Cell Communication & Adhesion

ISSN: 1541-9061 (Print) 1543-5180 (Online) Journal homepage: informahealthcare.com/journals/icac20

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To cite this article: Charles H. M. Castro, Joseph P. Stains, Sharmin Sheikh, Vera Lucia Szejnfeld, Klaus Willecke, Martin Theis & Roberto Civitelli (2003) Development of Mice with Osteoblast-Specific Connexin43 Gene Deletion, Cell Communication & Adhesion, 10:4-6, 445-450, DOI: 10.1080/cac.10.4-6.445.450

To link to this article: https://doi.org/10.1080/cac.10.4-6.445.450

Published online: 11 Jul 2009.

Cell Communication and Adhesion, 10: 445–450, 2003

ISSN: 1541-9061 print / 1543-5180 online
DOI: 10.1080/15419060390267700

Taylor & Francis
healthsciences

Development of Mice with Osteoblast-Specific Connexin43 Gene Deletion

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Genetic deficiency of Cx43 in vivo causes skeletal developmental defects, osteoblast dysfunction and perinatal lethality. To determine the role of Cx43 in the adult skeleton, we developed two models of osteoblast-specific Cx43 gene deletion using Cre mediated replacement of a "floxed" Cx43 allele with a LacZ reporter gene. Cre recombinase expression in osteoblasts was driven by either the osteocalcin OG2 promoter or the 2.3 kb fragment of the Colα1(I) promoter. Homozygous $Cx43^{fl/fl}$ mice, in which the Cx43 coding region is flanked by two loxP sites, were crossed with Cre expressing mice in a heterozygous Cx43-null background [$Cx43^{\pm}$; Colα1(I)-Cre or $Cx43^{\pm}$; OG2-Cre]. Cx43 gene ablation was demonstrated in tissues by selective X-gal staining of cells lining the endosteal surface, and in cultured osteoblastic cells from calvaria using different approaches. Although no LacZ expression was observed in proliferating calvaria cells, before osteoblast differentiation begins, post-proliferative cells isolated from conditional knockout mice [$Cx43^{fl/-}$; Colα1(I)-Cre or $Cx43^{fl/-}$; OG2-Cre] developed strong LacZ expression as they differentiated, in parallel to a progressive disappearance of Cx43 mRNA and protein abundance relative to controls. Selective Cre mediated Cx43 gene inactivation in bone forming cells will be useful to determine the role of Cx43 in adult skeletal homeostasis and overcome the perinatal lethality of the conventional null model.

Keywords. Bone mineral density, connexin43, gap junctions, osteoblast differentiation, transgenic mice

INTRODUCTION

Bone-forming cells are highly coupled by gap junctions formed primarily by connexin43 (Cx43) and, to a lesser degree, connexin45 (Cx45) (1–3). Previous work from our lab has demonstrated that lack of Cx43 alters differentiation, gene expression, and function of osteogenic cells, indicating that gap junctions play a fundamental role in the elaboration of a full osteoblast phenotype (4, 5). Reflecting this critical regulatory function in osteogenic cells, chemical inhibition of gap junctional communication alters bone cell differentiation and function *in vitro* (6, 7). Specifically, Cx43 is required for normal bone development since targeted ablation of Cx43 gene in the mouse leads to a skeletal

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phenotype characterized by retarded intramembranous and endocrondral ossification, craniofacial abnormalities, and autonomous osteoblast dysfunction (8). Homozygous Cx43 null mice die shortly after birth because of severe cardiovascular malformations (9), thus precluding the use of this model to study the role of Cx43 in the adult skeleton.

To resolve this limitation of the conventional Cx43 null mutation, we developed osteoblast-specific Cx43 conditional knockout models using the Cre/*lox*P system. Cre recombinase expression driven by either the osteocalcin OG2 or the 2.3 kb fragment of Colα1(I) promoter was used to selectively delete a Cx43 "floxed" allele in osteoblasts. Effective Cx43 gene deletion in bone-forming cells was demonstrated in tissue sections and in cultured calvaria cells by LacZ staining and direct detection of the deleted allele.

