#80 MAD4, a new regulator of self-renewal in human keratinocyte precursor cells.

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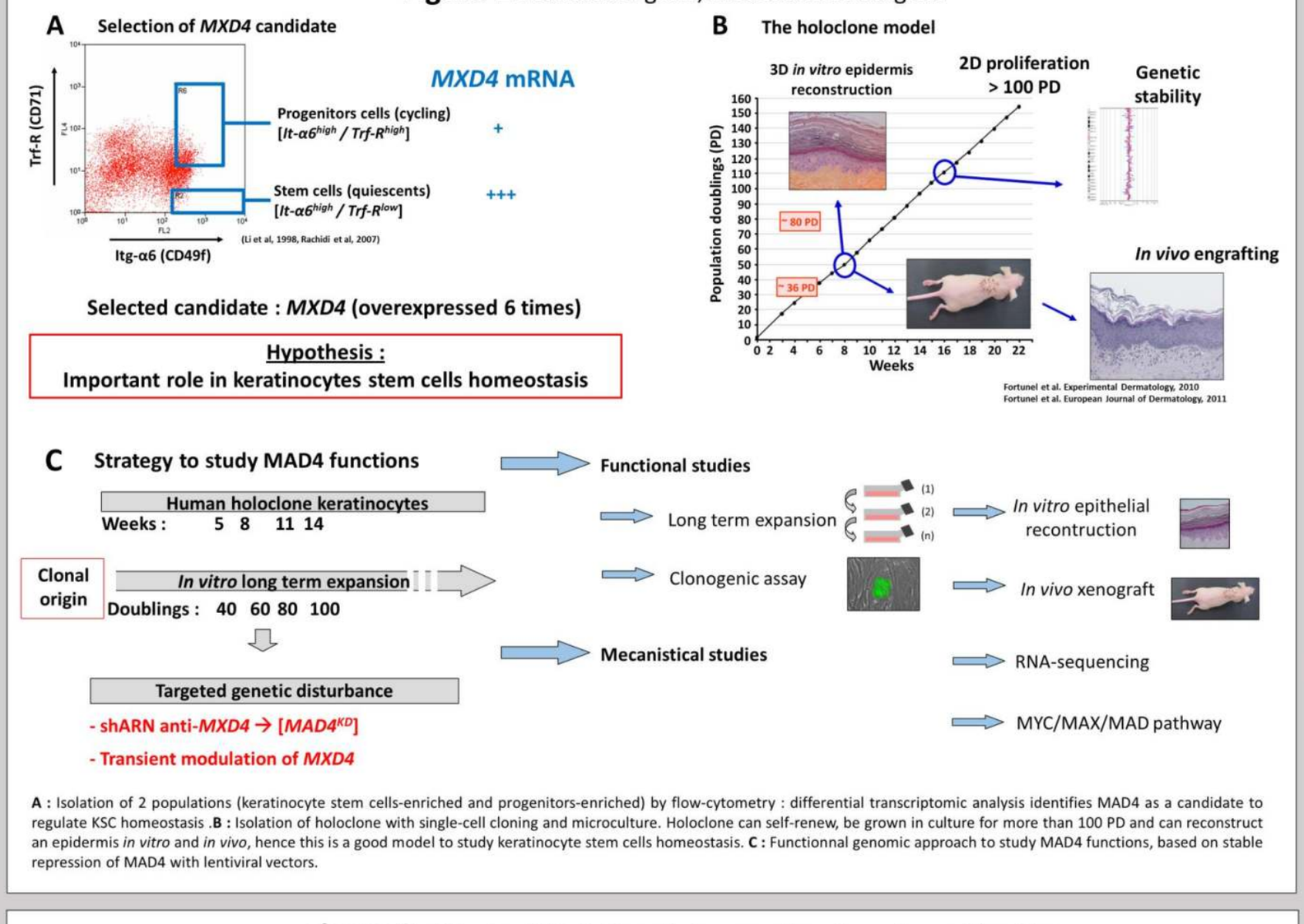
Abstract :

