, and the junctional conductance was calculated by dividing the measured current by the voltage difference, $G_j = I_j/(V_1 - V_2)$. To determine voltage-gating properties, transjunctional potentials (V_j) of opposite polarity were generated by hyperpolarizing or depolarizing one cell in 20 mV steps (range = ±120 mV) while clamping the second cell at −40 mV. Currents were measured at the end of the voltage pulse, at which time they approached steady-state (I_{jss}). Macroscopic conductance (G_{jss}) was calculated by dividing I_{jss} by V_j, normalized to the values determined at ±20 mV, and plotted against V_j. Data describing the relationship of G_{jss} as a function of V_j were analyzed using Origin 6.1 (Microcal Software, Northampton, MA) and fit to a Boltzmann relation of the form: $G_{jss} = (G_{jmax} - G_{jmin})/1 + \exp[A(V_j - V_0)] = G_{jmin}$, where G_{jss} is the steady-state junctional conductance, G_{jmax} (normalized to unity) is the maximum conductance, G_{jmin} is the residual conductance at large values of V_j, and V_0 is the transjunctional voltage at which $G_{jss} = (G_{jmax} - G_{jmin})/2$. The constant $A = nq/kT$ represents the voltage sensitivity in terms of gating charge as the equivalent number (n) of electron charges (q) moving through the membrane, k is the Boltzmann constant, and T is the absolute temperature.

2.6. Electrophysiological recording of hemichannel currents

Macroscopic recordings of hemichannel currents were recorded from single *Xenopus* oocytes using a GeneClamp 500 amplifier controlled by a PC-compatible computer through a Digidata 1320 interface (Axon Instruments). pClamp 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms. To obtain hemichannel *I–V* curves, cells were initially clamped at −40 mV and subjected to 5 s depolarizing voltage steps ranging from −30 to +60 mV in 10 mV increments. The effects of calcium on hemichannel currents was analyzed by recording hemichannel currents from cells incubated in either MB media without calcium, or MB supplemented with 2 mM $CaCl_2$.

3. Results

3.1. All missense alleles form gap junction channels

Mutant connexins may affect the formation of gap junction channels by failing to assemble into connexons and/or failing to target to the plasma membrane [15,16]. To determine if any of the alleles of zebrafish Cx43 exhibit such defects, expression of EGFP-tagged alleles was examined in HeLa cells. Gap junction channels aggregate in plaques (~100–1000 channels) and are visualized as puncta or lines in areas where cells are in contact with one another. Wild-type zebrafish Cx43-EGFP as well as each mutant allele were identified in gap junction plaques at the plasma membranes of adjacent transfected cells (Fig. 1B), indicating that all three alleles are capable of assembly into connexons and targetting to the plasma membrane.

Next, the function of the Cx43 mutant alleles was examined using dye coupling assays. Propidium iodide (MW 668) was microinjected into one cell of a transfected pair and dye transfer was monitored. In addition to evaluating coupling of the zebrafish Cx43 alleles, the human variants P1 and P3 were also tested. These variants form gap junction plaques but do not permit dye coupling [17], and therefore served as negative controls. Dye was successfully transferred to neighboring cells in the majority of cell pairs for each zebrafish allele (and dye was not transferred in cells transfected with either human P1 or P3), suggesting that the assembled gap junctions are functional (Fig. 1). Differences in dye coupling compared to wild-type was not observed ($P = 0.81$ for Cx43-F30V; $P = 0.72$ for Cx43-P191S; $P = 0.67$ for Cx43-F209I). Therefore, zebrafish missense mutations in Cx43 retain coupling activity by this measure.

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