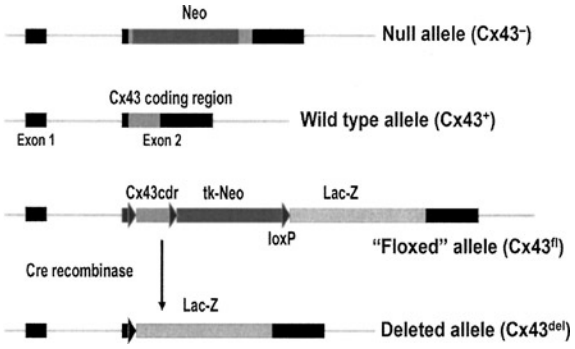

Figure 1. Cx43 gene replacement by LacZ reporter gene. Schematization of the "null" Cx43 allele (9), and the "floxed" allele with the loxP sites (11), compared to the wild type locus. Cre-mediated recombination of the "floxed" allele results in the replacement of the Cx43 coding region by the silent Lac Z cassette, which now can be translated, leading to expression of β-galactosidase under the control of the natural Cx43 promoter.

RESULTS AND DISCUSSION

Strategy for Deleting the Cx43 Gene Selectively in Osteoblasts

We used a Cre/*lox*P approach to delete the Cx43 gene selectively in osteoblasts (10). A mouse line was previously generated, in which the coding region of the Cx43 gene, entirely comprised within exon 2, had been flanked by two *lox*P sites ($Cx43^{fl}$) (11). In addition, a silent β-galactosidase (Lac-Z) cassette had been inserted downstream of the Cx43 coding region. This modification allows Cre mediated replacement of the floxed Cx43 coding region with the LacZ reporter gene (Figure 1). Such gene replacement strategy facilitates direct detection of Cx43 ablation by simple X-gal staining in tissues or cells (12, 13). This is an important feature that helps address one potential limitation of the Cre/*lox*P method, since demonstration of gene deletion in selected cells may be difficult.

Two different mouse lines expressing Cre recombinase under control of either the osteocalcin (OG2-Cre) or a 2.3 kb fragment of the type I collagen [Colα1(I)-Cre] promoter were used to express Cre selectively in osteoblasts (14). The OG2 gene is expressed almost exclusively in fully differentiated osteoblasts, whereas Colα1(I) is present at earlier stages of osteoblast differentiation, well before mineralization occurs (15). Furthermore, the 2.3 kb fragment of the Colα1(I) promoter has a more restricted expression distribution relative to the larger 3.2 kb region, which is believed to contain most if not all the regulatory elements of the gene (16). The shorter fragment is active almost exclusively in osteoblasts, without detectable expression in other tissues, including the heart during embryogenesis (16). Therefore, the two promoters provide two different stage-specific expression patterns of Cre in cells of the osteoblastic lineage.

One of the potential limitations of the Cre/lox method is that Cre recombination may be incomplete. To facilitate full gene deletion in osteoblasts, we introduced the $Cx43^{fl}$ allele into a Cx43 null background, with the goal of generating $Cx43^{fl/-}$ heterozygous mice. With only one floxed allele available for recombination, positive X-gal staining becomes a direct index of complete gene inactivation. To obtain the desired genotype, we first introduced the Cx43 null mutation into the Cre expressing transgenic mice. Thus, OG2-Cre or Colα1(I)-Cre mice (generous gift of Dr. Gerard Karsenty,

Baylor College of Medicine) were first mated with heterozygous $Cx43^{\pm}$ mice (9). The resulting crosses [$Cx43^{\pm}$; OG2-Cre or $Cx43^{\pm}$; Colα1(I)-Cre] were then bred with mice homozygous for the "floxed" Cx43 allele ($Cx43^{fl/fl}$). These crosses generate, in approximately equal numbers, the Cx43 conditionally deleted mice [$Cx43^{fl/-}$; OG2-Cre or $Cx43^{fl/-}$; Colα1(I)-Cre], as well as 3 additional genotypes [$Cx43^{fl/+fl/+}$, $Cx43^{fl/-}$, and $Cx43^{fl/+}$; OG2-Cre or $Cx43^{fl/+}$; Colα1(I)-Cre] which served as controls.