During the lifespan of individuals, epidermis integrity is maintained by the keratinocyte precursor cells (KPC), present within the basal layer. Inside this population, the keratinocytes stem cells (KSC) sub-population is able to self-renew, proliferate and give rise to progenitors cells (PC) that will differentiate as they migrate in the upper layers of the epidermis. A finely regulated balance between quiescence, proliferation and differentiation is crucial to ensure stem cell fate and epidermis homeostasis, but the mechanisms involved need further understanding. From freshly isolated human basal keratinocytes sorted based on integrin-α6 and transferrin-receptor expression, we realized a differential transcriptomic analysis comparing a sub-population enriched in quiescent KSC ([*Itg-α6+;Trf-R-*]) with a sub-population enriched in cycling progenitors ([*Itg-α6+;Trf-R+*]). We selected the *MXD4* gene, encoding the transcription factor MAD4, as a potential candidate regulating the KSC self-renewal as it was overexpressed 6 times in the KSC-enriched fraction. To investigate the role of MAD4, we developed a RNA interference approach and analyzed the impact of the loss-of-function of MAD4 (knock-down, KD) in keratinocytes precursor cells. Lentiviral vectors encoding a shRNA anti-*MXD4* were used to generate a cellular context where MAD4 is stably repressed ([$MAD4^{KD}$]), and siRNA anti-*MXD4* were used to test the effect of its transient repression. The repression of MAD4 has a positive effect on short- and long-term cell proliferation and increases their clonogenic potential. Moreover, the repression of MAD4 increases the expression of two immaturity markers, integrin-α6 and ΔNp63α, an isoform of p63 specifically expressed in basal keratinocytes. This results indicate that MAD4 has important role in the control of proliferation and immaturity, and therefore support the hypothesis that it might be involved in self-renewal mechanisms.

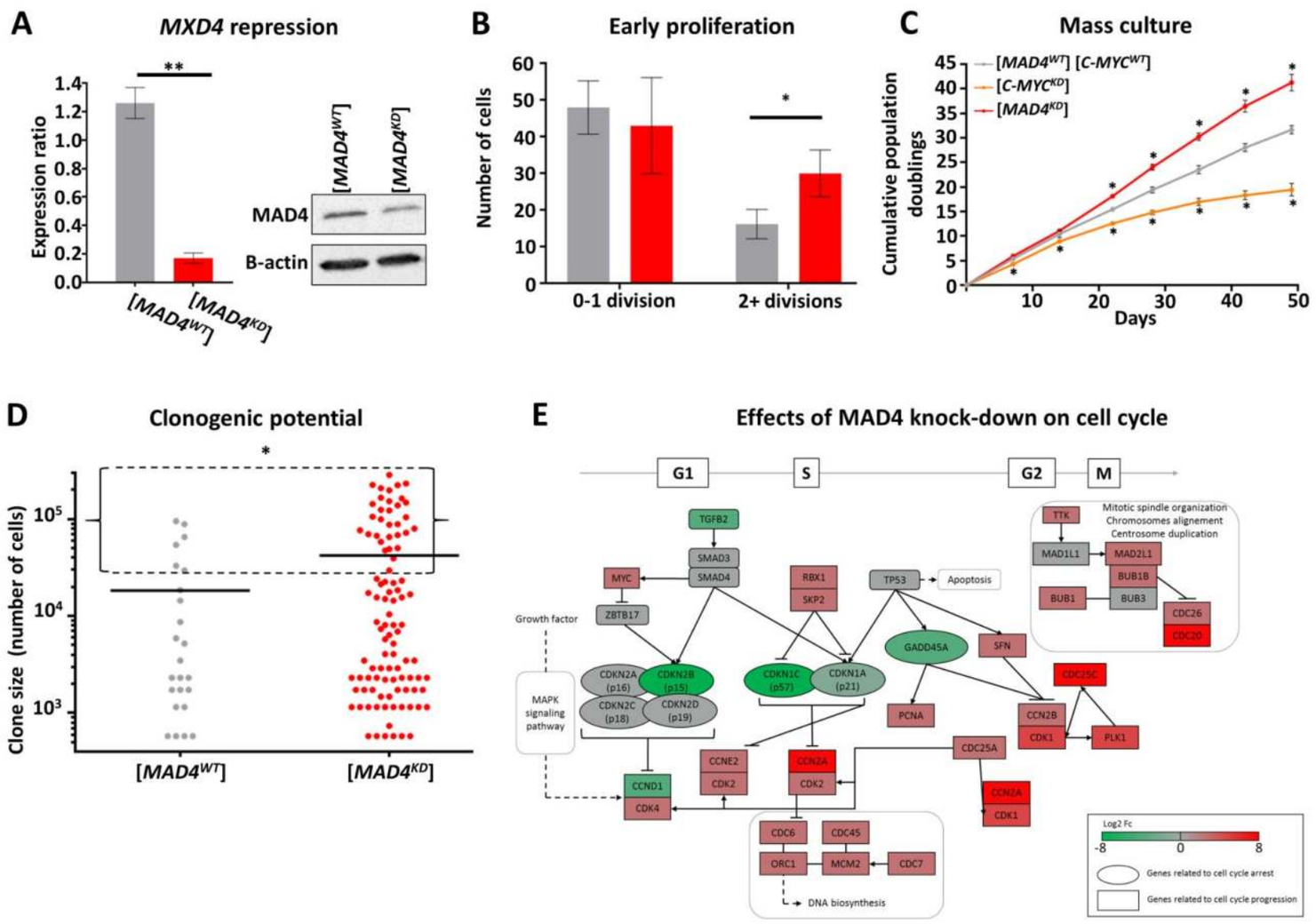
Material and methods :