The availability of these littermate controls is critical for a correct interpretation of the data, considering a possible haploinsufficiency of the $Cx43^{fl}$ allele (11), and the wide variability in bone mass among different mice strains (17). Thus, $Cx43^{fl/+}$ represents the main comparator (closest to a wild type), $Cx43^{fl/-}$ will allow testing the effect of single allele deletion (heterozygous), and $Cx43^{fl/+}$; OG2-Cre or $Cx43^{fl/+}$; Colα1(I)-Cre will allow testing for effects of hemizygosity for the Cre transgene, such as strong expression of Cre recombinase in cells, or heterozygosity for a possible gene deletion due to the random insertion of the Cre transgene in the genome. The latter group can also provide information on potential strain background effects, although all the three lines used in this project have been developed in a mixed 129/J-C57/BL background.

Detection of Selective Cx43 Gene Ablation in Osteoblasts

Whole mount X-gal staining of newborn mice demonstrated the presence of intense blue staining throughout the mineralized portion of the skeleton of conditional knockout animals, in a pattern exactly overlapping the mineralized skeleton, without detectable stain in other tissues (not shown). Histologic sections of tibiae isolated from $Cx43^{fl/-}$; OG2-Cre also demonstrated X-gal staining only in cells lining the endosteal surface, typical of bone-forming osteoblasts thus confirming the efficacy and selectivity of Cre mediated ablation of the $Cx43^{fl}$ allele (Figure 2A). Osteoblast-specific Cx43 gene deletion was also determined in cultures of osteoblastic cells isolated from newborn mice calvarium, an established method for generating bone-forming cells in culture.

After they reach confluence, calvaria cells undertake a differentiation program leading to timed expression of specific genes and matrix production and mineralization, which is usually complete after 3–4 weeks in culture (15). While cells isolated from calvaria of $Cx43^{fl/-}$; OG2-Cre mice did not show positive staining during the proliferative stage, blue stain developed with time to become intense 4 weeks post-confluence (Figure 2C). A similar pattern of X-gal staining was observed in $Cx43^{fl/-}$; Colα1(I)-Cre cells, although blue stain was observed as early as 1 week post-confluence in these cells, consistent with earlier expression of the Colα1(I) promoter relative to OG2 (data not shown). This time-related activation of LacZ reflects the expected, though distinct progressive up-regulation of both the OG2 and Colα1(I) genes during osteoblast differentiation, and demonstrates that at the time these cultures become fully differentiated (after 3–4 weeks post-confluence), the Cx43 gene is largely deleted.

As a third method to determine Cx43 gene deletion by Cre, we also directly amplified the Cx43 "deleted" allele by PCR, using primers designed to encompass a region including the most distal area of intron 1 and the most proximal sequence of the LacZ cassette (see Figure 1). A PCR product of the expected size (670 bp) was obtained only from genomic DNA extracted from confluent $Cx43^{fl/-}$; OG2-Cre or $Cx43^{fl/-}$; Colα1(I)-Cre calvaria cells, but not from DNA of $Cx43^{+/-}$; OG2-Cre or $Cx43^{fl/-}$ cells (Figure 3), thus demonstrating the effectiveness and selectivity of Cre driven Cx43 gene ablation. Interestingly, the intensity of the 670 bp band was stronger in the $Cx43^{fl/-}$; Colα1(I)-Cre than in the $Cx43^{fl/-}$; OG2-Cre lanes, suggesting that Colα1(I)-Cre may yield a more effective gene deletion compared to OG2-Cre, as previously shown (14).

Finally, to verify that gene deletion in osteoblasts resulted in reduced gene expression, the abundance of Cx43 protein was determined in whole cell lysates of calvaria cells isolated from conditionally deleted mice. Virtually no Cx43 specific bands were detected

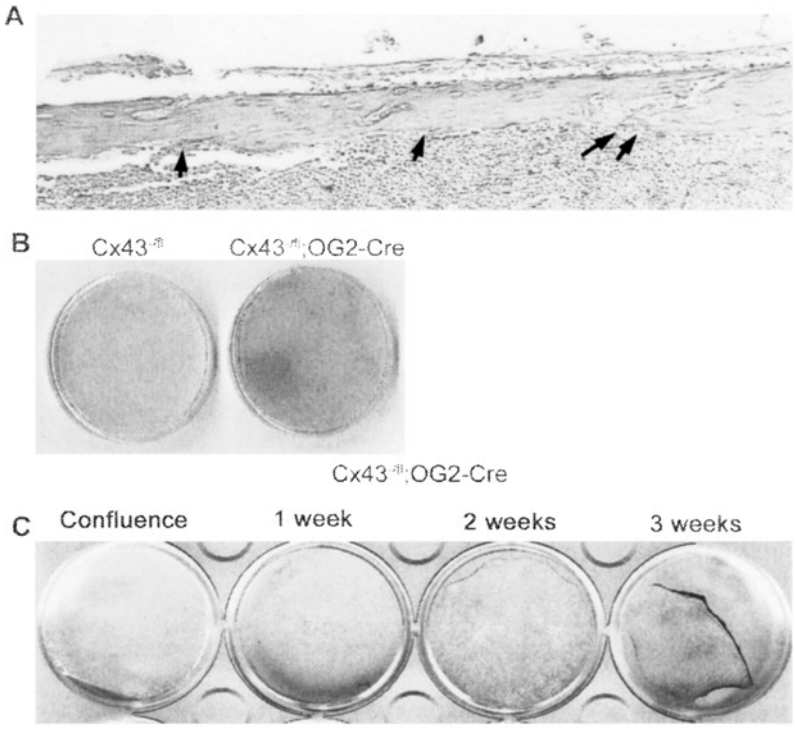

Figure 2. (A) Xgal staining of osteoblasts (arrows) on the endosteal surface of a tibia of $Cx43^{fl/-}$; OG2-Cre mice, demonstrating positive and selective expression of the reporter gene. (B) Intense Xgal staining is present in calvarial cells isolated from $Cx43^{fl/-}$; OG2-Cre animals, but not from $Cx43^{fl/-}$ littermates, after 2 weeks in culture. (C) Xgal staining was demonstrated only post-confluence in calvarial cells isolated from the conditional knockout mice ($Cx43^{fl/-}$; OG2-Cre) and became more and more intense with time in culture.

in cultures of $Cx43^{fl/-}$; OG2-Cre cells, whereas fainter but clearly detectable bands were present in extracts from $Cx43^{fl/+}$; OG2-Cre and $Cx43^{fl/-}$ cells (Figure 4). Likewise, Cx43 specific immunoreactivity was absent in immunoblots of $Cx43^{fl/-}$; Colα1(I)-Cre cells, and fainter bands were observed in $Cx43^{fl/+}$; Colα1(I)-Cre and $Cx43^{fl/-}$ cells, exactly as it occurred in the OG2-Cre model. The lower abundance of Cx43 protein in $Cx43^{fl/+}$; Colα1(I) or $Cx43^{fl/+}$; OG2-Cre cells reflects a gene dosage effect, since after Cre-mediated gene deletion these cells become heterozygous null, and loss of one allele leads to lower Cx43 abundance (8). Likewise, reduced expression of Cx43 protein in $Cx43^{fl/-}$ cells

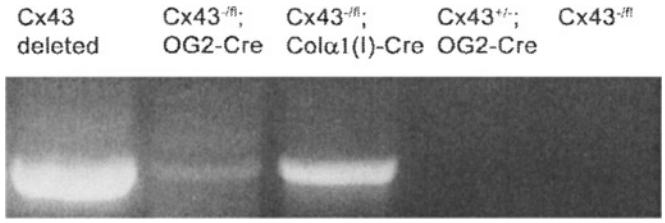

Figure 3. Detection of the Cx43 "deleted" allele. Genomic DNA extracted from calvarial cells was used as template in PCR using primers encompassing the most distal area of Cx43 Intron 1 and the most proximal sequence of the LacZ cassette. A 670 bp band was observed only in calvarial cells extracted from the conditional knockout mice ($Cx43^{fl/-}$; OG2-Cre or $Cx43^{fl/-}$; Colα1(I)-Cre), but not in their control littermates. DNA from ROS 17/2.8 cells transfected with the Cx43 deleted allele was used as control (first lane).