Figure 1 : candidate gene, model and strategies.



A : Isolation of 2 populations (keratinocyte stem cells-enriched and progenitors-enriched) by flow-cytometry : differential transcriptomic analysis identifies MAD4 as a candidate to regulate KSC homeostasis .**B :** Isolation of holoclone with single-cell cloning and microculture. Holoclone can self-renew, be grown in culture for more than 100 PD and can reconstruct an epidermis *in vitro* and *in vivo*, hence this is a good model to study keratinocyte stem cells homeostasis. **C :** Functionnal genomic approach to study MAD4 functions, based on stable repression of MAD4 with lentiviral vectors.

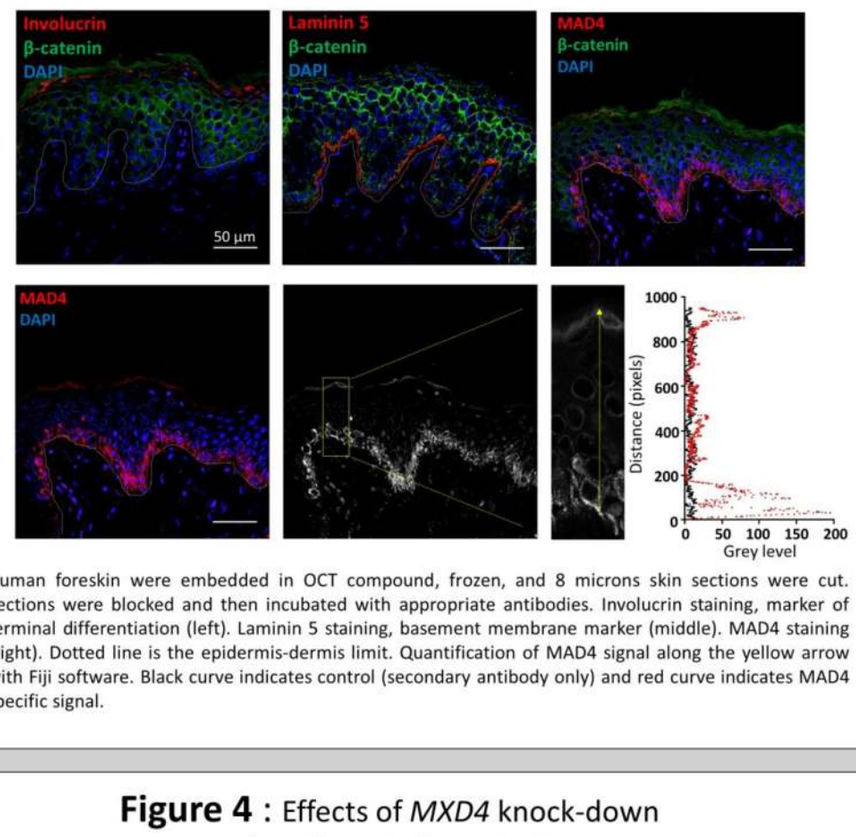
Figure 3 : Effects of *MXD4* knock-down on keratinocyte proliferation.



Keratinocyte precursor cells were transduced with lentiviral vectors driving expression of GFP alone ([$MAD4^{WT}$] and [$C\text{-}MYC^{WT}$] cellular context) or GFP and a specific anti-*MAD4* shRNA ([$MAD4^{KD}$] cellular context) or anti-*C-MYC* shRNA ([$C\text{-}MYC^{KD}$] cellular context). **A :** Verification of *MXD4* repression by q-RT-PCR and Western Blot. **B :** Keratinocyte precursor cells were seeded at the density of 500/cm², and observed 48h after seeding to follow the early proliferation. Cells that have divided once, twice or more, or not divided were observed under microscope and manually counted (n~ 900 for each cellular context, from 3 independent transductions). **C :** Long-term follow-up of [$MAD4^{WT}$], [$MAD4^{KD}$] and [$C\text{-}MYC^{KD}$] cell proliferation (n = 24 independent cultures for each cellular context from 3 independent transductions). **D :** Freshly transduced basal keratinocytes [$MAD4^{WT}$] and [$MAD4^{KD}$] were cloned in 96-wells plates and their clonogenic potential characterized after 4 weeks of culture. **E :** Effects of *MXD4* repression on cell cycle. Cell cycle inhibitors are repressed and cell cycle activators are induced upon *MXD4* knock-down.