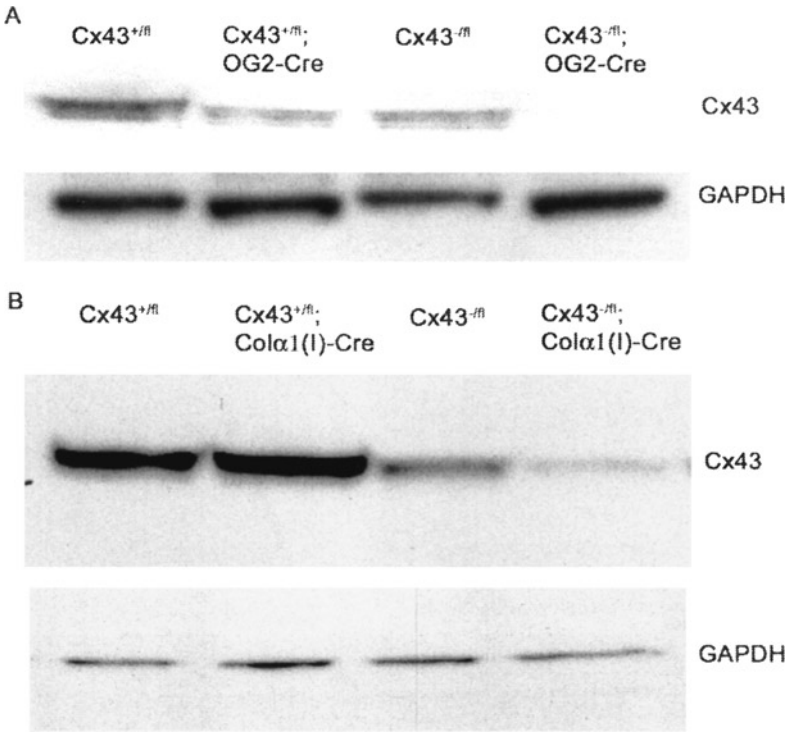

Figure 4. Cx43 protein abundance in calvarial cells. Whole cell lysates extracted from calvarial cells were separated by SDS-PAGE and immunoblotted with an anti-Cx43 polyclonal antibody (Sigma, St. Louis, MO). GAPDH expression was used as internal control. (A and B) After 3 weeks in culture, Cx43 protein was almost undetectable in $Cx43^{fl/-}$;OG2-Cre and $Cx43^{fl/-}$;Colα1(I)-Cre calvarial cells, whereas fainter but clearly detectable bands were present in extracts from $Cx43^{fl/+}$;OG2-Cre or $Cx43^{fl/+}$;Colα1(I)-Cre and $Cx43^{fl/-}$ cells.

is presumably the result of both loss of one allele and haploinsufficiency of the floxed allele. In fact, densitometric assessment of these immunoblots indicated that the intensity of Cx43-specific bands relative to GAPDH was lower in $Cx43^{fl/-}$ lanes than in $Cx43^{fl/+}$; Colα1(I) or $Cx43^{fl/+}$; OG2-Cre extracts (not shown).

CONCLUSIONS

In summary, we have established two models of osteoblast-specific Cx43 gene ablation *in vivo* using the Cre/*lox*P approach. Both result in effective ablation of the gene in osteoblasts when they reach a differentiated state. Lower abundance of Cx43 protein corresponds to single allele loss of Cx43. These new models of tissue specific gene ablation will be useful to understand the contribution of Cx43 junctional communication provided to adult skeletal homeostasis.

ACKNOWLEDGEMENTS

This work was supported by NIH-NIAMS grant R01 AR41255 (to R. Civitelli) and T32 AR07033 (to J. Stains), and in part by the National Aeronautics and Space Administration Grant NRA 99-HEDS-02-110 (to R. Civitelli). Work in Bonn was supported by grants of the German Research Association (SFB 400/E3 and Wi270/22-3.4) and the Funds of the Chemical Industry (to K. Willecke). C. H. M. Castro was a post-doctoral Fellow of CAPES Foundation, Ministry of Education, Brazil. M. Theis received a stipend of the GraduiertenKolleg: Pathogenese von Krankheiten des Nervensystems.

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