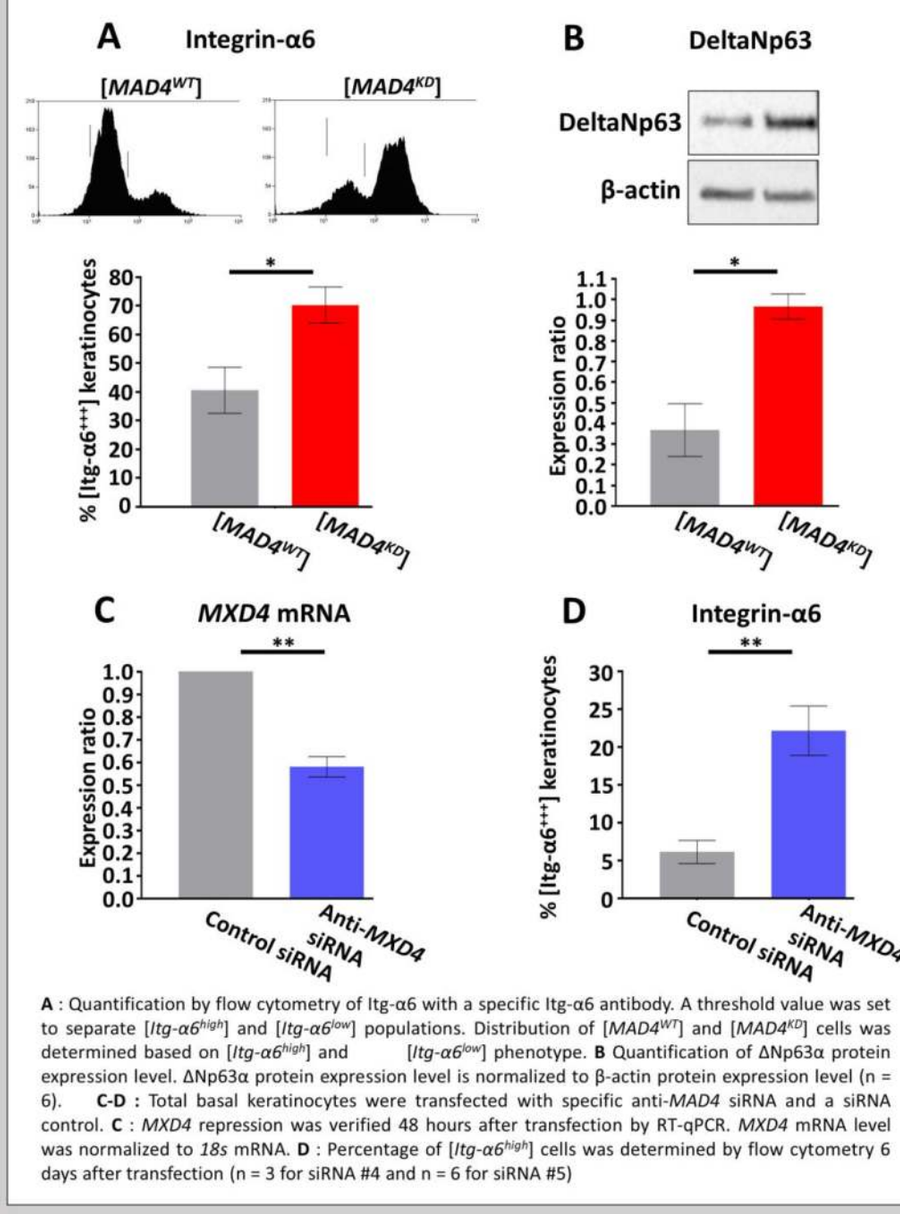
Results :

Figure 2 : MAD4 localization in normal human skin.



Human foreskin were embedded in OCT compound, frozen, and 8 microns skin sections were cut. Sections were blocked and then incubated with appropriate antibodies. Involucrin staining, marker of terminal differentiation (left). Laminin 5 staining, basement membrane marker (middle). MAD4 staining (right). Dotted line is the epidermis-dermis limit. Quantification of MAD4 signal along the yellow arrow with Fiji software. Black curve indicates control (secondary antibody only) and red curve indicates MAD4 specific signal.

Figure 4 : Effects of *MXD4* knock-down on keratinocyte immaturity.



A : Quantification by flow cytometry of Itg-α6 with a specific Itg-α6 antibody. A threshold value was set to separate [*Itg-α6high*] and [*Itg-α6low*] populations. Distribution of [$MAD4^{WT}$] and [$MAD4^{KD}$] cells was determined based on [*Itg-α6high*] and [*Itg-α6low*] phenotype. **B** Quantification of ΔNp63α protein expression level. ΔNp63α protein expression level is normalized to β-actin protein expression level (n = 6). **C-D :** Total basal keratinocytes were transfected with specific anti-*MAD4* siRNA and a siRNA control. **C** : *MXD4* repression was verified 48 hours after transfection by RT-qPCR. *MXD4* mRNA level was normalized to *18s* mRNA. **D** : Percentage of [*Itg-α6high*] cells was determined by flow cytometry 6 days after transfection (n = 3 for siRNA #4 and n = 6 for siRNA #5)

Conclusion :

MAD4 repression impacts on two important biological parameters in keratinocyte precursor cells: proliferation and cellular immaturity. A next important aspect that will be investigated is the impact of MAD4 knock-down on regenerative capacities *in vitro* and *in vivo*. To complete this functional genomic approach, we will focus on interactions between MAD4 and its major partners, MAX and C-MYC, in order to understand the cellular mechanisms by which MAD4 is regulating the growth potential of human keratinocytes precursor cells.

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